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Bax Does Not Directly Participate in the Ca^{2+}-induced Permeability Transition of Isolated Mitochondria*

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The mitochondrial permeability transition pore and Bax have both been proposed to be involved in the release of pro-apoptotic factors from mitochondria in the "intrinsic" pathway of apoptosis. The permeability transition pore is widely thought to be a supramolecular complex including or interacting with Bax. Given the relevance of the permeability transition *in vivo*, we have verified whether Bax influences the formation and/or the properties of the Ca^{2+}/P_i-induced permeability transition by using mitochondria isolated from isogenic human colon cancer *bax*$^{+/-}$ and *bax*$^{-/-}$ HCT116 cell lines. We used mitochondria isolated from both types of cells and from Bax$^+$ cells exposed to apoptotic stimuli, as well as Bax-less mitochondria into which exogenous Bax had been incorporated. All exhibited the same behavior and pharmacological profile in swelling and Ca^{2+}-retention experiments. Mitochondria from a *bax*$^-$/*bak*$^-$ cell line also underwent an analogous Ca^{2+}/P_i-inducible swelling. This similarity indicates that Bax has no major role in regulating the Ca^{2+}-induced mitochondrial permeability transition.

The release of cytochrome *c* and other pro-apoptotic factors from the intermembrane space of mitochondria is a key step in most models of apoptosis (for reviews see Refs. 1–5). The exact mechanism of this release is still debated. Most schemes assign a role to the pro-apoptotic Bcl2 family protein Bax (and/or Bak). Suggestions have been made, for example, that Bax may act by destabilizing the lipid bilayer (6, 7) or by inducing lipid oxidation (8). A well supported scheme envisions the regulated migration and insertion of Bax into the outer mitochondrial membrane where, alone or in cooperation with resident proteins such as VDAC[1] or components of the mitochondrial fusion/fission machinery, it would form pores capable of allowing cytochrome *c* escape.

The formation of Bax pores in the mitochondrial outer membrane does not seem to account for all available data. In particular, considerable evidence points to a role of the permeability transition pore (PTP) in cytochrome *c* release in a number of apoptosis model systems (reviewed in Refs. 9–17). The PTP (for review see Refs. 13 and 18–20) is a large channel that develops in the mitochondrial envelop, connecting the matrix and extramitochondrial space. Its properties have been deduced mostly from experiments with isolated mitochondria (most often from rat liver) in suspension. In such experiments, PTP formation (or opening) generally requires the accumulation of Ca^{2+} in the mitochondrial matrix, and it is favored by a variety of inducing agents (the "classical" one being phosphate) and/or stressful conditions such as oxidation of thiol groups and/or depolarization. Under the appropriate conditions, *i.e.* in the presence of an external pore-permeating osmolite, such as sucrose, opening of the PTP leads to colloidosmotic osmolite and water influx and swelling, with ensuing rupture of the outer membrane and formation of a mitoplast still bearing remnants of the outer membrane.

The catalogue of PT inhibitors includes cyclosporin A (CSP) and divalent cations other than Ca^{2+}, protons, and ADP. The sensitivity of the apoptotic process and of cytochrome *c* release to CSP has been the cue leading to the proposal that PTP activation is involved in many cases, including various chemotherapeutic treatments. Given its properties, PTP involvement would seem particularly likely in cases involving disruption of Ca^{2+} homeostasis and uptake of large amounts of the ion by mitochondria, such as in endoplasmic reticulum stress-induced apoptosis and ER-mitochondria Ca^{2+}-mediated "cross-talk" (16).

The molecular composition of the PTP has not been definitely established, but biochemical, electrophysiological, and pharmacological experiments have led to a model envisioning a supramolecular complex (the PTPC) of proteins from all compartments of the mitochondrial envelope, including the adenine nucleotide translocator, porin (VDAC), cyclophilin D, and other proteins, localized at contact sites between the inner and outer membrane (19, 21, 22). Bax has been reported to interact with the adenine nucleotide translocator (23–26), with VDAC (27–29), and with the complexes they form (22, 30) (Bcl-x_L also interacts with VDAC (28, 31)). These interactions reportedly result in the appearance of channels different from those formed by the individually isolated and reconstituted proteins. A recent report (32) suggests that the insertion of Bax in the outer membrane may result in the *de novo* formation of contact sites. These and other (33–37) findings suggest that Bax (and perhaps its homologues Bak and Bid) may induce the mitochondrial PT and cytochrome *c* release by interacting with the

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[1] The abbreviations used are: VDAC, voltage-dependent anion channel (mitochondrial porin); BMK, baby mouse kidney; CSP, cyclosporin A; DKO, double knock-out; HCT, human colon tumor; PIPES, 1,4-piperazinediethanesulfonic acid; PT, permeability transition; PTP, permeability transition pore; PTPC, PTP complex; RLM, rat liver mitochondria; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; TPP, tetraphenyl phosphonium; GFP, green fluorescent protein; h, human.

PTP to either form or regulate it (or both). Anti-apoptotic Bcl-2 family members antagonize the permeability transition (38) and inhibit activity by reconstituted PTPC (23, 39), but this antagonistic action takes place regardless of the presence of Bax in the complex (23). The possibility of a migration of Bax to the inner mitochondrial membrane has been raised (23, 24). The results of Marzo *et al.* (23) are particularly relevant with regard to the relationship between Bax and the PTP. These authors found that Bax strongly favored PTP opening by atractyloside but that it was not needed when permeabilization was induced by oxygen radicals or thiol cross-linking. Ca^{2+}-induced permeabilization was not examined from the point of view of the Bax requirement. These observations suggest that the permeabilization process differs depending on the inducing agent, *i.e.* different types of permeability transition can occur, but this aspect has not received much attention. The notion seems instead to have taken hold that Bax is a component, or a key regulator, of the PTP, regardless of the mode of induction.

The participation of Bax to PTP formation may lead to the unification of the major mechanistic hypotheses concerning cytochrome *c* efflux, yet it does not rest on firm ground. Two groups (40, 41), at variance with others (42, 43), have reported that Bax cannot be detected in Western blots of rat brain and rat liver mitochondria, two prominent models for PT studies. Some results (44, 45) suggest caution in automatically identifying CSP-inhibitable processes as being downstream of the PT. Several studies have concluded that Bax-mediated release of cytochrome *c* is independent of the PTP and takes place without permeabilization of the inner membrane (4).

Whether PT induction by atractyloside is an appropriate model for PT-inducing processes within living cells is uncertain. Radical species (or, in general, redox processes) and Ca^{2+} may be considered to be the most relevant among the several known PT inducers in pathophysiological processes and in apoptosis. Whether Bax is involved in these processes is in fact unknown. We have therefore looked into the relationship between Bax and the Ca^{2+}-induced PT by comparing the behavior of mitochondria isolated from two isogenic human colon carcinoma HCT116 cell lines, expressing either normal amounts of Bax or completely lacking the protein (46). These cells have been used to advantage in recent investigations into the mechanism of apoptosis, which have led to the conclusion that Bax is a necessary component of the intrinsic (*i.e.* involving mitochondrial release of pro-apoptotic factors) apoptotic pathway. Bak may partially substitute for its homologue Bax in mediating the release of mitochondrial pro-apoptotic factors, and it might conceivably take its place also in PTP formation. In unchallenged baby mouse kidney (BMK) cells, Bak appears to be a component of large complexes (47) and to interact with VDAC2, a low abundance porin isoform (48). We proceeded therefore to also investigate mitochondria isolated from a cell line expressing neither protein, namely DKO Bax- and Bak-less BMK cells (49).

EXPERIMENTAL PROCEDURES

Cells—Human colon tumor (HCT116) $bax^{+/-}$ and $bax^{-/-}$ cells (46) (kindly provided by B. Vogelstein) and the Bax-GFP-expressing line (kindly provided by X. Wu) (50) were grown in McCoy's 5A 10% fetal calf serum (Invitrogen). Baby mouse kidney (BMK) cells, DKO for *bax* and *bak* and controls (49) (kindly provided by E. White), were grown in Dulbecco's modified Eagle's medium, 5% fetal calf serum (Invitrogen).

Proteins—Oligomeric and monomeric full-length hBax with a His_6 tag at the N terminus was obtained as described previously (51); His-tagged hBcl-X_L lacking 24 amino acids at the C terminus was expressed and isolated as in Ref. 52; t^c-Bid (Bid residues 60–195) was obtained as in Ref. 53.

Isolation of Mitochondria—Approximately 80% confluent cells from four 75-cm^2 flasks were washed once with phosphate-buffered saline or Hepes saline buffer, detached by trypsinization and gentle scraping, and spun down in a table centrifuge at room temperature. The pellet was resuspended either in sucrose/TES (300 mM sucrose, 10 mM TES, 0.5 mM EGTA, pH 7.4) or in mannitol/PIPES (220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM 1,4-dithiothreitol, 50 mM PIPES/K^+, 10 μM cytochalasin, mini complete protease inhibitor mixture; pH 7.4) buffer (Roche Applied Science). After standing for 1 h on ice, cells were lysed in a glass homogenizer, and the lysate was centrifuged at 200 × *g* for 10 min at 4 °C. The pellet was again processed in the same way to maximize recovery. The combined supernatants were centrifuged once or twice at 200 × *g*, and the pellet was discarded. The mitochondria-containing supernatant from the last step was centrifuged at 6,000 × *g*. The pellet was gently homogenized and suspended in a small volume of buffer by suction/ejection with a Pasteur pipette, and the protein concentration was assayed by the Biuret or Bradford methods, using bovine serum albumin for the calibration curve. Rat liver mitochondria were prepared by standard differential centrifugation and rat brain mitochondria according to Ref. 54.

Assessment of Cytochrome c Release—Nearly confluent cells (one 75-cm^2 flask), exposed or not to apoptogenic agents for the desired times, were detached by scraping, washed, and resuspended in 300 μl of cold mannitol buffer + 5 mM succinate. After 30 min on ice, the cells were lysed as above, and the lysate was centrifuged at 28,000 × *g* for 10 min at 4 °C. The supernatant was the cytosolic fraction. Protein concentration was measured with the Biuret method, and 50 μg of total protein were used for Western blotting as described below.

Experiments with Mitochondrial Suspensions—Unless otherwise indicated, the experiments were initiated by the dilution of the mitochondrial suspension into 300 mM sucrose, 10 mM Hepes/K^+, 5 mM succinate/K^+, 1.25 μM rotenone, 1 mM P_i/K^+, pH 7.4 (suspension buffer), supplemented with the appropriate probes and/or inhibitors. Temperature was 20 °C. For transmembrane potential, variations in the potential maintained by the mitochondria (1 mg of protein/ml) were monitored by measuring the concentration of tetraphenylphosphonium (TPP) ion in the suspension buffer, using a TPP-selective electrode prepared according to Ref. 55. For Ca^{2+} uptake and release, experiments such as those in Fig. 2, *C—F,* extramitochondrial Ca^{2+} concentration was measured fluorimetrically essentially as described previously (56). Calcium Green 5-N (1 μM; Molecular Probes, Eugene, OR) fluorescence was excited at 506 nm, and emission was recorded at 532 nm in an LS-50B spectrofluorimeter (PerkinElmer Life Sciences). Serial additions of 10 μM (20 nmol/mg of protein) $CaCl_2$ were made at 90-s intervals. For swelling, mitochondrial volume changes were followed as a pseudo-absorbance decrease at 540 nm in a PerkinElmer Life Sciences λ5 UV-visible spectrophotometer. Mini-cuvettes and a suspension volume of 200 μl (1 mg of mitochondrial protein/ml) were employed to minimize the material used in each experiment. Experiments were initiated by the addition of mitochondria into the cuvettes, except when the mitochondria were preincubated with Bax, in which case swelling was initiated by the addition of Ca^{2+} (see below).

Electron Microscopy—Mitochondria were diluted to 1 mg of protein/ml in suspension buffer with or without Ca^{2+}, incubated for 60 min at 20 °C, and then spun down in a table centrifuge. The pellet was overlaid with 2% glutaraldehyde in 300 mM sucrose, 10 mM TES, 0.1% cacodylate, pH 7.4, and after standing overnight it was postfixed with 1% osmic acid in 0.1 M cacodylate buffer, dehydrated, and included in Epon 812. Thin sections were observed with a Philips EM 301 electron microscope. For fluorescence microscopy, cells expressing Bax-GFP were observed using an Olympus Biosystems apparatus comprising an Olympus IX71 microscope, MT20 light source, and Cell© software.

Incorporation of Bax into Mitochondrial Membranes—Isolated HCT116 Bax^- mitochondria were suspended in suspension buffer without Ca^{2+} at 1 mg of protein/ml in the presence of 20 nM (20 pmol/mg of protein) oligomerized Bax or of 20 nM monomeric Bax + 20 nM t^c-Bid in a spectrophotometer cuvette. When monomeric Bax and t^c-Bid were used, the two proteins were preincubated together at 30 °C for 30 min before addition of the mitochondria. Swelling experiments (see Fig. 7) were initiated by Ca^{2+} addition to the mitochondrial suspension after 15 min of incubation at 20 °C, during which time the absorbance was monitored to verify that no major variation of light scattering was taking place. The suspension medium was supplemented with 5 μM cytochrome *c*. To verify that Bax had become incorporated into the mitochondrial membrane, aliquots treated identically to those used for swelling experiments were processed as follow: after the 15-min incubation mitochondria were recovered by centrifugation at 28,000 × *g*; the pellet was resuspended in 150 μl of 0.1 M Na_2CO_3, pH 11.5, and incubated for 5 min at room temperature. The membranes were then recovered by centrifugation as above, washed once with 125 mM KCl, 20 mM Hepes/K^+, pH 7.4, and suspended in sample buffer for electrophore-

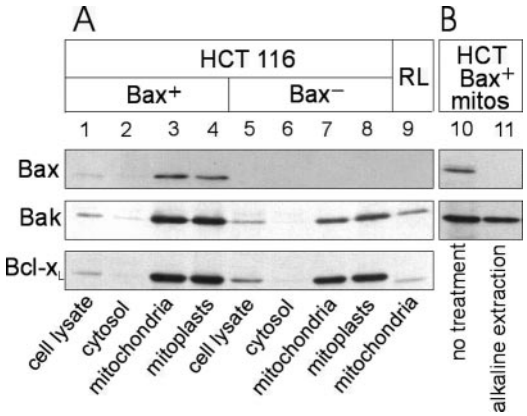

FIG. 1. **The relevant Bcl-2 family proteins in HCT116 and rat liver (*RL*) mitochondria.** *A*, Western blots of the indicated fractions. "Mitoplasts" indicate mitochondria suspended in 30 mM Tris-Cl for 15 min and then collected by centrifugation. 50 μg of total protein were loaded into each lane of the SDS-PAGE gels. *B* shows that alkaline treatment stripped off all Bax (but not Bak) associated with HCT116 Bax^+ mitochondria. *mitos*, mitochondria.

sis and blotting (this procedure reproducibly stripped all Bax from isolated HCT116 Bax^+ mitochondria). At the end of the swelling experiment the mitochondria were recovered and processed as above to double-check the presence of membrane-inserted Bax. The same alkaline extraction procedure was employed to verify the presence of integral membrane Bax in the mitochondria of cells exposed to apoptotic stimuli (Fig. 6).

Western Blots—Samples, dissolved in sample buffer and boiled for 5 min, were subjected to SDS-PAGE in 12% acrylamide minigels and transferred to a nitrocellulose sheet (Protran, Schleicher & Schuell). Primary antibodies used are as follows: anti-hBcl-X_L (S-18) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA; catalogue number sc-634); anti-hBax (N-20) rabbit polyclonal antibody (Santa Cruz Biotechnology; catalogue number sc-493); anti-hBak NT rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY; catalogue number 06-536); anti-pCyt c mouse monoclonal antibody (BD Biosciences, catalogue number 556433); anti-actin mouse monoclonal antibody (Sigma; catalogue number A 4700); and anti-cyclophilin D rabbit polyclonal antibody (Affinity Bioreagents; catalogue number PA1–028). Secondary antibodies (Calbiochem) were horseradish peroxidase-conjugated and used with chemiluminescence detection (Amersham Biosciences; catalogue number RPN 2106).

RESULTS

In agreement with others (40, 41), we were unable to detect Bax in RLM and rat brain preparations (Fig. 1, *lane 9*, and data not shown), two model systems widely employed for studies on the PT. Because only low amounts of the protein, which we may have missed, would presumably be required for the formation of one or a few pores per mitochondrion, we did not consider this negative result as definite evidence that Bax cannot be involved in the PT, and we proceeded to study organelles from HCT116 cells expressing normal levels of Bax or lacking it completely for genetic reasons. The absence or presence of Bax in cells and mitochondrial fractions was confirmed by Western blot (Fig. 1, *lanes 1–8*) (47, 50, 57–59). In untreated HCT116 cells Bax is cytosolic, as evident from the diffuse GFP fluorescence distribution pattern observed in $bax^{-/-}$ HCT116 cells expressing a Bax-GFP chimera (50) (not shown). However, stresses such as those inflicted in the initial stages of the preparation of mitochondria (detachment, washing, and cold incubation) induced a marked redistribution of Bax-GFP to the mitochondria (not shown). This process likely accounts for the presence of Bax in isolated mitochondria as a peripheral membrane protein, detachable by alkaline treatment (Fig. 1*B*) (47), as well as for its scarcity in the cytosolic fraction (Fig. 1*A*). An analogous mitochondrial localization has been observed in other cells as well, *e.g.* lymphocytes.[2] Mitoplasts produced by osmotic shock of isolated Bax^+ mitochondria retained most of the protein (Fig. 1, *lane 4*). Because one study (50) has reported Bak to be below detection limits in colon cancer cells, whereas others found it to be expressed (47, 58), we also verified the presence of this protein, which was indeed present in cell lysates and mitochondrial fractions. For reasons given below (see "Discussion"), we also compared the amounts of Bcl-x_L present in HCT116 mitochondria and RLM. HCT116 mitochondria turned out to contain much more Bcl-x_L per mg of protein than RLM (Fig. 1*A*, compare *lanes 3* and *7* with *lane 9*).

Isolated HCT116 mitochondria were characterized to verify whether they would undergo the permeability transition and whether any differences could be detected between mitochondria isolated from Bax-proficient and Bax-deficient cells. Experiments conducted using protocols commonly employed to study this phenomenon showed that both types of mitochondria underwent the PT. Its onset could be observed as an irreversible depolarization induced by high Ca^{2+} loads (Fig. 2, *A* and *B*) and as the eventual release of Ca^{2+} taken up in Ca^{2+} retention experiments (Fig. 2, *C–F*). Fig. 2 shows representative experiments; any differences between the two preparations (in the amount of TPP taken up initially, how much Ca^{2+} the mitochondria could import before depolarizing completely, or in the number of doses of the ion they could take up before releasing all of it) were not systematic and well within the range of variations for each type of preparation (Bax^+ or Bax^-), as shown by the *bars* in Fig. 2*G* for the Ca^{2+} uptake experiments.

The same result emerged from classical experiments assessing the decrease in light scattering due to Ca^{2+}/P_i-induced swelling (Fig. 3, *A* and *B*). A confirmation that mitochondria from both $bax^{+/-}$ and $bax^{-/-}$ cells could undergo the PT came from TEM photographs (Fig. 3, *C–F*). In both cases mitochondria became grossly swollen if incubated with Ca^{2+}/P_i (Fig. 3, *E* and *F*).

Again, the phenomenon took place essentially with the same characteristics for Bax^+ and Bax^- mitochondria, differences being well within the variability exhibited by each type of preparation from day to day. In all these experiments, HCT116 mitochondria seemed more resistant to Ca^{2+}/P_i than RLM, which are a standard for this field of study. In suspensions of RLM swelling is generally complete in a few minutes and requires lower $[Ca^{2+}]_{ext}$ than we routinely used. Differences in the sensitivity of mitochondria deriving from different cells to PT-inducing agents are well known to exist and may have various origins (see "Discussion"). For the purposes of this paper, the most relevant point is that no significant difference could be found between mitochondria from isogenic Bax-expressing and Bax-less cells, regardless of the Ca^{2+} dose administered.

The Ca-induced swelling of both types of HCT116 mitochondria turned out to be inhibited by a panel of well known inhibitors of the PT with similar dose dependence (Fig. 4, *A* and *B*).

No differences in the response of the two preparations to inhibitors could be identified, with the possible exception of sensitivity to cyclosporin A (Fig. 4, *C* and *D*). Swelling of Bax^- HCT116 mitochondria exposed to 100 or 200 μM Ca^{2+}/1 mM P_i could be completely and reproducibly inhibited for at least 1 h by 1–5 μM CSP (Fig. 4*D*), whereas even 20 μM CSP inhibited swelling of Bax^+ mitochondria only partially under the same conditions (Fig. 4*C*, *curves f* and *g*), suggesting the possible existence of a subpopulation of CSP-insensitive mitochondria in this latter case. This behavior was reproducible; inhibition was complete for 6 of 7 preparations of Bax^- mitochondria,

[2] E. Gulbins, personal communication.

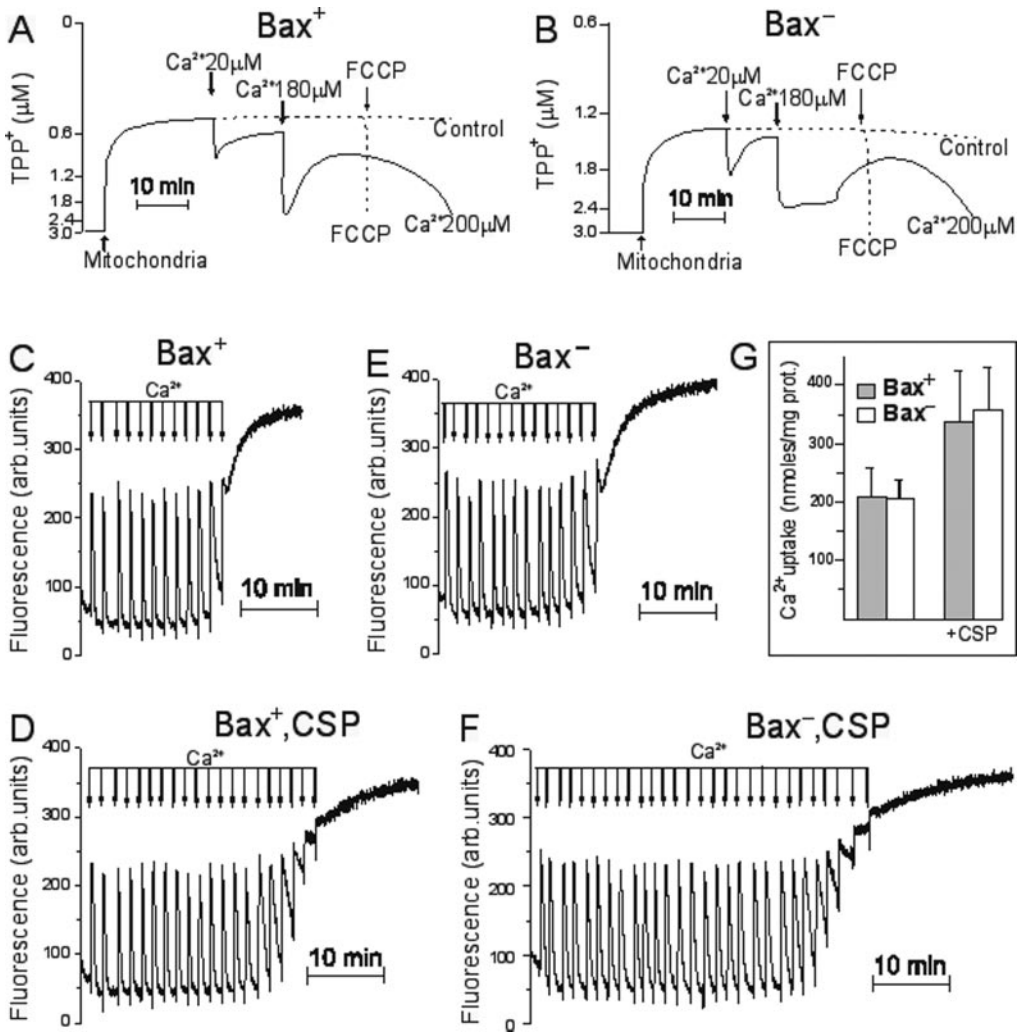

FIG. 2. **Ca^{2+}/P_i induces depolarization and Ca^{2+} release in both Bax^+ and Bax^- HCT116 mitochondria.** *A* and *B*, Ca^{2+}-induced depolarization. Representative experiments. The concentration of TPP^+ in the medium is plotted. Mitochondria in suspension buffer maintain a potential that is transiently reduced by the addition of 20 nmol of Ca^{2+}/mg of protein and eventually dissipated completely by the addition of higher amounts of the ion. *Dashed traces* show the behavior of controls and the effect of an uncoupler. *C–F*, cyclosporin-sensitive Ca^{2+}-induced Ca^{2+} release is similar for Bax^+ and Bax^- HCT116 mitochondria. Medium [Ca^{2+}] was followed using a fluorescent probe. 20 nmol/mg of protein doses of $CaCl_2$ were added sequentially. *G*, *shaded bars*, the average amount of Ca^{2+} taken up before onset of PT-associated release was the same, within experimental error, for Bax^+ and Bax^- mitochondria. *Open bars*, the similarity persisted in the presence of CSP, *i.e.* the protective efficacy of CSP was the same in the two cases. *Error bars* represent standard error ($n = 4$ in all cases).

whereas this was never the case with 9 preparations of Bax^+ organelles. Inhibition of PT in Bax^+ preparations was, however, complete if CSP was added together with ADP (not shown) or Mg^{2+} (Fig. 4*C, curves h* and *i*) at concentrations having, by themselves, little effect. As mentioned above (Fig. 2*G*), this difference in behavior was not observed when using other experimental protocols. The two types of mitochondria possessed similar levels of cyclophilin D, as assessed by Western blotting (not shown).

Because HCT116 cells express Bak (Fig. 1), we proceeded to verify whether mitochondria isolated from Bax^-/Bak^- (DKO) BMK cells would undergo the PT. The absence of Bax and Bak in DKO cells was checked by Western blot (not shown). Mitochondria isolated from the DKO cells showed the same Ca^{2+}-induced persistent depolarization, Ca^{2+}-induced Ca^{2+} release, and swelling as mitochondria isolated from the wild type BMK cells expressing both proteins and HCT116 mitochondria, with similar dependence on Ca^{2+} loads (not shown and Fig. 5). TEM micrographs confirmed that the pseudo-absorbance decrease was associated with swelling (Fig. 5*E*). This result indicates that the occurrence of the PT in Bax-less mitochondria cannot be attributed to a substituting function of Bak in PTP formation or regulation. Remarkably, CSP up to 10 μM had little effect on the swelling in either Bax^+/Bak^+ (Fig. 5*C, curve c*) or DKO (Fig. 5*A, curve c*) BMK mitochondria. ADP, Mg^{2+}, and acidification were instead able to prevent swelling (Fig. 5, *A* and *C, curves d–f*).

Taken together, the results of these experiments with suspensions of mitochondria did not support the idea of an involvement of Bax in the PT. However, Bax might interact with other components of the PTP only after becoming integrated into the mitochondrial membrane system in cells committed to apoptosis and act as a regulator of the PT. We therefore proceeded to test mitochondria isolated from Bax-proficient HCT116 cells exposed to etoposide (whose pro-apoptotic effectiveness is decreased in Bax-less HCT116 cells (60) and has been reported to cause Bax translocation and the mitochondrial PT in fibroblasts (61)) and to staurosporine (58). Western blots (not shown) were first performed to evaluate the kinetics of cytochrome *c* release, confirming that this process was well advanced or completed after 2 h with 1 μM staurosporine (62) and after overnight incubation with 50 μg/ml etoposide (61). Mitochondria isolated from cells thus treated did not show significant differences in PT-assessing experiments (not shown and

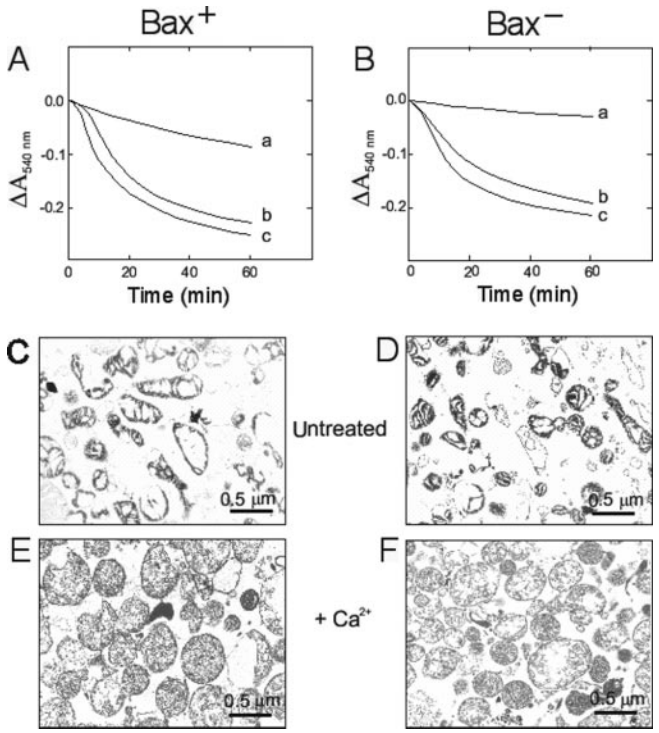

FIG. 3. **Ca^{2+}/P_i-induced swelling.** Bax^+ (*A*, *C*, and *E*) or Bax^- (*B*, *D*, and *F*) HCT116 mitochondria. *A* and *B*, the pseudo-absorbance at 540 nm of parallel incubations of mitochondria in suspension buffer was followed. For *curves b* and *c* the medium was supplemented with 100 or 200 μM $CaCl_2$, respectively. *C–F*, TEM photographs of HCT116 mitochondria untreated (*C* and *D*) or exposed to 200 μM Ca^{2+}/1 mM P_i for 60 min (*E* and *F*).

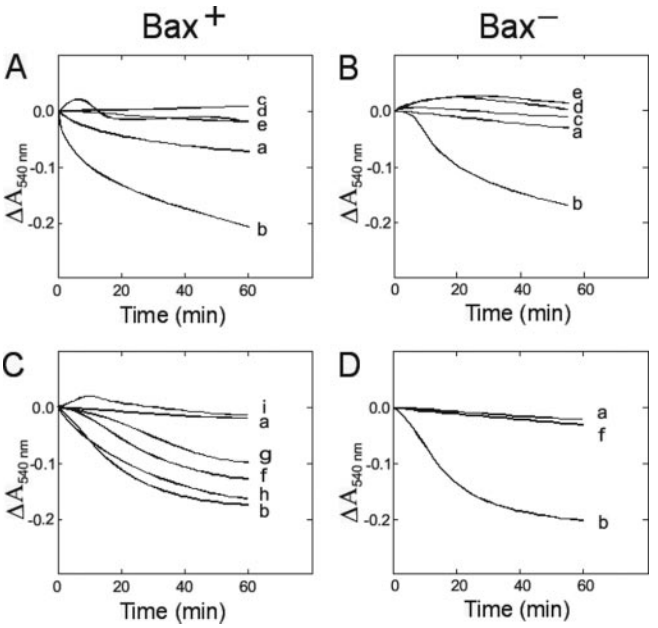

FIG. 4. **PT inhibitors affect Ca^{2+}/P_i-induced swelling of mitochondria regardless of Bax expression.** Representative experiments with Bax^+ (*A* and *C*) or Bax^- (*B* and *D*) HCT116 mitochondria. The pseudo-absorbance curves were recorded after the addition of 1 mg/ml of mitochondrial protein to suspension buffer plus the following: *curve a*, nothing (control); *curve b*, 200 μM $CaCl_2$; *curve c*, 200 μM $CaCl_2$ and 5 mM $MgCl_2$; *curve d*, 200 μM $CaCl_2$ and 2 mM ADP; *curve e*, 200 μM $CaCl_2$, pH 5.5; *curve f*, 200 μM $CaCl_2$ and 5 μM CSP; *curve g*, 200 μM $CaCl_2$ and 10 μM CSP; *curve h*, 200 μM $CaCl_2$ and 250 μM $MgCl_2$; and *curve i*, 200 μM $CaCl_2$, 250 μM $MgCl_2$, and 5 μM CSP. *A* and *B*, Ca^{2+}, Mg^{2+}, ADP, and H^+ have the expected effects regardless of the presence of Bax. *C*, complete inhibition of Ca^{2+}-induced swelling of HCT116 Bax^+ mitochondria by cyclosporin A requires cooperation with a low concentration of another PT inhibitor. *D*, inhibition of Ca^{2+}-induced swelling of HCT116 Bax^- mitochondria can be achieved by cyclosporin A alone.

Fig. 6), even though a large portion of Bax had become an integral membrane protein after induction of apoptosis, as shown by the resistance to alkaline extraction (Fig. 6, *inset, lane 2 versus lane 1*).

We considered the possibility that only part of the mitochondria might have been affected by the apoptotic process and that this portion might not be able to swell (see "Discussion") or might have been selectively lost during isolation. We therefore proceeded to expose isolated Bax^- HCT116 mitochondria to purified 20 nM monomeric Bax + t^c-Bid as described under "Experimental Procedures." Under these conditions Bax became incorporated into the mitochondrial membrane system: alkaline extraction followed by SDS-PAGE of the washed membranes, and Western blotting indicated that it had indeed become in considerable part an integral membrane protein (not shown). Bax + t^c-Bid-treated mitochondria were used for Ca^{2+}-induced swelling experiments in parallel with untreated mitochondria from the same preparations. Fig. 7 shows representative experiments. In each experiment mitochondria were incubated in suspension buffer, in their cuvettes, for 15 min, without additions (Fig. 7, *a, c,* and *c′*) or, in these examples, with 20 nM monomeric Bax and t^c-Bid (Fig. 7, *b*, *d*, and *d′*). No significant variation of light scattering took place during this period (not shown). Swelling was then initiated by the addition of $CaCl_2$ (100 (Fig. 7, *c* and *d*) or 50 μM (Fig. 7*c′* and *d′*)) to two of the samples. Pretreatment with Bax did not result in any significant difference between the pseudo-absorbance curves (n = 7). At the end of the experiment shown in Fig. 7*A*, the mitochondria were recovered, and each sample was divided into 2 aliquots, one of which was subjected to alkaline extraction. The subsequent Western blot (Fig. 7*A, inset*) confirmed that Bax was present as an integral membrane protein throughout the swelling experiment.

DISCUSSION

Our results strongly suggest that Bax and Bak are not involved in the formation or regulation of the PT in calcium- and P_i-loaded mitochondria. The PT takes place in mitochondria isolated from HCT116 or BMK cells regardless of whether the parent cells expressed or not Bax (and Bak for BMK cells). The Ca^{2+} dose dependence, the kinetics of PT spreading throughout the mitochondrial population, and the characteristics of inhibition by a panel of classical PT inhibitors were very similar in the presence and absence of Bax and Bak. These proteins, when expressed by the cells, were present in the mitochondrial fraction and were thus well positioned for interaction with other putative PTP components had this been their mission. Upon induction of apoptosis, Bax became largely an integral membrane protein, but the properties of the PT did not change (Fig. 6). No alteration of the control behavior was observed even when exogenous Bax was incorporated *in vitro* into isolated mitochondria (Fig. 7). In these latter experiments the amount of Bax used (20 nM, *i.e.* 20 pmol/mg of mitochondrial protein, ~0.4 μg/mg of mitochondrial protein) was close to the physiological range (63) (treatment of mitochondria with 0.25 or 0.5 μM Bax in a Ca^{2+}-free medium did result in a slow cyclosporin A-sensitive swelling, in partial agreement with some reports in the literature (27, 34, 45, 64) (data not shown)). The lack, or extreme scarcity (Fig. 1) (40, 41), of Bax in rat liver and brain mitochondria also supports the conclusion that this protein is irrelevant for the Ca^{2+}/P_i-induced permeability transition. The same conclusion is suggested by recent work (65) showing that Bax^-/Bak^- mouse embryonic fibroblast cells are still capable of undergoing CSP-inhibitable mitochondrial de-

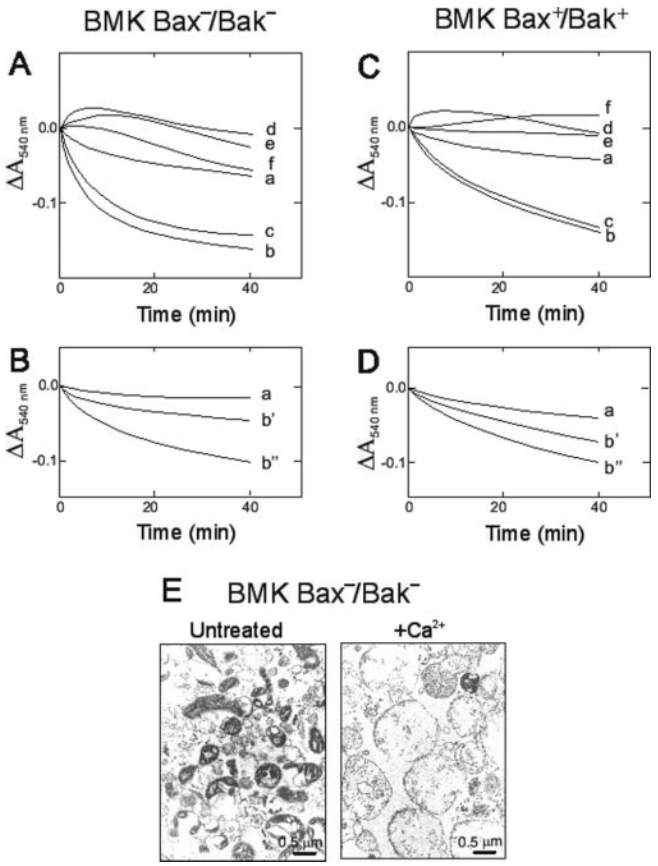

FIG. 5. **DKO (Bax^-, Bak^-) BMK mitochondria undergo the Ca^{2+}/P_i-induced permeability transition.** *A–D*, light scatter (swelling) curves as in Figs. 3 and 4. *A*, *B*, and *E*, mitochondria isolated from Bax- and Bak-less BMK cells. *C* and *D*, mitochondria from wild type BMK cells. *Curve a*, control (no addition); *curves b–f*, in the presence of 100 μM $CaCl_2$, plus the following: *curve c*, 5 μM CSP; *curve d*, pH 5.5; *curve e*, 5 mM $MgCl_2$; *curve f*, 6 mM ADP; *curve b′*, $CaCl_2$ was 25 μM; *b″*, $CaCl_2$ was 50 μM. *E*, TEM photographs of BMK DKO mitochondria untreated or exposed to 100 μM Ca^{2+}/1 mM P_i for 60 min.

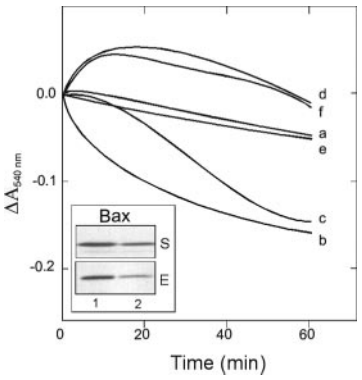

FIG. 6. **Swelling experiments fail to reveal differences between mitochondria isolated from untreated and pre-apoptotic HCT116 Bax^+ cells.** Light scattering traces from representative experiments with mitochondria isolated from HCT116 Bax^+ cells exposed to 1 μM staurosporine for 2 h. Conditions as in Figs. 3–5. *Trace a*, control (no addition). *Traces b–f*, in the presence of 200 μM $CaCl_2$ plus the following: *c*, 5 μM CSP; *d*, pH 5.5; *e*, 5 mM $MgCl_2$; *f*, 2 mM ADP. *Inset*, Bax is present as an integral membrane protein in mitochondria isolated from Bax^+ cells exposed to 1 μM staurosporine (*S*) for 2 h or to 50 μg/ml etoposide (*E*) overnight. *Lane 1*, untreated mitochondria. *Lane 2*, mitochondria subjected to alkaline extraction. 50 μg of protein were loaded on the SDS-polyacrylamide gel in each case.

polarization and apoptotic death upon exposure to H_2O_2, a process presumably involving induction of the PT by oxygen radicals.

The experimental approaches used in this paper (the classical tools for studies on the PT) provide information on the activation of sucrose-permeable pores spanning the mitochondrial inner membrane but no details about their biophysical characteristics. The possibility thus remains that the properties of these pores, when considered as individual channels, might differ depending on the presence of Bax. This point will be addressed in another paper.[3]

Bax might interact with other proteins to form or regulate the PTP only upon induction of the apoptotic process. As already mentioned, we addressed this question by performing experiments with mitochondria isolated from cells exposed to etoposide or staurosporine, which did not exhibit any peculiarity. This outcome calls for comment. For obvious reasons, isolation of the mitochondria was performed after cytochrome *c* release had started (however, in our hands the mitochondrial fraction retained a considerable portion of cytochrome *c* even at the longest exposure times we tested). Mitochondria that had lost all or nearly all of their cytochrome *c* would not be expected to respire, maintain a potential, and take up Ca^{2+} in the subsequent PT-assessing experiments. The results of our experiments might therefore reflect mostly the properties of a fraction of mitochondria not yet affected by the apoptotic process and therefore still cytochrome *c*-rich (whereas another fraction, completely depleted of cytochrome *c*, would not have been able to swell). Alternatively, most mitochondria might have retained a sufficient amount of cytochrome *c* to respire and maintain a potential even though they had entered the apoptotic chain of events. Cytochrome *c* in the periplasmic space is in vast excess over the needs of the respiratory chain, as shown by determinations of respiration control coefficients (66, 67) and experiments with permeabilized cells (68). A partial release, even if substantial, would therefore have a limited effect on the ability to generate a transmembrane potential. In agreement with the second hypothesis, Mootha *et al.* (69) concluded that mitochondria isolated from hepatocytes activated through Fas retained most of their cytochrome *c* even though their outer membrane had become permeable to it. Other authors (63, 64, 70–72) have concluded that cytochrome *c* is largely and rapidly lost by a fraction of the mitochondria of a pre-apoptotic cell population, *i.e.* there is heterogeneity in the apoptotic response. In patch clamp experiments, performed on nonrespiring, depolarized mitoplasts, the complications mentioned above do not apply. Unpublished experiments[3] of this type concur with swelling experiments in suggesting that application of pre-apoptotic stimuli does not alter the frequency of observation and properties of the PTP.

Whereas essentially Bax-independent, the PT exhibited by the mitochondria from these cell lines differed in some quantitative respects from that of RLM. Inducing the PT required higher amounts of Ca^{2+} to be taken up. Thus, in the experiments reported in Fig. 2 of Ref. 55, the PT occurred after the RLM had taken up some 50 nmol of Ca^{2+}/mg of protein and *versus*, on average, 200 nmol/mg of protein in our analogous experiments. At comparable Ca^{2+} concentrations, swelling propagated in the suspension more slowly in the case of HCT116 and BMK mitochondria than in RLM, with a difference of up to an order of magnitude in the time required for the phenomenon to reach completion (compare, for example, Fig. 3 above with Fig. 3 in Ref. 73). The propensity of mitochondria to undergo the PT is known to depend on the cell or organ of origin as well as on factors such as the age of the preparation or the substrate being oxidized by the respiratory chain. Although we

[3] S. Campello, U. De Marchi, I. Szabò, F. Tombola, J.-C. Martinou, and M. Zoratti, manuscript in preparation.

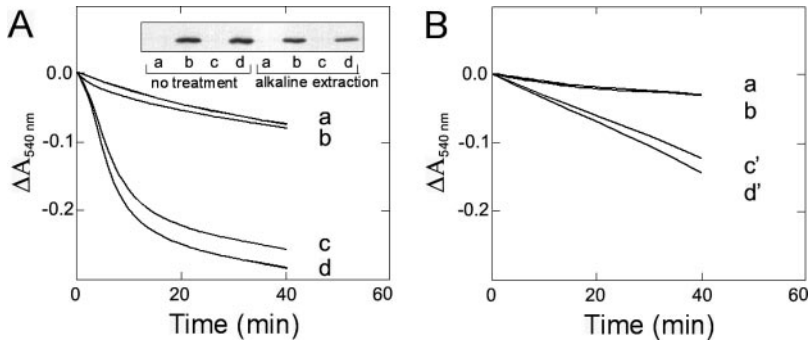

FIG. 7. **Incorporation of exogenous Bax into the membranes of HCT116 Bax^- mitochondria does not affect their swelling response to Ca^{2+}/P_i.** Representative swelling experiments with HCT116 Bax^- mitochondria exposed (*traces b*, *d*, and *d′*) or not (*traces a*, *c*, and *c′*) to Bax as detailed under "Experimental Procedures" and text. 100 (*A*, *traces c* and *d*) or 50 μM $CaCl_2$ (*B*, *traces c′* and *d′*) was added to two of the four cuvettes in each experiment after a 15-min monitoring period, thus initiating swelling. *A*, *inset*, Western blot of the mitochondria recovered from the experiment showing that Bax had largely become an integral membrane protein. 50 μg of total protein from each sample were loaded onto the SDS-polyacrylamide gel for Western blotting. The *same letter* identifies corresponding swelling traces and blot lanes.

are not aware of an in-depth study on the matter, this variability is often ascribed to different contents of PTP-antagonizing molecules or ions such as ADP or to different redox levels of NADH and glutathione pools. Mitochondria from tumors or cancer-derived cell lines seem to be particularly resistant, a characteristic that may depend on various factors, including an elevated content of Mg^{2+} (74) or a high expression of anti-apoptotic proteins such as Bcl-2 (75). In our case the latter reason may apply, because Bcl-x_L is present at much higher levels in HCT116 mitochondria than in RLM (Fig. 1*A*), and Bcl-2 and Bcl-x_L inhibit the permeability transition.

Although quantitative comparisons are made difficult by the complex interactions between the various protective and inducing factors, prevention of the PT in HCT116 mitochondria also seemed to require somewhat higher concentrations of inhibitors than in the case of RLM perhaps, at least in part, because of the high concentrations of Ca^{2+} used to induce the PT in the first place (Ca^{2+} and various inhibitors display a competitive-type behavior (76)). This difficulty of inhibition parallels the difficulty of induction noted above. The inability of CSP, used by itself, to completely inhibit swelling of Bax^+ mitochondria was the only characteristic we could identify that distinguished them from Bax^- organelles (Fig. 4, *C* and *D*). As already mentioned, this difference did not emerge in Ca^{2+} retention experiments, and the presence of a low concentration of another inhibitor, having by itself only a small effect on swelling, was sufficient for full inhibition (Fig. 4*C*). This is an example of synergy among inhibitors, an often-described behavior exhibited particularly by CSP (19). Ca^{2+}-induced swelling of BMK mitochondria is hardly inhibited by CSP at all, irrespective of the expression of Bax and Bak (Fig. 6). Although it is considered to be a classical inhibitor of the PT, cyclosporin is notoriously fickle, and it has been reported to be ineffective under various circumstances, such as high Ca^{2+} loads, loss of endogenous coadjuvants such as ADP or Mg^{2+}, and possibly the oxidation of relevant residues in the PTP complex or the presence of free fatty acids in the membrane (19, 77). The incompleteness of CSP inhibition in Bax^+ mitochondria might be due to a Bax-linked, but collateral, characteristic of the organelles or it might signal an interference by Bax in the interactions between the membrane pore complex and cyclophilin D. When viewed in the general context of the results, however, this appears in any case to be a secondary effect.

In conclusion, our investigation strongly suggests that Bax and Bak play no direct role in the induction and/or modulation of the Ca^{2+}-induced PT. The PT observed in mitochondria isolated from unchallenged HCT116 Bax-deficient cells and DKO BMK cells was indistinguishable from that of the counterparts expressing these proteins. No relevant difference was found in comparison with the phenomena exhibited by organelles obtained from pre-apoptotic cells or with the behavior of mitochondria into whose membrane system reasonable amounts of oligomeric exogenous Bax had been introduced.

The interaction of Bax with the adenine nucleotide translocator and its relevance for PT induction by atractyloside have been documented (23, 24). It now emerges that the necessity for Bax is restricted to that particular mode of PT induction, differentiating it from the reactive oxygen species-, SH reagent-, and Ca^{2+}/P_i-induced PT variants, which are more likely to be relevant for apoptotic cell death. The possibility remains to be explored that in intact cells Bax migration to mitochondria may induce the PT indirectly, rather than by molecular interactions with PTPC components.

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REFERENCES

1. Hengartner, M. O. (2000) *Nature* **407,** 770–776
2. Kroemer, G., and Reed, J. C. (2000) *Nat. Med.* **6,** 513–519
3. Desagher, S., and Martinou, J. C. (2000) *Trends Cell Biol.* **10,** 369–377
4. Martinou, J. C., and Green, D. R. (2001) *Nat. Rev. Mol. Cell. Bio.* **2,** 63–67
5. Newmeyer, D. D., and Ferguson-Miller, S. (2003) *Cell* **112,** 481–490
6. Basanez, G., Nechustan, A., Drozhinin, O., Chanturiya, A., Choc, E., Tutt, S., Wood, K. A., Hsu, Y., Zimmerberg, J., and Youle, R. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5492–5497
7. Basanez, G., Sharpe, J. C., Galanis, J., Brandt, T. B., Hardwick, J. M., and Zimmerberg, J. (2002) *J. Biol. Chem.* **277,** 49360–49365
8. Priault, M., Bessoule, J. J., Grelaud-Coq, A., Camougrand, N., and Manon, S. (2002) *Eur. J. Biochem.* **269,** 5440–5450
9. Petit, P. X., Susin, S. A., Zamzami, N., Mignotte, B., and Kroemer, G. (1996) *FEBS Lett.* **396,** 7–13
10. Kroemer, G., Zamzami, N., and Susin, S. A. (1997) *Immunol. Today* **18,** 44–51
11. Kroemer, G., Dallaporta, B., and Resche Rigon, M. (1998) *Annu. Rev. Physiol.* **60,** 619–642
12. Lemasters, J. J., Nieminen, A. L., Qian, T., Trost, L. C., Elmore, S. P., Nishimura, Y., Crowe, R. A., Cascio, W. E., Bradham, C. A., Brenner, D. A., and Herman, B. (1998) *Biochim. Biophys. Acta* **1366,** 177–196
13. Ichas, F., and Mazat, J.-P. (1998) *Biochim. Biophys. Acta* **1366,** 33–50
14. Crompton, M. (1999) *Biochem. J.* **341,** 233–249
15. Zamzami, N., and Kroemer, G. (2001) *Nat. Rev. Mol. Cell. Bio.* **2,** 67–71
16. Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003) *Nat. Rev. Mol. Cell. Bio.* **4,** 552–565
17. Borutaite, V., and Brown, G. C. (2003) *FEBS Lett.* **541,** 1–5
18. Gunter, T. E., and Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258,** C755–C786
19. Zoratti, M., and Szabò, I. (1995) *Biochim. Biophys. Acta* **1241,** 139–176
20. Bernardi, P. (1999) *Physiol. Rev.* **79,** 1127–1155
21. Crompton, M., Virji, S., and Ward, J. M. (1998) *Eur. J. Biochem.* **258,** 729–735
22. Crompton, M., Barksby, E., Johnson, N., and Capano, M. (2002) *Biochimie (Paris)* **84,** 143–152
23. Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C., and Kroemer, G. (1998) *Science* **281,** 2027–2031
24. Brenner, C., Cadiou, H., Vieira, H. L. A., Zamzami, N., Marzo, I., Xie, Z., Leber, B., Andrews, D., Duclohier, H., Reed, J. C., and Kroemer, G. (2000) *Onco-*

gene **19,** 329–336
25. Belzacq, A.-S., Vieira, H. L. A., Kroemer, G., and Brenner, C. (2002) *Biochimie* (*Paris*) **84,** 167–176
26. Belzacq, A. S., Vieira, H. L., Verrier, F. C., Cohen, I., Prevost, M., Larquet, E., Pariselli, F., Petit, P. X., Kahn, A., Rizzuto, R., Brenner, C., and Kroemer, G. (2003) *Cancer Res.* **63,** 541–546
27. Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H., and Tsujimoto, Y. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 14681–14686
28. Shi, Y., Chen, J. J., Weng, C. J., Chen, R., Zheng, Y. H., Chen, Q., and Tang, H. (2003) *Biochem. Biophys. Res. Commun.* **305,** 989–996
29. Tsujimoto, Y., and Shimizu, S. (2002) *Biochimie* (*Paris*) **84,** 187–193
30. Marzo, I., Brenner, C., Zamzami, N., Susin, S. A., Beutner, G., Brdiczka, D., Rémy, R., Xie, Z.-H., Reed, J. C., and Kroemer, G. (1998) *J. Exp. Med.* **187,** 1261–1271
31. Vander Heiden, M. G., Li, X. X., Gottlieb, E., Hill, R. B., Thompson, C. B., and Colombini, M. (2001) *J. Biol. Chem.* **276,** 19414–19419
32. He, L., Perkins, G. A., Poblenz, A. T., Harris, J. B., Hung, M., Ellisman, M. H., and Fox, D. A. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 1022–1027
33. Pastorino, J. G., Chen, S.-T., Tafani, M., Snyder, J. W., and Farber, J. L. (1998) *J. Biol. Chem.* **273,** 7770–7775
34. Pastorino, J. G., Tafani, M., Rothman, R. J., Marcinevicicute, A., Hoek, J. B., and Farber, J. L. (1999) *J. Biol. Chem.* **274,** 31734–31739
35. Pastorino, J. G., Shulga, N., and Hoek, J. B. (2002) *J. Biol. Chem.* **277,** 7610–7618
36. Tafani, M., Karpinich, N., Hurster, K. A., Pastorino, J. G., Schneider, T., Russo, M. A., and Farber, J. L. (2002) *J. Biol. Chem.* **277,** 10073–10082
37. Zamzami, N., El-Hamel, C., Maisse, C., Brenner, C., Munoz-Pinedo, C., Belzacq, A. S., Costantini, P., Vieira, H., Loeffler, M., Molle, G., and Kroemer, G. (2000) *Oncogene* **14,** 6342–6350
38. Susin, S. A., Zamzami, N., and Kroemer, G. (1998) *Biochim. Biophys. Acta* **1366,** 151–165
39. Marzo, I., Brenner, C., and Kroemer, G. (1998) *Biomed. Pharmacother.* **52,** 248–251
40. Polster, B. M., Kinnally, K. W., and Fiskum, G. (2001) *J. Biol. Chem.* **276,** 37887–37894
41. Brustovetsky, N., Dubinsky, J. M., Antonsson, B., and Jemmerson, R. (2003) *J. Neurochem.* **84,** 196–207
42. Doran, E., and Halestrap, A. P. (2000) *Biochem. J.* **348,** 343–350
43. Appaix, F., Guerrero, K., Rampal, D., Izikki, M., Kaambre, T., Sikk, P., Brdiczka, D., Riva-Lavieille, C., Olivares, J., Longuet, M., Antonsson, B., and Saks, V. A. (2002) *Biochim. Biophys. Acta* **1556,** 155–167
44. Wu, C. H., Rastegar, M., Gordon, J., and Safa, A. R. (2001) *Oncogene* **20,** 7006–7020
45. Jürgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D., and Reed, J. C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4997–5002
46. Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. (2000) *Science* **290,** 989–992
47. Mikhailov, V., Mikhailova, M., Degenhardt, K., Venkatachalam, M. A., White, E., and Saikumar, P. (2003) *J. Biol. Chem.* **278,** 5367–5376
48. Cheng, E. H., Sheiko, T. V., Fisher, J. K., Craigen, W. J., and Korsmeyer, S. J. (2003) *Science* **301,** 513–517
49. Degenhardt, K., Sundararajan, R., Lindsten, T., Thompson, C., and White, E. (2002) *J. Biol. Chem.* **277,** 14127–14134
50. Deng, Y., Lin, Y., and Wu, X. (2002) *Genes Dev.* **16,** 33–45
51. Montessuit, S., Mazzei, G., Magnenat, E., and Antonsson, B. (1999) *Protein Expression Purif.* **15,** 202–206
52. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999) *J. Cell Biol.* **144,** 891–901
53. Kudla, G., Montessuit, S., Eskes, R., Berrier, C., Martinou, J. C., Ghazi, A., and Antonnson, B. (2000) *J. Biol. Chem.* **275,** 22713–22718
54. Ciman, M., Rascio, N., Pozza, D., and Sartorelli, L. (1992) *Neurosci. Res. Commun.* **11,** 87–92
55. Kamo, N., Muratsugu, R., Hongoh, R., and Kobatake, Y. (1979) *J. Membr. Biol.* **49,** 105–121
56. Fontaine, E., Ichas, F., and Bernardi, P. (1998) *J. Biol. Chem.* **273,** 25734–25740
57. Yamaguchi, H., Bhalla, K., and Wang, H. G. (2003) *Cancer Res.* **63,** 1483–1489
58. Theodorakis, P., Lomonosova, E., and Chinnadurai, G. (2002) *Cancer Res.* **62,** 3373–3376
59. He, Q., Montalbano, J., Corcoran, C., Jin, W., Huang, Y., and Sheikh, M. S. (2003) *Oncogene* **22,** 2674–2679
60. LeBlanc, H., Lawrence, D., Varfolomeev, E., Totpal, K., Morlan, J., Schow, P., Fong, S., Schwall, R., Sinicropi, D., and Ashkenazi, A. (2002) *Nat. Med.* **8,** 274–281
61. Karpinich, N. O., Tafani, M., Rothman, R. J., Russo, M. A., and Farber, J. L. (2002) *J. Biol. Chem.* **277,** 16547–16552
62. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) *Science* **275,** 1129–1132
63. Polster, B. M., Basanez, G., Young, M., Suzuki, M., and Fiskum, G. (2003) *J. Neurosci.* **23,** 2735–2743
64. von Ahsen, O., Renken, C., Perkins, G., Kluck, R. M., Bossy-Wetzel, E., and Newmeyer, D. D. (2000) *J. Cell Biol.* **150,** 1027–1036
65. Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) *Science* **300,** 135–139
66. Korzeniewski, B., and Froncisz, W. (1992) *Biochim. Biophys. Acta* **1102,** 67–75
67. Moreno-Sanchez, R., Devars, S., Lopez-Gomez, F., Uribe, A., and Corona, N. (1991) *Biochim. Biophys. Acta* **1060,** 284–292
68. Waterhouse, N. J., Goldstein, J. C., von Ahsen, O., Schuler, M., Newmeyer, D. D., and Green, D. R. (2001) *J. Cell Biol.* **153,** 319–328
69. Mootha, V. K., Wei, M. C., Buttle, K. F., Scorrano, L., Panoutsakopoulou, V., Mannella, C. A., and Korsmeyer, S. J. (2001) *EMBO J.* **20,** 661–671
70. Goldstein, J. C., Waterhouse, N. J., Juin, P., Evan, G. I., and Green, D. R. (2000) *Nat. Cell Biol.* **2,** 156–162
71. Krysko, D. V., Roels, F., Leybaert, L., and D'Herde, K. (2001) *J. Histochem. Cytochem.* **49,** 1277–1284
72. D'Herde, K., De Prest, B., Mussche, S., Schotte, P., Beyaert, R., Coster, R. V., and Roels, F. (2000) *Cell Death Differ.* **7,** 331–337
73. Petronilli, V., Cola, C., Massari, S., Colonna, R., and Bernardi, P. (1993) *J. Biol. Chem.* **268,** 21939–21945
74. Saris, N. E., Teplova, V. V., Azarashvili, T. S., Evtodienko, Y. V., and Virtanen, I. (1998) *Magnes. Res.* **11,** 155–160
75. Evtodienko, Y. V., Teplova, V. V., Azarashvily, T. S., Kudin, A., Prusakova, O., Virtanen, I., and Saris, N. E. (1999) *Mol. Cell. Biochem.* **194,** 251–256
76. Szabò, I., Bernardi, P., and Zoratti, M. (1992) *J. Biol. Chem.* **267,** 2940–2946
77. Brustovetsky, N., and Dubinsky, J. M. (2000) *J. Neurosci.* **20,** 8229–8237