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Oligomeric Bax Is a Component of the Putative Cytochrome *c* Release Channel MAC, Mitochondrial Apoptosis-induced Channel

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Bcl-2 family proteins regulate apoptosis, in part, by controlling formation of the mitochondrial apoptosis-induced channel (MAC), which is a putative cytochrome *c* release channel induced early in the intrinsic apoptotic pathway. This channel activity was never observed in Bcl-2–overexpressing cells. Furthermore, MAC appears when Bax translocates to mitochondria and cytochrome *c* is released in cells dying by intrinsic apoptosis. Bax is a component of MAC of staurosporine-treated HeLa cells because MAC activity is immunodepleted by Bax antibodies. MAC is preferentially associated with oligomeric, not monomeric, Bax. The single channel behavior of recombinant oligomeric Bax and MAC is similar. Both channel activities are modified by cytochrome *c*, consistent with entrance of this protein into the pore. The mean conductance of patches of mitochondria isolated after green fluorescent protein-Bax translocation is significantly higher than those from untreated cells, consistent with onset of MAC activity. In contrast, the mean conductance of patches of mitochondria indicates MAC activity is present in apoptotic cells deficient in Bax but absent in apoptotic cells deficient in both Bax and Bak. These findings indicate Bax is a component of MAC in staurosporine-treated HeLa cells and suggest Bax and Bak are functionally redundant as components of MAC.

INTRODUCTION

A key early event in the apoptotic cascade in many cell types is the release of cytochrome *c* and other proapoptotic factors from mitochondria (reviewed in Antonsson, 2004; Danial and Korsmeyer, 2004; Green *et al*., 2004; Reed *et al*., 2004). Once in the cytosol, cytochrome *c* and procaspase 9 bind the cytosolic protein apoptotic protease activating factor-1 and dATP to form apoptosomes that promote caspase activation and destruction of the cell (Liu *et al*., 1996). Proapoptotic factors are released from mitochondria early in apoptosis mediated by the intrinsic pathway by either of two mechanisms. The opening of the permeability transition pore of the inner membrane causes swelling of the matrix, which ruptures the outer membrane and spills cytochrome *c* and other proapoptotic proteins into the cytosol (Skulachev, 1996; Kroemer and Reed, 2000). However, cytochrome *c* release also has been shown to occur in the absence of mitochondrial depolarization and without loss of outer membrane integrity in some cell types, suggesting instead a more selective mechanism such as the formation of a pore in the outer membrane, i.e., the channel MAC (Liu *et al*., 1996; Pavlov *et al*., 2001; Wei *et al*., 2001; De Giorgi *et al*., 2002; Guo *et al*., 2004). These two mechanisms may act alone or in combination, depending on cell type and death stimulus.

MAC is defined as a high-conductance, voltage-independent channel activity in patch-clamp experiments. MAC forms in the mitochondrial outer membrane early in the intrinsic apoptotic pathway, about the time cytochrome *c* is released (Gross *et al*., 1998; Pavlov *et al*., 2001; Guo *et al*., 2004; Martinez-Caballero *et al*., 2004). A recent report indicates MAC activity has been detected only at a late stage of the extrinsic apoptotic pathway (Guihard *et al*., 2004). The difference in the time of MAC development may be due to differences in the intrinsic and extrinsic pathways or cell type. The observation that cytochrome *c* itself modifies the channel behavior of MAC provides strong evidence that cytochrome *c* enters the pore of MAC and likely uses MAC to exit mitochondria in early apoptosis (Guo *et al*., 2004). The diameter of MAC's pore is ~5 nm if estimated from a mean conductance of 4.5 nS. Hence MAC's pore should be large enough to release the ~3-nm cytochrome *c* from mitochon-

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Abbreviations used: GFP-Bax, green fluorescence protein-tagged Bax; IL-3, interleukin-3; MAC, mitochondrial apoptosis-induced channel; PTP, permeability transition pore; VDAC, voltage-dependent anion-selective channel.

dria. Although MAC activity is now better understood (Pavlov *et al*., 2001; Guo *et al*., 2004; Martinez-Caballero *et al*., 2004), the identity of MAC components is a subject of speculation and has not yet been defined.

The BCL-2 family of proteins regulates entrance into the cell death cascade, and many of these proteins are located in mitochondria (Cory *et al*., 2003; Antonsson, 2004; Danial and Korsmeyer, 2004; Green *et al*., 2004). This family can be divided into three groups: 1) antiapoptotic proteins represented by Bcl-2 and Bcl-xL; 2) the multidomain, proapoptotic proteins, including Bax and Bak; and 3) small proapoptotic proteins such as t-Bid and Bad that are only comprised of BH3, the so-called death domain. BH3-only proteins directly or indirectly activate Bax/Bak to induce apoptosis, whereas Bcl-2 and Bcl-xL prevent activation of Bax/Bak either by binding to Bax/Bak or by sequestering the BH3-only molecules (Cheng *et al*., 1997, 2003; Wei *et al*., 2001).

Overexpression of Bcl-2 suppresses MAC activity and several observations support a role for Bax in MAC activity (Pavlov *et al*., 2001; Guo *et al*., 2004; Martinez-Caballero *et al*., 2004). MAC is detected by patch clamping mitochondria at the time Bax translocates into the outer membrane of mitochondria during apoptosis. Recombinant Bax forms high-conductance channels in artificial membranes and induces the release of cytochrome *c* from isolated mitochondria and permeabilized cells (Antonsson *et al*., 1997; Schendel *et al*., 1997, 1998; Schlesinger *et al*., 1997; Saito *et al*., 2000; Pavlov *et al*., 2001). These lines of evidence suggest a mechanistic link between Bax translocation to mitochondria, cytochrome *c* release into the cytosol, and MAC formation. Although Bax is known to induce cytochrome *c* release, little evidence supports the presumption that Bax is a component of a cytochrome *c* release channel.

In this study, immunodepletion of MAC activity from lysates of apoptotic HeLa cells by Bax antibodies provides the first direct proof that oligomeric Bax is a component of MAC, the putative cytochrome *c* release channel. Recombinant Bax channels and MAC have similar single channel behaviors and responses to effectors such as cytochrome *c*, suggesting oligomeric recombinant Bax may be a model system for MAC activity. Finally, the results obtained upon examination of MAC onset in apoptotic parental, single Bax, and double Bax/Bak knockout cells suggest Bax and Bak are functionally redundant with respect to this channel.

MATERIALS AND METHODS

Cells

Both green fluorescent protein (GFP)-Bax–expressing HeLa-derived clone 10 and HeLa cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in 5% CO_2, and 1 μM staurosporine induced apoptosis (Antonsson *et al*., 2001; De Giorgi *et al*., 2002). FL5.12 cells were grown in improved minimum essential medium with 10% FBS and 10% WEHI-3B (interleukin-3 [IL-3]) supplement (Gross *et al*., 1998), and IL-3 withdrawal induced apoptosis. Mouse embryonic fibroblast (MEF) Bax−/− and Bax−/−Bak−/− cells were cultured in DMEM with 10% FBS, 1% nonessential amino acids, and 1% L-glutamine (Wei *et al*., 2001).

Cytochrome c Release Assays

Permeabilization of cells with digitonin allows separation of cytosolic proteins and total membranes (Cheng *et al*., 2003). Briefly, cells were harvested, resuspended in permeabilization buffer [70 mM sucrose, 230 mM mannitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1% (vol/vol) mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 5 mM HEPES-KOH, pH 7.4] and incubated on ice for 5 min in the presence of 3 μg of digitonin/million cells. Total membranes were pelleted at 12,000 × *g* for 2 min at 4°C. Cytochrome *c* released from mitochondria was determined by enzyme-linked immunosorbent assay (ELISA) in the supernatants following manufacturer's instructions (Quantikine; R&D Systems, Minneapolis, MN). Pellets were suspended in Laemmli loading buffer (Bio-Rad, Hercules, CA) before SDS-PAGE.

Preparation of Mitochondrial Extracts and Gel Filtration Analyses

Mitochondria were isolated and outer membranes were purified as described previously (Mannella, 1982; Lohret *et al*., 1997; Antonsson *et al*., 2001; Pavlov *et al*., 2001). Mitochondrial extracts of HeLa cells and subsequent gel filtration analyses were described previously (Antonsson *et al*., 2001). Briefly, mitochondria were lysed in the presence of isolation media with 2% (wt/vol) CHAPS, and gel filtrations were on a Superdex 200 column equilibrated in 300 mM NaCl, 0.2 mM dithiothreitol, and 25 mM HEPES-NaOH, pH 7.5 (AP buffer), with 2% (wt/vol) CHAPS. Bicinchoninic acid protein assay kits (Pierce Chemical, Rockford, IL) were used to estimate protein concentration.

Microscopy

Cells to be imaged were grown on coverslips and mounted in sealed Rose chambers (Khodjakov *et al*., 2004). Images were captured through 60× 1.4 or 40× PlanApo lenses with a Spot RT Monochrome charge-coupled device camera on a Nikon Eclipse TE300 microscope equipped with epifluorescence and Uniblitz shutters. Other images were acquired with a Zeiss 510 meta confocal microscope. The laser lines used were 405 nm (for Hoechst), 488 nm (for GFP-Bax), and 543 nm (for Texas Red-secondary antibody in conjunction with a primary antibody against cytochrome *c* [566432BD; Biosciences PharMingen, San Diego, CA]).

Immunoblotting

Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). The primary antibodies used were against Bax (N-20 sc-493; Santa Cruz Biotechnology, Santa Cruz, CA), voltage-dependent anion-selective channel (VDAC) (anti-porin 31HL; Calbiochem, San Diego, CA), ANT (N-19 sc-9299; Santa Cruz Biotechnology), and BAK antibodies (anti-NT; Upstate Biotechnology, Lake Placid, NY). The secondary antibodies were horseradish peroxidase-coupled anti-mouse (Jackson ImmmunoResearch Laboratories, West Grove, PA), anti-rabbit and anti-goat (Sigma-Aldrich). Bax was quantified by densitometry (Scion Imaging; National Institutes of Health, Bethesda, MD) using recombinant Bax as standards.

Bax Immunoprecipitation

Partially purified fractions (2–7 μg of protein, 2 ng of Bax) or total lysates (35–50 μg of protein, 40 ng of Bax) from mitochondria of control and apoptotic HeLa cells were resuspended in AP buffer. Samples were incubated overnight with 0.2 μg of anti-Bax (N-20 sc-493; Santa Cruz Biotechnology) or control (total rabbit IgGs; Sigma-Aldrich) antibodies and then 5 h with protein G-agarose beads (Calbiochem). Supernatants were collected after centrifugation (10 min; 10,000 × *g*). Pellets were washed twice with phosphate-buffered saline, suspended in sample buffer (Bio-Rad), incubated 5 min at 95°C, and protein G-Agarose was removed by centrifugation (10 min; 10,000 × *g*).

Preparation of Proteoliposomes

Proteoliposomes containing mitochondrial proteins from HeLa cells or FL5.12 mitochondrial outer membranes were formed as described previously (Criado and Keller, 1987; Lohret *et al*., 1997; Guo *et al*., 2004). CHAPS-solubilized fractions of HeLa cells (2–50 μg) were resuspended in AP buffer with liposomes at a ratio of ~1:10 protein/lipid. Samples were dialyzed times three (10 kDa; Harvard Apparatus, Holliston, MA) against AP buffer without CHAPS containing 1% (vol/vol) of Extracti-Gel detergent removing gel (Pierce Chemical). Liposomes were added (1/25 protein/lipid) for reconstitution (Criado and Keller, 1987; Lohret *et al*., 1997; Guo *et al*., 2004).

Recombinant Proteins

Monomeric human Bax, truncated for 20 amino acids at the C terminus (BaxΔC), and t-Bid tagged with six histidines at the N terminus, were expressed in *Escherichia coli* and purified as described previously (Montessuit *et al*., 1999; Antonsson *et al*., 2000, 2001). Oligomeric BaxΔC was made either by purifying the oligomers after disrupting bacteria in 1% Triton X-100 buffer (Antonsson *et al*., 2000) or by preincubating BaxΔC with t-Bid. Bax channel activity was studied using microelectrode tips filled with 170 ng/μl oligomeric BaxΔC or 380 ng/μl monomeric BaxΔC plus 35 ng/μl t-Bid. Microelectrodes were then backfilled with 150 mM KCl, 5 mM HEPES, pH 7.4, so the actual Bax concentrations are lower than that loaded in the tips. Seals were formed on liposomes prepared without protein with these microelectrodes.

Patch-Clamp Analysis

Patch-clamp procedures and analysis used were described previously (Lohret *et al*., 1997; Pavlov *et al*., 2001; Guo *et al*., 2004). Typically, the solution was symmetrical 150 mM KCl, 5 mM HEPES, pH 7.4. Voltage clamp was established with an Axopatch 200 amplifier, and voltages are reported as pipette

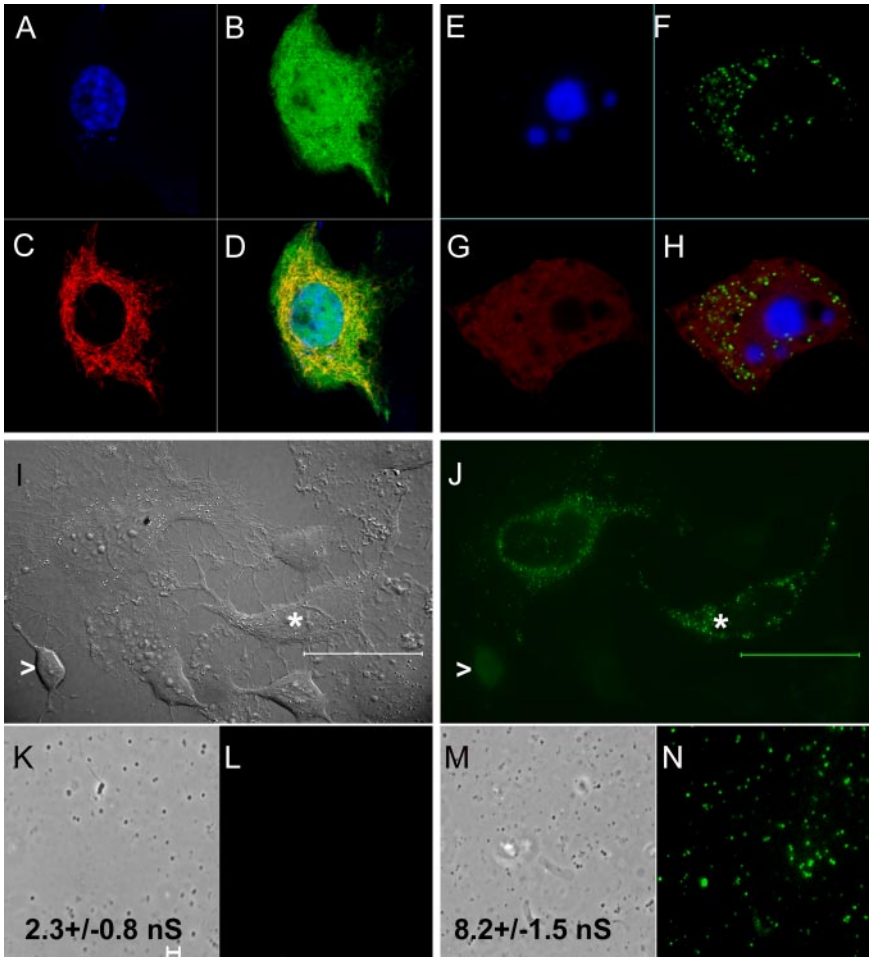

Figure 1. Staurosporine-induced GFP-Bax translocation coincides with an increase in outer membrane permeability. Confocal images of staurosporine-treated clone 10 cells show fluorescence of Hoescht in blue (A and E), cytochrome *c* immunofluorescence in red (C and G), and GFP-Bax in green (B and F), and are merged in D and H. Differential interference contrast (I) and fluorescence (J) images show Clone 10 cells expressing GFP-Bax after 5 h of treatment with 1 μM staurosporine. Diffuse GFP-Bax fluorescence (>) becomes punctate with time (*). Phase contrast (K and M) and fluorescence (L and N) images show mitochondria isolated from apoptotic (K and L) and control (M and N) cells at the time the GFP-Bax fluorescence became punctate in the staurosporine treated cells. The mean ($\pm$ SE) conductance of patches shown in panels K and M was measured by patch clamping these mitochondria. Bar, 20 μm (I and J) and 5 μm (K–N).

potentials. WinEDR version 2.3.3 was used for analysis (courtesy of J. Dempster; Strathclyde Software, Glasgow, United Kingdom). Sample rate was typically 5 kHz with 2-kHz filtration.

RESULTS

MAC Is Detected in Mitochondria Containing GFP-Bax

Cytochrome *c* release was previously associated with development of punctate GFP-Bax fluorescence in mitochondria of COS-7 cells (De Giorgi *et al*., 2002). The GFP fluorescence of clone 10 cells that stably express low levels of GFP-Bax increases during treatment with staurosporine and becomes punctate with time. Clone 10 cells with relatively diffuse GFP-Bax fluorescence show punctate cytochrome *c* immunofluorescence, consistent with localization of cytochrome *c* in mitochondria (Figure 1, A–D). In contrast, clone 10 cells with punctate GFP-Bax fluorescence show diffuse cytochrome *c* immunofluorescence, consistent with localization of cytochrome *c* in the cytoplasm (Figure 1, E–H). These observations suggest cytochrome *c* release is associated with development of punctate GFP-Bax fluorescence in mitochondria of clone 10 cells. Mitochondria were isolated from clone 10 cells treated with staurosporine when most cells showed punctate GFP-Bax fluorescence (Figure 1, I–N) and were directly patch clamped. Single channel recordings were typically not possible on isolated mammalian mitochondria, probably because of the high expression level of VDAC (Lohret *et al*., 1997; Pavlov *et al*., 2001).

The approach of measuring mean conductance of mitochondrial patches was applied to clone 10 cells to monitor MAC formation during Bax translocation, as we previously did with apoptotic FL5.12 cells (Pavlov *et al*., 2001). As shown in Figure 1, K–N, the fluorescence of the mitochondria isolated from the apoptotic clone 10 cells was much greater than that of untreated cells, indicating GFP-Bax had translocated to the mitochondria of staurosporine-treated cells. Patch clamping indicated the permeability of the outer membrane was higher in the mitochondria isolated from apoptotic cells, consistent with onset of MAC activity. The mean conductance of the mitochondria was 8.2 $\pm$ 1.5 nS for apoptotic and 2.3 $\pm$ 0.8 nS for control cells (mean $\pm$ SE; n = 29 and n = 16 independent patches, respectively). These results are similar to those obtained in FL5.12 cells after IL-3 withdrawal and suggest Bax translocation and oligomerization/clustering may play a role in MAC activity and cytochrome *c* release (Pavlov *et al*., 2001; De Giorgi *et al*., 2002).

Bax Content of Mitochondria Increases during Apoptosis

Bax translocates to mitochondria and is present in high-molecular-weight fractions of mitochondrial lysates of apoptotic but not control cells, as indicated by gel filtration studies (Antonsson *et al*., 2001; Cheng *et al*., 2001, 2003). In the present study, HeLa cells were and were not treated with staurosporine, and proteins were extracted from isolated mitochondria with 2% CHAPS, a detergent that does not modify the oligomeric or monomeric status of Bax (Hsu and

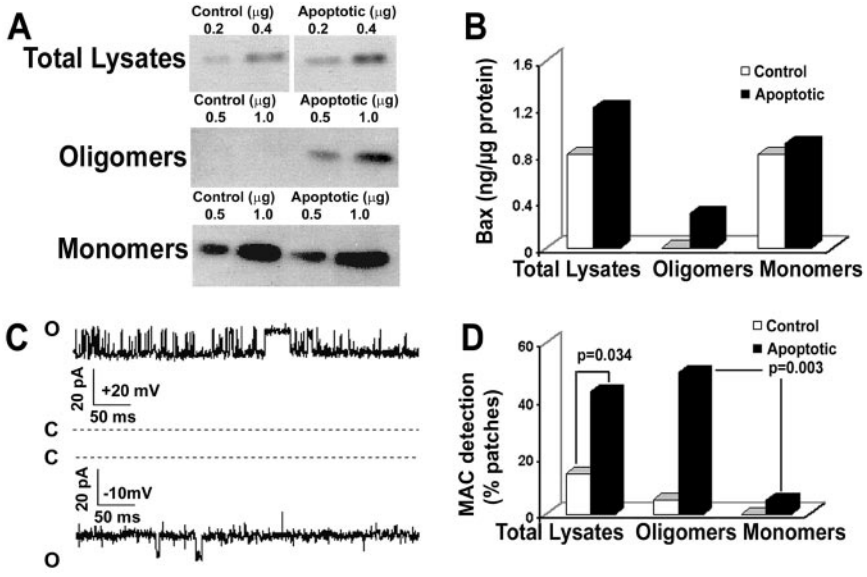

Figure 2. MAC detection is associated with Bax oligomers but not Bax monomers. Apoptosis was induced by treatment of HeLa cells with 1 μM staurosporine for 16 h, and solubilized total mitochondrial proteins were partially purified by gel filtration as described in *Materials and Methods*. (A) Western blots show Bax content of total lysates and gel filtration fractions containing Bax oligomers (oligomers) or monomers (monomers) from isolated mitochondria of control and apoptotic cells. The total protein loaded is indicated. (B) Histograms show the relative Bax content of total lysates (total), oligomeric Bax (oligomers), or monomeric Bax (monomers) fractions from isolated mitochondria of control and apoptotic cells. (C) Representative current traces are shown of MAC recorded at +20 and −10 mV with 2-kHz filtration displaying both rapid and slow kinetics (top and bottom, respectively). The total lysate of mitochondria of apoptotic cells was reconstituted into proteoliposomes and patch clamped. O and C correspond to open and closed current levels, respectively. (D) Histograms show the fraction of patches displaying MAC activity after reconstituting total lysates, oligomeric Bax (oligomers), or monomeric Bax (monomers) fractions of control or apoptotic cells in proteoliposomes. n = 20–23 independent patches for each condition. p values shown were calculated using the Fisher exact statistical test (Fisher, 1935).

Youle, 1998; Antonsson *et al*., 2000, 2001). Bax translocates to mitochondria during apoptosis as shown by higher Bax levels in the apoptotic compared with control lysates (Figure 2A), consistent with the results of others (Gross *et al*., 1998; Desagher *et al*., 1999; Antonsson *et al*., 2001; Pavlov *et al*., 2001). The total lysates of mitochondria were partially purified by gel filtration. Whereas Bax eluted in fractions corresponding to Bax monomers (20 kDa) under both conditions, Bax also eluted in the 96- and 260-kDa fractions of apoptotic, but not control, mitochondrial lysates (Antonsson *et al*., 2001). The higher molecular weight fractions contained oligomers of Bax as shown by cross-linking studies (Antonsson *et al*., 2001; Mikhailov *et al*., 2001, 2003). These findings suggest Bax may form complexes and/or oligomers in apoptotic but not control cells. The 20- and 260-kDa fractions were further examined for the presence of MAC activity and are referred to in this report as the monomeric and oligomeric fractions, respectively. The amount of Bax in the monomeric fractions of apoptotic and control cells was similar (Figure 2B). Finally, the oligomeric fraction of apoptotic cells contained 0.3 ng Bax/μg protein, whereas Bax was not detectable in the corresponding fraction of control cells.

MAC Activity Is Associated with Bax Oligomers

MAC activity was readily detected by patch-clamping proteoliposomes containing total mitochondrial lysates and oligomeric fractions from apoptotic HeLa cells but not monomeric fractions (Figure 2D). MAC has a high but variable conductance and was scored present if a voltage-independent conductance ≥1.5 nS was observed (Pavlov *et al*., 2001; Guo *et al*., 2004). Although occasional bursts were seen, MAC typically had a stable open state with relatively infrequent transitions (Figure 2C; Pavlov *et al*., 2001; Guo *et al*., 2004). The mean conductance of MAC of apoptotic cells was 3.3 ± 1.3 nS in the total mitochondrial lysates and 2.7 ± 1.4 nS in the oligomeric fraction (mean ± SD, n = 20–22 patches). As expected from an apoptosis-induced channel, the frequency of detecting MAC was threefold higher in proteoliposomes prepared with total lysates from apoptotic versus control mitochondria (Figure 2D; p = 0.03). Untreated cultures of HeLa cells have 10–12% apoptotic cells as indicated by annexin-V staining (Cirman *et al*., 2004), and this background of apoptotic cells presumably contributed to the basal detection of MAC in the total mitochondrial lysates from control cells (Figure 2D). Notably, MAC was found in half the patches of proteoliposomes containing the oligomeric fraction of apoptotic cells, but in only one of 20 patches of the control (Figure 2D). In contrast, MAC was rarely detected in the monomeric fractions of either apoptotic or control cells (5 and 0% of the patches, respectively; Figure 2D), suggesting MAC is associated with oligomeric, not monomeric, Bax (p = 0.003).

MAC Activity Is Immunodepleted by Antibodies against Bax Oligomers

A direct link between MAC activity and Bax oligomers was established through immunodepletion experiments in apoptotic HeLa cells. The Bax antibody used was directed against the N terminus of Bax and known to recognize oligomeric, but not monomeric, Bax (Desagher *et al*., 1999; Nechushtan *et al*., 1999). This antibody immunoprecipitated all the Bax in the oligomeric fraction of apoptotic HeLa cells, but none in the fraction containing Bax monomers (Figure 3A, top). About 20% of the Bax was immunoprecipitated in the total mitochondrial lysates from apoptotic cells and only a small amount of Bax was immunoprecipitated in the corresponding control lysates (Figure 3A, bottom). The anti-Bax antibodies were in excess because 0.2 μg of Bax antibody immunoprecipitated at least 40 ng of oligomeric recombinant Bax (our unpublished data), which is the amount of Bax in the immunoprecipitation assays for both the control and apoptotic total lysates (our unpublished data). These results indicate that only a minor fraction (~20%) of the total Bax formed oligomers in the mitochondria of apoptotic HeLa cells.

To determine whether MAC activity was immunodepleted by these anti-Bax antibodies, the supernatants derived from the immunoprecipitations were dialyzed and

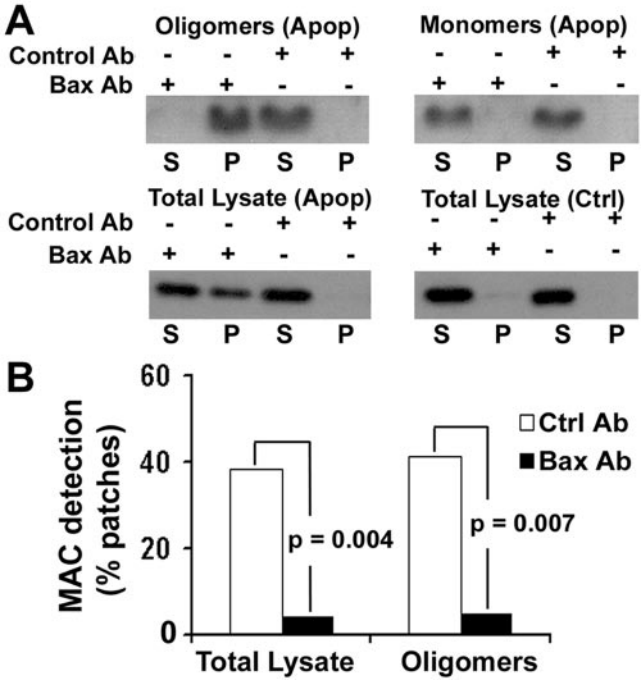

Figure 3. Immunoprecipitation of Bax oligomers causes depletion of MAC activity. (A) Total mitochondrial lysates and fractions containing oligomeric and monomeric Bax of HeLa cells were immunoprecipitated with anti-Bax antibodies (Bax antibody) or total rabbit IgG (control antibody). The pellets containing the immunoprecipitated proteins (P) and their supernatant (S) were subjected to SDS-PAGE, and the presence of Bax was assessed by Western blot. (B) The supernatants from the immunoprecipitation assays described above and corresponding to immunoprecipitates with anti-Bax antibodies (Bax antibody, closed) or with total rabbit IgG (Control antibody, open) were dialyzed and reconstituted in proteoliposomes. MAC detection frequency was determined by patch clamping. n = 20–23 independent patches/condition. Other conditions were as described in Figure 2.

reconstituted into proteoliposomes. Patch clamping was used to assay for MAC activity. Immunoprecipitation with a control antibody only slightly reduced the detection of MAC in either the total lysates or the oligomeric fraction (41 vs. 50% and 38 vs. 43%; compare Figures 2D and 3B). In contrast, anti-Bax antibodies immunodepleted the activity because MAC was detected in only one of 21 patches and one of 23 patches in the oligomeric fraction and total mitochondrial lysates, respectively (Figure 3B; $p < 0.01$ in both cases). The concomitant loss of MAC activity and Bax oligomers by immunoprecipitation with anti-Bax antibodies directly supports the hypothesis that Bax oligomers are components of MAC in this system.

MAC and Recombinant Bax Channels Have Similar Single Channel Characteristics

A comparison of MAC and recombinant BaxΔC channel activities was undertaken to further evaluate the role of Bax in MAC activity. In these studies, we extended the evaluation to MAC of apoptotic FL5.12 cells isolated 12 h after IL-3 withdrawal as this system has been used in several investigations and cytochrome *c* release was shown to occur after Bax translocation (Bojes *et al*., 1997; Gross *et al*., 1998; Pavlov *et al*., 2001; Guo *et al*., 2004). Furthermore, sufficient amounts of purified mitochondrial outer membranes can be isolated from these nonadherent cells compared with adherent HeLa cells. Oligomeric Bax spontaneously forms high-conductance channels in artificial membranes and causes release of trapped cytochrome *c* and dextrans (Antonsson *et al*., 1997; Lewis *et al*., 1998; Antonsson *et al*., 2000; Saito *et al*., 2000; Polster *et al*., 2001; Kuwana *et al*., 2002; Terrones *et al*., 2004). In this report, recombinant BaxΔC oligomers, formed by exposure to Triton X-100 or t-Bid, are referred to as rBax (see *Materials and Methods*). After seal formation on a liposome containing no protein, increases in patch conductance were observed with time if rBax was included in the microelectrode tip, as shown in the current traces of Figure 4A. These increases in current flow are consistent with the formation of large pores. Although small current transitions are observed, no high-conductance channel activity was recorded from t-Bid or monomeric BaxΔC. Like MAC of HeLa and FL5.12 cells, rBax channels displayed multiple conductance levels, a slight cation selectivity, single transitions of up to 2 nS, and were voltage independent (Figure 4B; Pavlov *et al*., 2001; Guo *et al*., 2004). MAC of FL5.12 cells and rBax channels are similarly heterogeneous with respect to their peak conductance, as shown in the detection frequency profiles of peak conductance of Figure 4B. The mean conductance of MAC of apoptotic FL5.12 cells and HeLa cells, and rBax channels was 4.5 ± 2.4, 3.3 ± 1.3, and 5.0 ± 3.0 nS, respectively (mean ± SD; n = 57, 22, and 35 patches, respectively; conductances below 1.5 nS were not considered). These findings indicate rBax forms channels with activity similar to MAC.

MAC and Recombinant Bax Channels Are Similarly Affected by Cytochrome c, Ribonuclease A, and Hemoglobin

Cytochrome *c* is a 12.5-kDa, positively charged protein that is presumably the physiological permeant ion for MAC. As shown in Figure 5, A and C, the current flow through MAC of FL5.12 cells and rBax channels decreased at least 10% upon perfusion with cytochrome *c* in 64% of the patches whose initial conductance was larger than ~2 nS but <5.4 nS; the conductance was reduced by cytochrome *c* in only 7% of the patches outside this range. As was found with MAC, 14-kDa ribonuclease A induced a ≥10% current decrease in 100% of the patches if the conductance of the rBax channels was 1.4–5.4 nS, and it had this effect on only 20% of the rBax channels outside this range (Figure 5, B and C). Finally, hemoglobin had no effect on rBax channels regardless of initial conductance (Figure 5, B and C). The summary in Figure 5C illustrates the similar effects of cytochrome *c*, ribonuclease A and hemoglobin on MAC and rBax channels.

Further examination of the effects of cytochrome *c* on MAC revealed two likely mechanisms for inducing the decrease in conductance, which are referred to by us as type 1 and type 2 effects (Guo *et al*., 2004). The type 1 effects on MAC are probably due to a partitioning of cytochrome *c* into MAC's pore, and the characteristics are a 5–50% decrease in conductance that is reversible, voltage dependent, and associated with an increase in noise (Guo *et al*., 2004). Like MAC, the type 1 effects of cytochrome *c* on rBax channels are a reversible decrease in the conductance and an increase in noise as shown in the current trace of Figure 5D. The type 2 effects of cytochrome *c* on MAC likely correspond to a destabilization of MAC's open state (Guo *et al*., 2004). The type 2 effects on rBax channels are a ≥50% decrease in

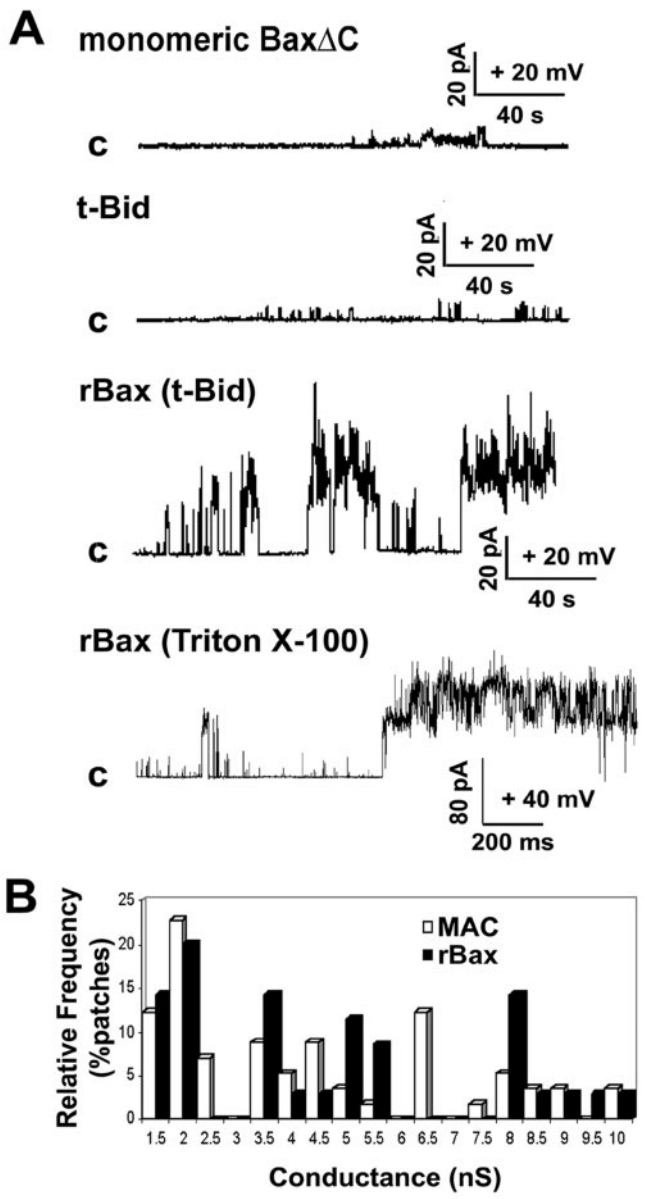

Figure 4. MAC and rBax display similar single channel characteristics. (A) Current traces show the channel activities recorded when monomeric BaxΔC, t-Bid, or oligomeric BaxΔC (rBax) were included in the tip of the micropipette and seals were formed on giant liposomes formed without protein. No channel activities developed when the tips of the microelectrodes were loaded with buffer only (our unpublished data). (B) Histogram compares the frequency profile of recording peak conductances for MAC of apoptotic FL5.12 cells (MAC) and channels formed by rBax. The peak conductances of MAC and rBax channels were determined from amplitude histograms and shown as a function of their frequency (n = 57 and 35 independent patches displaying MAC and rBax channels, respectively). Bin width was 0.5 nS and conductances below 1.5 nS were not considered. See *Materials and Methods* for formation of rBax (BaxΔC oligomers formed by exposure to Triton X-100 or t-Bid).

conductance (Figure 5A), which is typically not reversible. Like MAC, the type 2 effects of cytochrome *c* on rBax channels are dose dependent (our unpublished data), and occur without an increase in noise. Hence, cytochrome *c* has similar effects on MAC and Bax channel activities.

MAC Activity Is Detected in Mitochondria of Apoptotic Cells Lacking Bax but Not Those Lacking Both Bax and Bak

The multidomain proapoptotic proteins Bax and Bak are functionally redundant with respect to triggering apoptosis by different stimuli (Wei *et al*., 2001). Like Bax, Bak is known to form oligomers in the mitochondrial outer membrane early in apoptosis (Mikhailov *et al*., 2001, 2003; Cheng *et al*., 2003). A final test of the involvement of Bax in MAC activity used staurosporine-treated MEF cell lines that were and were not deficient in Bax, and both Bax and Bak. The mean conductance of mitochondria isolated from control and staurosporine-treated cells was measured using patch-clamp techniques. A significant increase in outer membrane permeability was observed in mitochondria of parental and Bax-deficient cells compared with those of untreated cells (Figure 6). This increase in mean conductance is consistent with the presence of MAC activity in apoptotic mitochondria as was found with HeLa cells in Figure 1 and with IL-3–starved FL5.12 cells in our previous studies (Pavlov *et al*., 2001). However, no increase in mean conductance was observed in mitochondria of staurosporine-treated cells deficient in both Bax and Bak. Furthermore, the same staurosporine treatment induced cytochrome *c* release from parental and Bax-deficient cells, but not cells deficient in both Bax and Bak. These findings indicate that deficiency of Bax is not sufficient to prevent MAC formation and cytochrome *c* release during intrinsic apoptosis. That is, Bax and Bak are functionally redundant with respect to these two events.

DISCUSSION

Bcl-2 family proteins regulate apoptosis, in part, by controlling formation of the MAC, which is a putative cytochrome *c* release channel induced early in the intrinsic apoptotic pathway. This report provides the first direct demonstration that Bax is a component of MAC in staurosporine-treated HeLa cells because immunoprecipitation of Bax depletes MAC activity from apoptotic lysates and fractions of mitochondria. The frequency of detecting MAC is higher in the mitochondrial lysates from apoptotic cells compared with control cells and MAC activity is preferentially associated with the oligomeric fraction, and not the monomeric fraction, of Bax. The channel activity of recombinant Bax is similar to MAC activity, and MAC and Bax channel activities are similarly modified by cytochrome *c*. The mean conductance of patches of mitochondria isolated from HeLa cells after GFP-Bax translocation is significantly higher than those from untreated cells, consistent with onset of MAC activity. In contrast, the mean conductance of patches of mitochondria indicate MAC activity is present in apoptotic cells deficient in Bax but absent in apoptotic cells deficient in both Bax and Bak. These findings indicate that Bax is a component of MAC in staurosporine-treated HeLa cells and suggest that Bax and Bak are functionally redundant as components of MAC.

Although many lines of evidence support the involvement of Bax in MAC activity (Antonsson *et al*., 1997; Schlesinger *et al*., 1997; Wei *et al*., 2001; De Giorgi *et al*., 2002; Kuwana *et al*., 2002; Mikhailov *et al*., 2003; Polster *et al*., 2003), we used immunodepletion studies to directly determine whether the multidomain BCL-2 family protein Bax is a component of MAC from staurosporine-treated HeLa cells. Anti-Bax antibodies immunodepleted almost all the MAC activity from the total lysates and the oligomeric fraction (Figure 3B; $p \leq 0.004$ and 0.007, respectively). These data indicate Bax is

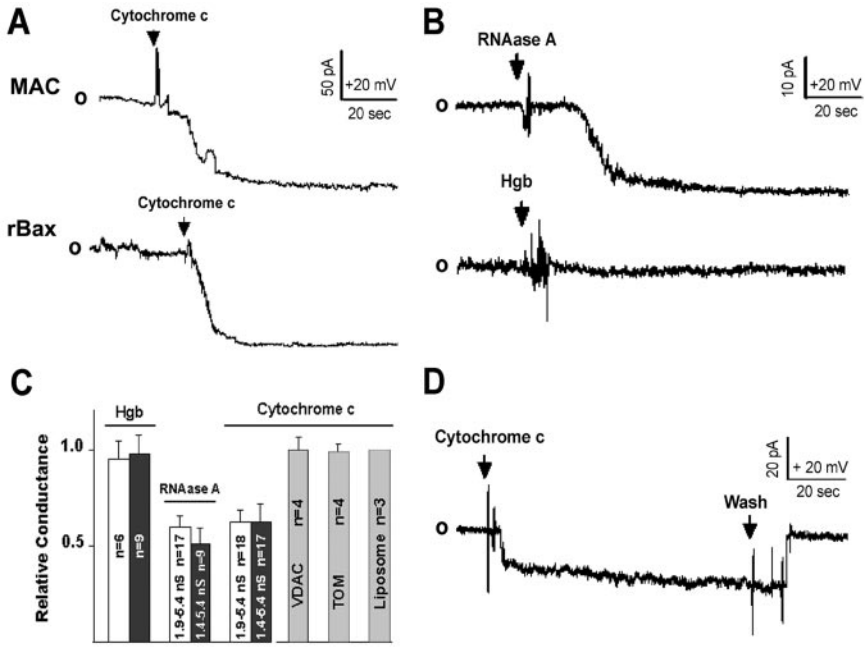

Figure 5. MAC and rBax channel activities are similarly affected by cytochrome *c*, ribonuclease A, and hemoglobin. (A) Current traces of MAC of apoptotic FL5.12 cells and oligomeric BaxΔC (rBax) channels show similar type 2 effects of perfusing 100 μM cytochrome *c* into the bath. (B) Current traces show the effects of perfusion of the bath with ribonuclease A (RNase A) or hemoglobin (Hgb) on rBax channels. (C) Histograms show the conductance of MAC (open) and rBax (filled) channels in the presence relative to the absence of 100 μM cytochrome *c*, 100 μM ribonuclease A, or 200 μM hemoglobin as indicated. Cytochrome *c* and ribonuclease A similarly exert a maximal effect on MAC and rBax channels with peak conductances of 1.9–5.4 and 1.4–5.4 nS, respectively. Similarly, the effects of 100 μM cytochrome *c* on VDAC, translocase outer membrane channels, and liposomes conductances are shown. The conductance of MAC is much greater than that of VDAC and translocase outer membrane channels (0.7–0.75 nS) as described previously (Pavlov *et al*., 2001). Mean ± SE is shown and n is the number of independent determinations. (D) Current trace of rBax channel shows the type 1 effect of 100 μM cytochrome *c* that is reversible upon washing of the bath with 150 mM KCl, 5 mM HEPES, pH 7.4, by perfusion. Voltage was +20 mV, and filtration was 2 kHz with 5-kHz sampling for all current traces. Other conditions were as described in Figure 4.

associated with the structure responsible for MAC activity in staurosporine-treated HeLa cells. Untreated cultures of HeLa cells have 10–12% apoptotic cells (Cirman *et al*., 2004). Hence, it was not surprising that a low level of MAC activity was detected and that a low level of Bax was immunoprecipitated in the control total lysates (Figures 3A, bottom, and 2D).

Two observations indicate Bax oligomers underlie MAC. First, MAC activity is preferentially detected in the oligomeric, not the monomeric, fraction (Figure 2; $p \leq 0.003$). Second, essentially all the MAC activity is depleted by antibodies that specifically immunoprecipitate Bax oligomers and not Bax monomers. These antibodies immunoprecipitated all the Bax in the oligomeric fraction, 20% of the Bax in the total mitochondrial lysates, and none of the monomeric Bax (Figure 3). These results are consistent with the report that the 260-kDa fraction contains oligomeric Bax (Antonsson *et al*., 2000, 2001) and indicate most of the Bax is in the monomeric, inactive form in mitochondria of apoptotic HeLa cells. These findings agree with the results of others reporting the formation of Bax oligomers in apoptotic fractions, detected using cross-linking agents (Antonsson *et al*., 2001; Cheng *et al*., 2003; Mikhailov *et al*., 2003; Guihard *et al*., 2004). Collectively, these findings support the hypothesis that oligomers of Bax are components of MAC. However, these results do not exclude the possible involvement of mitochondrial lipids and/or proteins as pore-forming components in MAC as suggested by other studies (Kuwana *et al*., 2002; Polcic and Forte, 2003; Antonsson, 2004).

Electrophysiological data show that oligomeric rBax, but not monomeric BaxΔC, mimics the single channel activity of MAC from apoptotic FL5.12 cells (Figures 4 and 5). Both MAC and oligomeric rBax channels are slightly cation selective and present similar, although not identical, frequency profiles of conductance (Figure 4C). Subtle differences in behavior may be due to a variety of factors, including additional components, e.g., lipids or proteins (Kuwana *et al*., 2002; Polcic and Forte, 2003; Antonsson, 2004), deletion of the last 20 amino acids in the recombinant Bax used in these experiments, and reconstitution. Note, however, BaxΔC induces cytochrome *c* release in isolated mitochondria and liposomes, and the C terminus is not needed for translocation to mitochondria (Tremblais *et al*., 1999; Appaix *et al*., 2000; Terrones *et al*., 2004). Similarly, MAC-like activity also is found in mitochondria of VDACless yeast forced to express hBax (Pavlov *et al*., 2001). As shown above, immunodepletion of oligomeric Bax from apoptotic fractions almost eliminates MAC activity. The recombinant protein and the immunoprecipitation experiments strongly link MAC activity to Bax oligomers.

MAC is a putative cytochrome *c* release channel. Cytochrome *c* is known to modify the current through MAC in a manner consistent with its permeation (Guo *et al*., 2004). The flow of currents through MAC and rBax channels is similarly modified by cytochrome *c* and ribonuclease A. Ribonuclease A is slightly larger and less positively charged at neutral pH than cytochrome *c*, but both molecules have the same effects on MAC and Bax channel activities (Figure 5). Finally, 10- and 17-kDa dextrans also block MAC and Bax channels consistent with their ability to be transported (Guo *et al*., 2004; our unpublished data). The mean conductances of MAC and Bax channels suggest their mean pore sizes are ~5 nm [calculated by the method of Hille (2001)], consistent with their ability to allow passage of cytochrome *c*. Finally, proteoliposomes containing MAC or Bax fail to retain cytochrome *c* (Antonsson *et al*., 2000; Saito *et al*., 2000; Pavlov *et al*., 2001). These findings are consistent with the work of several labs that implicate the channel forming properties of Bax with the release of cytochrome *c* (Antonsson *et al*., 1997, 2000; Schendel *et al*., 1997; Desagher *et al*., 1999; Cheng *et al*., 2001; Saito *et al*., 2000; Polster *et al*., 2001; Kuwana *et al*.,

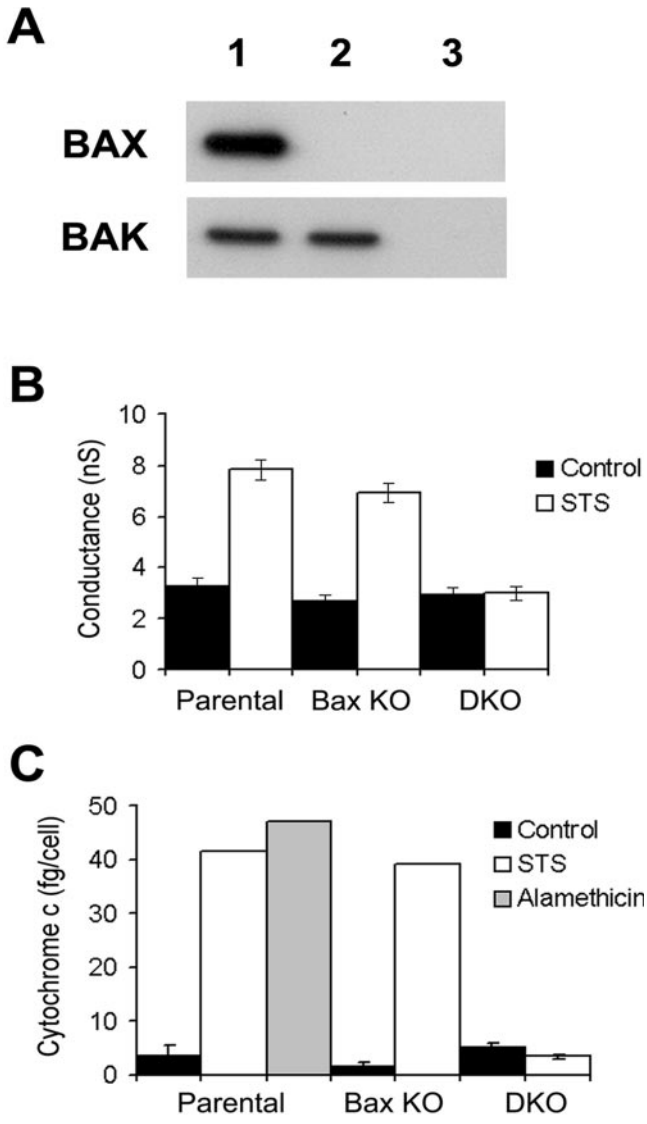

Figure 6. An increase in outer membrane permeability occurs in mitochondria of staurosporine-treated parental and Bax-deficient cells but not Bax/Bak deficient cells. (A) Western blots show expression of Bax and Bak in three MEF cell lines: lane 1, parental Bax+/+Bak+/+; lane 2, single Bax knockout (KO) Bax−/−Bak+/+; and lane 3, double KO (DKO) Bax−/− Bak−/−. Total membrane extracts containing 20 μg of protein were probed for Bax and Bak, respectively. The same results were obtained with whole cell extracts (our unpublished data). (B) Mitochondria were isolated from the indicated cell lines that were and were not treated with staurosporine for 16 h. The mean conductance of the outer membrane was measured by patch clamping isolated mitochondria (n = 20–23 patches per condition). (C) Cytochrome *c* release was assayed by ELISA in the supernatants after permeabilization of the cells with digitonin as described in *Materials and Methods*. When indicated, alamethicin (80 μg/ml) was added during digitonin treatment as a positive control for cytochrome *c* release (Polster *et al*., 2001).

2002). The association of MAC with Bax is further strengthened by recent pharmacological studies in which agents that block cytochrome *c* release induced by Bax and a BH-3 peptide also block MAC activity in patch-clamp experiments (Polster *et al*., 2003; Martinez-Caballero *et al*., 2004). Together, these data are consistent with the notion that cytochrome *c* is released during apoptosis by the Bax-containing channel MAC.

There are additional data linking MAC activity to cytochrome *c* release and the proapoptotic protein Bax. A statistically significant increase in mean conductance of patches of isolated mitochondria is associated with the onset of MAC activity about the time of cytochrome *c* release (Gross *et al*., 1998; Pavlov *et al*., 2001). Similarly, an increase in the mean conductance of patches of mitochondria isolated from clone 10 cells treated with staurosporine is observed when punctate GFP-Bax fluorescence appears in mitochondria (Figure 1). Furthermore, the release of cytochrome *c* from mitochondria occurs at the time that GFP-Bax fluorescence becomes punctate (Figure 1, A–H). These findings are consistent with the increase in whole-patch conductance marking onset of MAC activity when Bax forms oligomers and cytochrome *c* is released in apoptotic cells.

Other proteins have been examined for their possible role in MAC activity. No adenine nucleotide translocator (ANT) or VDAC is present in the oligomeric apoptotic fractions (Antonsson *et al*., 2001) containing MAC activity. Furthermore, the putative permeability transition pore (PTP) components ANT and VDAC were not coimmunoprecipitated with Bax in total lysates from apoptotic HeLa cells (our unpublished data). These findings indicate ANT and VDAC are not requisite to MAC activity and are consistent with the lack of effect of the PTP inhibitor cyclosporine A on MAC (Martinez-Caballero *et al*., 2004). Mikhailov *et al*. (2001, 2003) report that some Bak coimmunoprecipitates with Bax. In fact, some Bak coimmunoprecipitates with Bax (our unpublished data) in the oligomeric 260-kDa fractions of our system of HeLa cells. This observation may underlie the finding that essentially all the MAC activity can be immunodepleted with Bax antibodies in staurosporine-treated HeLa cells (Figure 4) and suggest Bak, like Bax, may be a constituent of MAC.

Previous studies suggest Bak and Bax are functionally redundant with respect to their role in apoptosis, e.g., in cytochrome *c* release. Cytochrome *c* release occurs in Bax−/−Bak+/+ and Bax+/+Bak−/− cells but not in Bax−/−Bak−/− cells during staurosporine treatment of MEF cells (Cheng *et al*., 2001, 2003; Wei *et al*., 2001). Hence, deletion of both Bax and Bak is needed to make these cells resistant to apoptosis. Using the same cells, our findings indicate that MAC was present and cytochrome *c* was released in apoptotic Bax−/−Bak+/+ cells. Further, MAC was absent and cytochrome *c* was not released in Bax−/−Bak−/− cells during staurosporine-treatment (Figure 6). Although alternative interpretations are possible and most of the findings presented in this study indicate that Bax is a component of MAC, the data of Figure 6 indicate that Bak may replace Bax as a structural component of MAC in Bax−/−Bak+/+ cells. That is, Bax and Bak are functionally redundant with respect to MAC.

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