BAX supports the mitochondrial network, promoting bioenergetics in nonapoptotic cells

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BAX supports the mitochondrial network, promoting bioenergetics in nonapoptotic cells. Am J Physiol Cell Physiol 300: C1466–C1478, 2011. First published February 2, 2011; doi:10.1152/ajpcell.00325.2010.—The dual functionality of the tumor suppressor BAX is implied by the nonapoptotic functions of other members of the BCL-2 family. To explore this, mitochondrial metabolism was examined in BAX-deficient HCT-116 cells as well as primary hepatocytes from BAX-deficient mice. Although mitochondrial density and mitochondrial DNA content were the same in BAX-containing and BAX-deficient cells, MitoTracker staining patterns differed, suggesting the existence of BAX-dependent functional differences in mitochondrial physiology. Oxygen consumption and cellular ATP levels were reduced in BAX-deficient cells, while glycolysis was increased. These results suggested that cells lacking BAX have a deficiency in the ability to generate ATP through cellular respiration. This conclusion was supported by detection of reduced citrate synthase activity in BAX-deficient cells. In nonapoptotic cells, a portion of BAX associated with mitochondria and a sequestered, protease-resistant form was detected. Inhibition of BAX with small interfering RNAs reduced intracellular ATP content in BAX-containing cells. Expression of either full-length or COOH-terminal-truncated BAX in BAX-deficient cells rescued ATP synthesis and oxygen consumption and reduced glycolytic activity, suggesting that this metabolic function of BAX was not dependent upon its COOH-terminal helix. Expression of BCL-2 in BAX-containing cells resulted in a subsequent loss of ATP measured, implying that, even under nonapoptotic conditions, an antagonistic interaction exists between the two proteins. These findings infer that a basal amount of BAX is necessary to maintain energy production via aerobic respiration.

AN UNDERSTANDING of the apoptotic process has revealed that many of the important mediators, such as cytochrome c, can have dual roles: a nonapoptotic function and an apoptotic function (21). The nonapoptotic roles of such proteins can range from contributing to energy production (21) to maintaining pH homeostasis (8). During apoptosis, these proteins’ housekeeping functions are subverted, committing the cell to a death program. When first discovered, the members of the B-cell lymphoma (BCL-2) family of apoptotic modulators were presented as regulators of cell death (3), and the detrimental effects upon their deletion were attributed to loss of their apoptotic activity (17, 32). While these proteins remain critical effectors of apoptosis, recent findings suggest that functional duality may also exist for multiple BCL-2 proteins.

Conventionally, the BCL-2 family is segregated into two subgroups: pro- and antiapoptotic proteins. Each of the proteins in this family shares at least one and up to four BCL-2 homology domains (BH1–4) (4, 37). The antiapoptotic group includes such proteins as BCL-2, BCLXL, and MCL-1. Proapoptotic proteins include the multidomain proteins BAX and Bak and the BH3-only proteins BIM, BAD, and BID (16). In the mitochondrial BH3 domain, arrange to form a prominent hydrophobic groove. In its cytosolic state the COOH-terminal α9-helix of BAX, a putative transmembrane domain, occupies this hydrophobic groove (27). Although the apoptotic roles of these BCL-2 family members are the focus of much research, only a small number of nonapoptotic functions have been described. The BH3-only protein BAD was reported to complex with hexokinase 4 in order to regulate intracellular glucose metabolism in nonapoptotic hepatocytes (5). BCL-2 was shown to modulate cell cycle progression (19, 31). BAX was found to affect the production of reactive oxygen species (ROS) by the mitochondria in nonapoptotic neurons (13), and the absence of BAX and Bak in T cells altered Ca2+ release, resulting in a defect in antigen-specific proliferation (10). These findings are intriguing and suggest that possible nonapoptotic activities of the BCL-2 family merit further examination.

Current studies on the apoptotic activity of BAX revealed many interesting features that enable the protein to transition from a soluble, cytosolic form to a membrane-bound form. But do these same features enable BAX to have a nonapoptotic function? While BAX is known as a tumor suppressor, it is mutated but rarely completely ablated in certain types of tumors (2), suggesting that the complete loss of all function could be detrimental. As an example, HCT-116 colorectal cells were treated with a mutagen to produce cells deficient in BAX, with the result that only 4% of the cells had two mutant BAX alleles (−/−) while 94% mutated one allele (+/−) (39). Studies of BAX-deficient mice revealed conflicting results: the loss of BAX produced either hyperplasia or hypoplasia in a tissue-specific manner (14). These data indicate that BAX may have an unrecognized activity in healthy, normal cells. To study this, we examined mitochondrial bioenergetics in BAX-containing (BAX+/+) and BAX-deficient (BAX−/−) HCT-116 cells as well as hepatocytes from BAX-deficient or wild-type mice. We found that in BAX−/− cells intracellular ATP and aerobic respiration were significantly reduced, while glycolysis was increased. We inferred that a small amount of mitochondrion-associated BAX was needed to support cellular respiration. We thus describe a novel nonapoptotic activity of BAX that sustains mitochondrial metabolism and could mitigate the complete loss of the protein by a tumor cell.
MATERIALS AND METHODS

Cell lines and reagents. BAX−/− and BAX−/−/HCT-116 colorectal cancer cell lines (a kind gift from Dr. Bert Vogelstein, John Hopkins University) were grown and maintained in McCoy’s 5A Medium (GIBCO), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, Sigma) was used at a concentration of 5 μM as described below. This was determined to be the optimal concentration after performance of a titration experiment (FCCP 0–25 μM) and measurement of effects on cell viability and ATP production as described below.

Isolation of primary mouse hepatocytes. The animal use protocol used in this study was approved by the University of Central Florida Institutional Animal Care and Use Committee (IACUC) and conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996). Hepatocytes were isolated from the livers of adult BAX knockout (KO) and wild-type (WT) C57BL/6 mice (Jackson Labs). The livers were perfused with Ca2+−free Krebs buffer by insertion of a cannula into the hepatic portal vein, severing the inferior vena cava, and injection of buffer to flush red blood cells from the liver. The buffer was injected at a constant flow rate of 700 μl/min for 10 min with a syringe pump (ColeParmer). After 10-min perfusion, the caudate lobe was removed for sectioning and the livers were then perfused with PBS-5% collagenase (MPBiomedicals) for 10 min or until the tissue became spongy. The isolated tissues were then run through a 0.2-μm cell strainer (BD Biosciences) with ice-cold Krebs buffer, and residual red blood cells were eliminated by osmotic lysis. The cells were then washed three times in Krebs buffer to remove any residual collagenase. Isolated hepatocytes were resuspended in DMEM, 10% FBS, 1% penicillin-streptomycin and assayed immediately.

Plasmids, mutagenesis, and transfection. PCR-directed deletion of the COOH terminus of BAX to generate the COOH-terminal-truncated form of BAX (BAX-DCT) was performed with untagged primer sets and was confirmed by sequencing. BAX was PCR amplified from the template pEGFP-BAX (a gift from Dr. Richard Youle, National Institutes of Neurological Disorders and Stroke, National Institutes of Health). To examine the transient expression of both the full-length (BAX-FL) and BAX-DCT recombinant BAX constructs, bicistronic ProToScreen vectors (CloneTech) containing BAX insert and green fluorescent protein (GFP) separated by an internal ribosome entry site (IRES) were transfected into BAX−/−/HCT-116 cells. Expression of the BAX construct was induced upon the addition of Shield (CloneTech) at a concentration of 1 μM. The plasmid DNA was delivered at a concentration of 1 μg/ml with the TransIT-LT1 transfection reagent (Mirus) according to the manufacturer’s protocol. Transfection efficiency was determined microscopically by visualizing GFP expression after 18 h and ranged from 50% to 70%. Experiments were performed within defined time frames to ensure optimal cell viability, before any induction of apoptosis.

Measurement of oxygen consumption and extracellular acidification rates. BAX−/− and BAX−/−/HCT-116 cells, as well as isolated hepatocytes, were cultured in a BD Oxygen Biosensor plate at a concentration of 40,000 cells/well in 1% penicillin-streptomycin, 10% fetal bovine serum, and 20 mM HEPES. The cells were then washed three times in PBS to remove any residual collagenase. Isolated hepatocytes were resuspended in DMEM, 10% FBS, 1% penicillin-streptomycin and assayed immediately.

BAX-FL and BAX-DCT BAX constructs were transfected into BAX−/− cells for measurement as well. The transfected cells were treated with Shield 3 h before reading to induce expression of the protein. Measurements were taken on the Seahorse XF24 plate reader (Seahorse Bioscience). Values were normalized as changes relative to the initial reading.

Mitochondrial staining: fixed imaging, live cell imaging, and tissue sectioning. For fixed cells, BAX−/−/HCT-116 cells were plated onto coverslips coated with 10 μg/ml laminin (Invitrogen). Cells were plated at a density of 50,000 cells/well and were fixed by methanol fixation. The cells were then probed with anti-BAX (N-20; Santa Cruz) and anti-heat shock protein (HSP)60 (H-300; Santa Cruz) antibodies, followed by FITC and Cy3 secondary antibodies. Images were scanned with the LSM 510 (Zeiss) with a ×100/1.4 plan-apochromat objective. The scanned image was processed with Zen 2009 software (Zeiss). For live cell imaging, BAX−/− and BAX−/−/HCT-116 cells were grown and plated in 24-well glass-bottom dishes (MatTek) that had been pretreated with 1 N HCl and coated with 10 μg/ml laminin. The cells were plated at 10,000 cells/well and allowed to grow overnight. BAX-FL or BAX-DCT constructs were transfected with the TransIT-LT1 transfection reagent (Mirus) and expressed as described above. At 18 h after transfection, cells were incubated with 1 μM MitoTracker Red 580 or 5 μM 10-nonyl acridine orange (NAO) (Molecular Probes) in McCoy’s complete medium for 30 min before imaging. Treatments with 5 μM FCCP were done 15 min before imaging.

The caudate lobes of BAX KO and C57BL/6 mice were frozen and sectioned with a Leica CM 1850 cryostat. Tissues were cut into 12-μm sections. The sections were rehydrated with PBS and then stained with either 1 μM MitoTracker Red or 5 μM NAO.

Fluorescent images were acquired with the UltraView spinning disk confocal system (PerkinElmer) with an AxioObserver.Z1 (Carl Zeiss) stand and a Plan-Apochromat 63/1.4 Oil differential interference contrast (DIC) objective. Z-stack projections of the scanned images were generated and modified within the Volocity image processing program (PerkinElmer).

Subcellular fractionation and immunoblotting. BAX−/− and BAX−/−/HCT-116 cells were plated in 75-cm² flasks and grown to 70–80% confluency. Cells were transiently transfected with the TransIT-LT1 transfection reagent (Mirus) and expressed as described above. At 18 h after transfection, the cells were lifted and pelleted. The pellets were resuspended and lysed according to the protocol from the Mitochondrial Enrichment Kit (Pierce). It should be noted that a low-speed centrifugation step in the mitochondrial enrichment process ensures that only intact and unfragmented mitochondria are isolated. The enriched mitochondria were layered on an iodixanol gradient (6%, 10%, 15%, 20%, 23%, and 27%) and subjected to ultracentrifugation with an Optima L-100XP Ultracentrifuge for 2 h at 145,000 relative centrifugal force (rcf) in a SW55.1 swing bucket rotor (Beckman Coulter). After centrifugation, each sample was unloaded in 500-μl fractions with a fraction recovery system (Beckman Coulter) and washed in ice-cold PBS twice (18,000 rcf, 30 min) to remove residual iodixanol. The pellets from each of these fractions were resuspended in 1X loading buffer and run on 8–16% Tris-glycine gradient gels (Invitrogen). The gels were transferred to polyvinylidene difluoride (PVDF) membranes and probed with primary antibodies to BAX (N-20; Santa Cruz), prohibitin (Abcam), and GRP78 (Santa Cruz), incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz, Cell Signaling), and developed by chemiluminescence (Pierce).

Proteinase K digestion of membrane-bound proteins. BAX−/− and BAX−/−/HCT-116 mitochondria were isolated as described above. The mitochondria were then treated with proteinase K (Sigma) (10 μg/ml) for 5, 10, 15, or 20 min in order for the outer mitochondrial membrane (OMM)-associated proteins to be digested. The reaction was terminated with PMSF (12). The mitochondria were then washed with isotonic buffer, pelleted, resuspended in 1X sample buffer, and
run on 12% acrylamide gels in parallel with untreated mitochondria. The gels were transferred to PVDF membranes, which were then probed with BAX (N-20; Santa Cruz), as well as Beclin (2762, Cell Signaling) primary antibodies, followed by incubation with anti-mouse or anti-rabbit IRDye 800CW secondary antibodies (Licor). The membranes were imaged with the Licor Odyssey Infrared Imaging system. Densitometry measurements were made with ImageJ.

**Measurement of ATP production and mitochondrial membrane potential.** Cells were seeded and treated in 24-well plates overnight. The cells were then lifted, counted, and seeded in black 96-well flat-bottom plates at a density of 4,000−5,000 cells/well. To uncouple oxidative phosphorylation, 5 μM FCCP was added to the cells 2 h before analysis of ATP concentration. ATP levels were quantified with the ATPLite 1-Step assay kit (PerkinElmer). Luciferase activity, reported as relative luminescence units (RLU), was measured on an EnVision (PerkinElmer) plate reader. The luminescence was normalized to the number of cells per well. Additionally, a standard curve of known ATP concentrations was established to ensure that the experimental values were within an accurate range. Background signal was corrected for by subtracting the values of an empty plate. In addition, as the fluorescence of MitoTracker Red 580 (Invitrogen) is a result of oxidation of the compound within the mitochondrial matrix, changes in MitoTracker fluorescence intensity were used to assess the qualitative and quantitative efficiency of the electrochemical potential (the relative oxidative capability of actively respiring mitochondria) (23).

Measurements of mitochondrial content, membrane potential, and viability were done by staining the cells with both ethidium bromide (EtBr; Fisher Scientific) and NAO (6). Briefly, 500,000 cells/sample were stained with 25 ng/ml NAO and 40 ng/ml EtBr for 10 min. The cells were then washed three times with 0.1% BSA in PBS at 4°C for 10 min at 1,000 g. The cells were read with the C6 flow cytometer (Accuri) using the FL1 and FL3 channels. Data were analyzed with FCExpress (DeNovo).

**Measurement of citrate synthase activity.** Isolated mitochondria from BAX+/+ and BAX−/− HCT-116 cells as well as primary hepatocytes from BAX KO and C57BL/6 mice were assayed for citrate synthase activity. The enzyme citrate synthase catalyzes the reaction:

\[
\text{CoA-SH} + \text{DTNB} \rightarrow \text{TNB} + \text{CoA-S-S-TNB}
\]

where DTNB is dithionitrobenzoic acid and TNB is thionitrobenzoic acid. The rate of TNB formation is linear and has a measurable absorbance at 412 nm. The rate of TNB generation (MTATP)8 gene were as follows:

\[
\Delta C = (C_{\text{naDNA}}/C_{\text{mdDNA}}) - (C_{\text{mdDNA}}/C_{\text{naDNA}}), \text{ where } C_i \text{ is the cycle threshold for each condition.}
\]

**Surface plasmon resonance binding experiments.** Recombinant BCL-2, BAX-FL, and BAX-ΔCT were generated with the Human In Vitro Protein Expression Kit for DNA Templates (Pierce). A SR7000DC (Reichert) dual-channel surface plasmon resonance (SPR) system and a SR7000 gold sensor slide (Reichert) with a surface composition of 10% COOH-(PEG)6-C11-SH, 90% OH-(PEG)3-C11-SH were used to test the binding affinities of BAX-FL and BAX-ΔCT to BCL-2. Either BAX-FL or BAX-ΔCT was covalently linked to the sensor slide at a concentration of 1.4 μg/ml and a flow rate of 25 μl/min. The recombinant BCL-2 was then passed over the bound BAX derivatives at five serially diluted concentrations (1.56, 0.78, 0.39, 0.195, and 0.0975 mg/ml) at a flow rate of 25 μl/min.

**RESULTS**

**Mitochondrial bioenergetics requires BAX.** To determine whether BAX has a previously unrecognized nonapoptotic activity, we examined mitochondrial structure, function, and bioenergetics in BAX+/+ and BAX−/− HCT-116 colon cancer cells. MitoTracker Red 580 fluorescence intensity was used as an indicator of oxidative capacity and to visualize actively respiring mitochondria (23). Live-cell imaging was performed to ensure that fixatives would not disrupt MitoTracker uptake. In Fig. 1A, when BAX was present, we observed respiring mitochondria that appeared elongated and tubular in shape with a loosely dispersed branching network. In contrast, when imaging the BAX−/− cells, we observed that in the absence of BAX respiring mitochondria were numerous but appeared less elongated (Fig. 1B). These observed differences in functional morphology upon MitoTracker staining were not due to alterations in the fission/fusion process. Treatment with Mdivi-1, a chemical inhibitor of DRP1 that prevents fission by blocking the fission/fusion process. Treatment with MitoTracker staining were not due to alterations in the fission/fusion process. Treatment with Mdivi-1, a chemical inhibitor of DRP1 that prevents fission by blocking the fission/fusion process. Treatment with Mdivi-1, a chemical inhibitor of DRP1 that prevents fission by blocking the fission/fusion process. Treatment with Mdivi-1, a chemical inhibitor of DRP1 that prevents fission by blocking the fission/fusion process. Treatment with Mdivi-1, a chemical inhibitor of DRP1 that prevents fission by blocking the fission/fusion process.

1 Supplemental Material for this article is available online at the Journal website.
116 cells. This was confirmed by the measurement of total NAO fluorescence in each cell population (Fig. 1C). Hence, total mitochondrial density or content was independent of BAX expression. To confirm results from NAO staining, we performed a quantitative experiment measuring total mtDNA relative to nDNA. These results are shown in Table 1. We found no detectable differences in mtDNA content between BAX+/+ and BAX−/− HCT-116 cells: P values were not significant. In total, these results suggest that the morphological differences detected by mitochondrial tracker staining (Fig.
2–4% of the BAX/H11011 cant BAX-dependent differences in overall viability. Only is the reason for the observation made with BAXing has been reported with FCCP, we do not think this artifact). Although relaxation of MitoTracker fluorescence quench-

ATP levels in BAXing was revealed by MitoTracker staining, we examined the elec-

dissipation of the chemiosmotic gradient. A titration of FCCP was consistent with the data shown in Fig. 1D.

The viability of cell lines used in all experiments reported here was due more to a functional defect than to decreased mitochondrial content. To demonstrate that BAX+/+ and BAX−/− HCT-116 cells were equally viable, we stained cells with NAO and EtBr. EtBr stains DNA only when the cell membrane is ruptured, as it is in cultured apoptotic cells. Representative data displayed in Fig. 1D revealed no significant BAX-dependent differences in overall viability. Only ~2–4% of the BAX+/+ and BAX−/− HCT-116 cells were apoptotic, that is, double-stained for both NAO and EtBr, and ~30% of the cells were viable (negative EtBr staining) but had low NAO fluorescence that was unrelated to BAX expression. The viability of cell lines used in all experiments reported here was consistent with the data shown in Fig. 1D.

To explore a possible functional defect upon BAX loss that was revealed by MitoTracker staining, we examined the electrochemical properties of the mitochondria in the two HCT-116 BAX variants, using the uncoupling agent FCCP. FCCP is a hydrophobic compound that carries protons across the IMM, releasing these protons into the matrix and allowing for the dissipation of the chemiosmotic gradient. A titration of FCCP was previously performed to determine the optimal concentra-
tion of FCCP for use, that is, the concentration that caused the least effect on cell viability and the most effect on ATP production (data not shown). When treated with FCCP, BAX+/+ mitochondria displayed a stronger intensity of MitoTracker fluorescence compared with untreated cells (Fig. 2, A and C). This was not observed in BAX−/− cells (Fig. 2, B and C). Although relaxation of MitoTracker fluorescence quench-
ing has been reported with FCCP, we do not think this artifact is the reason for the observation made with BAX+/+ cells, since the increase in MitoTracker fluorescence was not re-

Quantification of the effect of FCCP treatment revealed a twofold increase in MitoTracker fluorescence in BAX+/+ cells compared with untreated cells that was not observed with BAX−/− cells (Fig. 2C). We propose that in BAX+/+ cells the FCCP-mediated uncoupling of the electron transport system results in the rapid oxidation of matrix substrates, such as MitoTracker Red 580, which can be detected as an increase in MitoTracker fluorescence (6). This does not occur in BAX−/− cells, suggesting that loss of BAX may cause a defect in mitochondrial oxidative capacity.

BAX−/− cells also had significantly decreased levels of intracellular ATP compared with BAX+/+ cells (Fig. 2D). Furthermore, FCCP treatment resulted in a marked drop in ATP levels in BAX+/+ cells but not in BAX−/− cells (Fig. 2D). It should be noted that a previous titration of FCCP concentrations correlated with ATP amounts detected, indicat-
ing that we were primarily assessing changes in ATP produc-
tion (data not shown). To determine whether the loss of ATP in BAX−/− cells was due to abnormal bioenergetics, we mea-
sured glycolysis and oxygen consumption with an XF analyzer. Metabolic activity in BAX+/+ and BAX−/− HCT-116 cells was assessed by plotting the OCR, a measure of mitochondrial respiration, against the ECAR, an indicator of glycolysis. Comparison of changes that occur in aerobic and glycolytic metabolism (Fig. 2E) revealed that BAX+/+ cells had increased oxygen consumption (OCR) and decreased glycolytic activity (ECAR) relative to BAX−/− cells; hence BAX−/− cells were more dependent on glycolysis for their energy needs. A separate measure of oxygen consumption, using an alternative method, confirmed these findings (Fig. 2F).

In support, we had previously observed that BAX−/− cells were more susceptible to glucose deprivation, acquiring a shrunken morphology with decreased cytosolic content compared with BAX+/+ cells (Supplemental Fig. S2). From our experimental findings, we concluded that the decreased levels of ATP observed in BAX−/− cells were likely due to depressed respi-
ration, indicating that cells lacking BAX had a significant defect in oxidative phosphorylation that was in part compensated by glycolytic energy production.

BAX contributes to ATP production. Having identified a nonapoptotic function for BAX in the regulation of mitochon-
drial metabolism, we determined whether BAX localized to mitochondria in the absence of induced apoptosis. We performed an immunofluorescence experiment, using BAX+/+ HCT-116 cells, and detected partial overlay of BAX with mito-
chondria (HSP60 used as a mitochondrial marker) (Fig. 3A). To better quantitate the interaction of BAX with mitochondria under standard growth conditions, ultracentrifugation of en-
riched mitochondria was performed to examine the localization of BAX. It should be noted that lysates were prepared from cells that were viable (Fig. 1D) and the isolation procedure ensured that only intact mitochondria were used. The enriched mitochondrial lysate was layered on an iodixanol gradient and subjected to ultracentrifugation as described in MATERIALS AND METHODS. Separation of organelles was based on density, which is a function of the protein-to-lipid ratio of the organelles. Western blot analysis of the gradient fractions for prohibitin (mitochondrial content) (Fig. 3B) showed that the distribution of the mitochondria between BAX+/+ and BAX−/− HCT-116 cells was similar (across the 20–23% fractions). While no BAX was detected in BAX−/− cells, ~7% of the total endog-

Table 1. Level of mtDNA content in BAX+/+ and BAX−/− HCT-116 cells

<table>
<thead>
<tr>
<th></th>
<th>HCT-116 BAX+/+</th>
<th>HCT-116 BAX−/−</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔCt</td>
<td>−0.355</td>
<td>−0.318</td>
<td>0.4044</td>
</tr>
<tr>
<td>ΔCt mtDNA</td>
<td>0.782</td>
<td>0.802</td>
<td>0.4047</td>
</tr>
<tr>
<td>mtDNA/nDNA</td>
<td>r = 0.644</td>
<td>r = 0.681</td>
<td>0.1967</td>
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Values are averages of 3 experiments. mtDNA, mitochondrial DNA; nDNA, nuclear DNA; C, threshold cycle. P values were determined by unpaired t-test with Welch’s correction.

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BAX contributes to ATP production. Having identified a nonapoptotic function for BAX in the regulation of mitochondrial metabolism, we determined whether BAX localized to mitochondria in the absence of induced apoptosis. We performed an immunofluorescence experiment, using BAX+/+ HCT-116 cells, and detected partial overlay of BAX with mitochondria (HSP60 used as a mitochondrial marker) (Fig. 3A). To better quantitate the interaction of BAX with mitochondria under standard growth conditions, ultracentrifugation of enriched mitochondria was performed to examine the localization of BAX. It should be noted that lysates were prepared from cells that were viable (Fig. 1D) and the isolation procedure ensured that only intact mitochondria were used. The enriched mitochondrial lysate was layered on an iodixanol gradient and subjected to ultracentrifugation as described in MATERIALS AND METHODS. Separation of organelles was based on density, which is a function of the protein-to-lipid ratio of the organelles. Western blot analysis of the gradient fractions for prohibitin (mitochondrial content) (Fig. 3B) showed that the distribution of the mitochondria between BAX+/+ and BAX−/− HCT-116 cells was similar (across the 20–23% fractions). While no BAX was detected in BAX−/− cells, ~7% of the total endogenous BAX detected in the nonapoptotic BAX+/+ cells was associated with the mitochondrial fractions (Fig. 3B, Table 2). With the appropriate loading controls, the percentage of BAX localized to the mitochondria was determined by densitometry, calculating the relative band intensity in each lane compared with the amount of BAX detected in a Western blot of whole cell lysates (data not shown). This finding suggested that the amount of mitochondrion-associated BAX that supports energy production is considerably less than is required for the BAX-mediated perpetuation of the apoptotic cascade.

It remained to determine whether BAX was loosely associated with mitochondria (i.e., bound to the OMM) or whether it was sequestered within an inner membrane compartment (i.e., IMM). Enriched mitochondrial lysates, such as those used for the ultracentrifugation experiment, were treated with protease K for 5–20 min. Digested lysates were analyzed by SDS-PAGE and immunoblotted with antibodies to detect BAX and, as a control, BCL-XL. BCL-XL is mainly an OMM-localized protein and sensitive to protease digestion. We found
that treatment with proteinase K resulted in 50–60% lysis of BAX, compared with BCL-XL, in which 77–100% of the protein was lysed (Fig. 3C). Although in the same sample less BCL-XL protein was detected compared with BAX, an increase in the amount of BCL-XL digested occurred over time, while BAX was mostly digested within 5 min. These results suggest that a significant amount of BAX was sequestered within the mitochondria and not accessible to protease treat-
ment. Hence BAX has both outer and inner mitochondrial localization, supporting its proposed role mediating mitochondrial respiration. That BAX could be directly regulating mitochondrial metabolism was further demonstrated by measurements of citrate synthase activity. Citrate synthase, which is localized to the mitochondrial matrix, is one of the first enzymes of the tricarboxylic acid (TCA) cycle. We found that BAX+/− cells had reduced levels of citrate synthase activity compared with BAX+/+ cells (Fig. 3D). It should be noted that background levels of citrate synthase activity from ruptured mitochondria were not significantly different in the BAX variants (Fig. 3D).

To confirm that loss of BAX is sufficient to inhibit ATP production and is not due to a defect inherent to BAX−/− cells, BAX+/+ HCT-116 cells were treated with SMARTPool BAX siRNA. Previously, using Cy3-tagged nonspecific control
Table 2. Association of membrane-bound BAX with different density fractions recovered after differential ultracentrifugation

<table>
<thead>
<tr>
<th>BAX Construct</th>
<th>Total Protein Membrane Associated, %</th>
<th>Nonmitochondrial peak fractions, %</th>
<th>Mitochondrial peak fractions, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous BAX (+/+ )</td>
<td>7.21</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>BAX (-/- )</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Full length</td>
<td>2.73</td>
<td>0.9</td>
<td>91</td>
</tr>
<tr>
<td>ΔCt</td>
<td>9.46</td>
<td>67</td>
<td>33</td>
</tr>
</tbody>
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Data shown were determined by quantification of the mean pixel intensity of the BAX and prohibitin bands from blots shown Fig. 3B and Fig. 6, A and B, and are representative of 3 independent experiments.

siRNA, we established uptake efficiency and the absence of nontarget effects in HCT-116 cells (data not shown). After BAX siRNA treatment, cells were lysed and protein lysates analyzed by Western blot for inhibition of BAX expression and measured for ATP production. BAX protein expression was reduced ~50% by treatment with BAX siRNA, as shown in a representative blot (Fig. 4A). This correlated to a reduction in ATP as well, indicating that the amount of available BAX dictated the effect on the ATP produced (Fig. 4B). We repeated this experiment using a lung cancer epithelial line, which is highly dependent on oxidative phosphorylation, to ensure that this phenomenon was not isolated to HCT-116 cells. Western blot and ATP analysis of these lung cancer cells showed that a reduction in BAX expression correlated with the measured decrease in ATP levels (Fig. 4).

The work presented indicates that cell lines lacking BAX had reduced ATP production due to depressed respiration. To show that these results were not due to cell line or culture artifacts, we examined mitochondrial metabolism in primary hepatocytes freshly isolated from BAX KO mice. We observed that livers from BAX KO mice were slightly enlarged compared with those from aged WT mice. Staining of liver sections from BAX KO and WT mice with MitoTracker and NAO revealed findings very similar to those observed with HCT-116 cells (in Fig. 1). MitoTracker fluorescence was dim in liver sections from BAX KO mice compared with WT mice, while NAO staining was comparable (Fig. 5, A and B). Hence there appeared to be diminished mitochondrial respiration in BAX KO liver cells. This was confirmed by measuring oxygen consumption of BAX KO hepatocytes compared with WT hepatocytes (Fig. 5C). ATP production was likewise reduced in BAX KO hepatocytes (Fig. 5D) as well as citrate synthase activity (Fig. 5E). While, because of the limitations of cell numbers, biochemical assay results were less striking in freshly isolated hepatocytes, the differences between BAX KO and WT cells are statistically significant and follow patterns similar to those demonstrated with HCT-116 cells.

Having found that inhibition of BAX replicated the loss of respiration seen in cells that were genetically ablated (Figs. 4 and 5), we next determined whether restoring BAX to deficient cells would rescue mitochondrial metabolic activity. Overexpression of BAX-FL in BAX/- cells resulted in recovery of respiring mitochondria morphology (as seen in BAX/+ cells in Fig. 1A) observed upon MitoTracker staining (Fig. 6A). Western blot analysis of density gradients showed that ~2.5% of the total expressed protein associated with mitochondrial fractions (Table 2, Fig. 6B). As a result, we observed a threefold increase in ATP production upon the expression of BAX-FL that was inhibited by treatment with FCCP (Fig. 6E). This indicated that BAX-FL could rescue ATP production through oxidative phosphorylation. ECAR/OCR measurements confirmed that expression of BAX-FL could increase oxygen consumption of BAX-FL hepatocytes compared with WT hepatocytes (Fig. 6C). ATP production was likewise reduced in BAX KO hepatocytes (Fig. 5C). ATP as well, indicating that the amount of available BAX dictated the effect on the ATP produced. We repeated this experiment using a lung cancer epithelial line, which is highly dependent on oxidative phosphorylation, to ensure that this phenomenon was not isolated to HCT-116 cells. Western blot and ATP analysis of these lung cancer cells showed that a reduction in BAX expression correlated with the measured decrease in ATP levels (Fig. 4).

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Fig. 5. Liver hepatocytes from BAX-deficient mice have reduced metabolic activity, resulting in decreased ATP and oxygen consumption. A: sections of the caudate lobe of the liver from BAX knockout (KO) and C57BL/6 mice were stained with MitoTracker Red or NAO. B: isolated hepatocytes from these mice were stained with NAO and measured by flow cytometry to determine mitochondrial content. Data are representative of 3 replicates, and n = 2 for each group. C: oxygen consumption of 50,000 isolated hepatocytes per well was measured over 24 h. Data are representative of 2 experiments each with 46 replicates/group. D: ATP content was measured in isolated hepatocytes. Luminescence was normalized to 5,000 cells per well, and each of 3 experiments consisted of 42 replicates per condition. E: citrate synthase activity was examined in isolated mitochondria by measuring the absorbance of TNB over time. ATP and citrate synthase assay analysis was done by 1-way ANOVA with Dunnett’s post-test analysis. *P < 0.05, ***P < 0.001.

consumption, while reducing glycolytic activity, indicating that BAX was a critical regulator of these activities (Fig. 6F).

Overexpression of BAX-ΔCT in BAX−/− cells was performed to determine the contribution of the COOH-terminal helix of BAX to mitochondrial bioenergetics. Expression of BAX-ΔCT did not restore the respiring mitochondrial morphology seen in the presence of BAX (Fig. 6C). This activity of BAX appears to require the COOH-terminal domain. Predictably, the removal of this COOH-terminal helix resulted in a scattered localization of BAX-ΔCT, as exhibited by the altered banding pattern in the gradient fractions (Fig. 6D). Even though mitochondrial targeting was deregulated in this mutant, some BAX-ΔCT was found in the mitochondrial fractions (~9%; Table 2, Fig. 6D) and, surprisingly, was sufficient to restore ATP production as effectively as BAX-FL (Fig. 6E). Moreover, measurements of ECAR/OCR showed that expression of BAX-ΔCT was just as effective in restoring metabolic balance in BAX−/− cells as BAX-FL (Fig. 6F). The differences between BAX−/− cells and BAX−/− cells expressing BAX-FL or BAX-ΔCT were statistically significant. These results suggest that there are multiple regulatory domains of BAX, such as the COOH-terminal helix or the BH3 domain, which can operate independently of each other to affect the function of mitochondria.

Interaction with BCL-2 blocks BAX’s nonapoptotic activity. BAX’s known antagonist is the antiapoptotic protein BCL-2, which resides anchored in the OMM (36). BAX and BCL-2 exist in a balance—each counteracting the other’s activity (20). This balance is likely dependent on interactions mediated through each of the proteins’ BH3 domains (38). Since expression of BAX-ΔCT could rescue mitochondrial respiration and ATP synthesis, we postulated that this activity was being mediated through BAX’s BH3 domain, which could be inhibited by binding to BCL-2. To test this, the binding capabilities of the BAX constructs with BCL-2 were first measured by SPR. Recombinant BAX-FL or BAX-ΔCT proteins were generated and covalently linked by amine coupling to gold SPR sensor plates. The recombinant BCL-2 protein was then passed over the bound BAX constructs. Binding kinetics was determined by calculating the association constant (ka) and dissociation constant (kd) of serially diluted BCL-2 binding to immobilized BAX. The binding profiles of BCL-2 to both BAX proteins were similar, but based on the kinetics BCL-2 bound with twice the affinity to BAX-ΔCT compared with BAX-FL (Fig. 7, A and B). This disparity in binding affinities was exemplified by the twofold difference in the calculated equilibrium constant (Kd) between BAX-FL and BAX-ΔCT. This outcome is likely due to a greater exposure of a BCL-2 interacting domain in BAX-ΔCT resulting from removal of the COOH terminus of the BAX protein.

Overexpression of BCL-2 in BAX+/+ cells resulted in decreased mitochondrial ATP production (Fig. 7C), indicating that BCL-2 was impeding the normal function of BAX in mitochondria. This reduction was equivalent to the reduction
observed upon FCCP treatment of BAX+/+ cells. Adding FCCP to the BCL-2-overexpressing cells further amplified the reduction of ATP (Fig. 7C). Because of the effect of binding to BCL-2 that is observed in both BAX-FL and the BAX-ΔCT mutant (Fig. 7, A and B), it can be inferred that BCL-2 also acts as an antagonist to BAX in its activity promoting bioenergetics. Since BAX still binds BCL-2 in the absence of the COOH terminus, it is possible that the regulatory region, likely the BH3 domain, for this interaction becomes more accessible upon deletion of the COOH-terminal helix, and that this domain could associate with other mitochondrial membrane proteins to support ATP synthesis.

**DISCUSSION**

Our findings suggest that BAX has an essential, nonapoptotic, homeostatic activity. Although the major portion of BAX in nonapoptotic cells resides in a soluble, cytosolic form (27), we and others (26) have observed that under normal conditions BAX can also associate with mitochondria. Here, we report that BAX has a supporting role in mitochondrial energy production. Cells deficient in BAX had decreased mitochondrial oxidative capacity and reduced levels of intracellular ATP. Such cells were dependent on energy produced through glycolytic activity. The localization of a small fraction of BAX to the outer and inner mitochondrial compartments could enable ATP production through respiration, a process reversed when BAX was inhibited by specific siRNAs or genetically ablated, such as in KO mice. By introducing BAX-FL into cells lacking endogenous BAX, we were able to restore mitochondrial respiration and increase the amount of ATP. Expression of a BAX mutant lacking the COOH-terminal α-helix also restored aerobic metabolism and ATP production, indicating that
BAX is required for mitochondrial bioenergetics in manner that is independent of the COOH-terminal transmembrane helix. Coexpression of BAX with BCL-2 resulted in reduced ATP detection, suggesting perhaps that the interaction of BAX with a mitochondrial protein(s) (inhibited by BCL-2) is required for regulation of ATP levels.

In the current model of apoptosis, the cytosolic, monomeric (inactive) form of BAX must undergo significant conformational changes (activation) that enable transitioning to mitochondria (25). We may infer from our results that, under nonapoptotic conditions, equilibrium could exist between the two conformations, inactive and active. A small amount of “active” BAX could associate with healthy mitochondria, contributing to the regulation of functional architecture and bioenergetics, but at a concentration well under that needed to induce apoptosis. This leads to the idea that the movement of BAX to mitochondria under nonlethal conditions could be restricted by the lipid and protein composition of the organelle membrane (18,29). Apoptosis induces changes in mitochondria that could help recruit BAX, while in the absence of apoptosis the OMM environment is less likely to support the translocation of BAX. In healthy mitochondria, a few BAX monomers or dimers may insert into mitochondrial membranes to form small pores, but this process needs to be tightly regulated. It is more feasible that BAX interacts with existing mitochondrial proteins. This idea is supported by our findings that a portion of BAX was sequestered within the mitochondria, perhaps interacting with an IMM protein. Hence, the amount of BAX associated with mitochondria in nonapoptotic cells would be constrained by the availability of the binding partner(s), a process we demonstrated by the inhibition of ATP production with coexpression of a known inhibitor (BCL-2) with the BAX constructs.

A review of current literature reveals a number of possible mitochondrial binding partners for BAX, although most were discovered in the context of an apoptotic scenario. Examples include the adenine nucleotide translocator (ANT) (30) and the voltage-dependent anion channel (VDAC). VDAC could serve as a mitochondrial receptor for BAX (22). In addition to ANT and VDAC, many other proteins are known to interact with BAX, including factors that mediate changes in the mitochondrial network such as fission or fusion proteins like Drp1 (11), Mfn-2 (1), or endophilin B1 (Bif-1) (28) as well as other regulatory proteins such as cyclophilin D or Ku70 (reviewed in Ref. 15). Although this may seem speculative, the excess of circumstantial evidence for the interaction of BAX with healthy mitochondria highlights the need for further study to determine whether some of these proteins act to facilitate BAX’s role in mitochondrial bioenergetics.

In a recent proteome-wide quantification of proteins that are differentially expressed between BAX-containing and BAX-deficient HCT-116 cells (33), a number of mitochondrial proteins were found to be downregulated upon loss of BAX, including VDAC. Significantly, two essential enzymes for glucose metabolism and ATP production were also decreased in BAX-deficient cells: glucose-6-phosphate isomerase was reduced 10.2±0.3-fold, and citrate synthase was reduced 7-fold. This supports our own observation of reduced citrate synthase activity in the absence of BAX. Loss of critical metabolic components exacerbates the inability of BAX-deficient cells to produce energy from glucose. That the reintroduction of BAX into these cells dramatically increased respiration and ATP production, as we showed, strongly supports the concept that BAX plays an important homeostatic role supporting growth and metabolism.

Given the decisive role of BAX in apoptosis, it would seem that the mutation rate in cancerous cells would be equivalent to...


