Assembly of the Mitochondrial Apoptosis-induced Channel, MAC*§

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Although Bcl-2 family proteins control intrinsic apoptosis, the mechanisms underlying this regulation are incompletely understood. Patch clamp studies of mitochondria isolated from cells deficient in one or both of the pro-apoptotic proteins Bax and Bak show that at least one of the proteins must be present for formation of the cytochrome c-translocating channel, mitochondrial apoptosis-induced channel (MAC), and that the single channel behaviors of MACs containing exclusively Bax or Bak are similar. Truncated Bid catalyzes MAC formation in isolated mitochondria containing Bax and/or Bak with a time course of minutes and does not require VDAC1 or VDAC3. Mathematical analysis of the stepwise changes in conductance associated with MAC formation is consistent with pore assembly by a barrel-stave model. Assuming the staves are two transmembrane α-helices in Bax and Bak, mature MAC pores would typically contain ~9 monomers and have diameters of 5.5–6 nm. The mitochondrial permeability data are inconsistent with formation of lipidic pores capable of transporting megadalton-sized macromolecules as observed with recombinant Bax in liposomes.

Permeabilization of the mitochondrial outer membrane is the commitment step in intrinsic apoptosis. This process is tightly regulated by Bcl-2 family proteins that control formation of the megachannel mitochondrial apoptosis-induced channel (MAC) in this membrane. MAC formation correlates with release of pro-apoptotic factors, including cytochrome c from the intermembrane space into the cytosol, and initiates apoptosis (1–7).

MAC is absent from normal mitochondria but forms in the outer membrane early in apoptosis, reaching peak conductances of 1.5–5 nS. This channel is formed in the presence of the multidomain pro-apoptotic proteins Bax and/or Bak (8–13), and may be composed of these proteins along with other components (14, 15). Unlike Bax, Bak is normally a resident of the mitochondrial outer membrane and is bound to VDAC2, another outer membrane protein (16). However, Bak is not available for oligomerization until another pro-apoptotic protein, like t-Bid, disrupts the interaction of Bak with VDAC2. In contrast, most Bax is located in the cytoplasm until an apoptotic signal induces the translocation of Bax to the outer membrane of mitochondria and eventual Bax oligomerization in this same membrane (14, 17).

Bax and Bak have multiple putative transmembrane domains; the amphipathic helices 5 and 6 of Bax are predicted to form, at least in part, the pore of the cytochrome c release channel (18). Bax lacking helices 5 and 6 does not translocate to mitochondria nor cause cytochrome c release (19, 20). Given the structural similarities between Bax and Bak, the same helices may be important in formation of the MAC pore by both proteins (21). Although Bax and Bak are certainly involved in MAC formation, the exact molecular composition of this channel remains unknown.

In this study we report that Bax and Bak are functionally redundant with regard to MAC formation and cytochrome c release in mouse embryonic fibroblasts (MEF). This is true despite the fact that Bak normally resides in the outer membrane, whereas Bax is generally translocated to this membrane to induce MAC formation. Our experimental design bypasses Bax translocation and any underlying autocatalytic mechanism that might be involved (22). Instead, it focuses on formation of the MAC pore. Early MAC-associated conductance increments are relatively small, suggesting that Bax-dependent formation of the cytochrome c-permeable pore does not occur prior to membrane insertion of Bax. Mathematical modeling of the conductance changes indicates that, if MAC is a circular pore assembled by sequential addition of helices 5 and 6 from Bax and/or Bak monomers, the mature, cytochrome c transport–competent pore is likely a 9–10-mer of these proteins.

**EXPERIMENTAL PROCEDURES**

*Cell Lines and Isolation of Mitochondria—Parental (MEF) and derivative (Bax<sup>−/−</sup>Bak<sup>+/+</sup>, Bax<sup>+/+</sup>Bak<sup>−/−</sup>, Bax<sup>−/−</sup>Bak<sup>−/−</sup>, and VDAC1<sup>−/−</sup>/VDAC3<sup>−/−</sup>) cell lines were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% nonessential amino acids, and 1% 1-glutamine (23,
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24). Cells at 80% confluence were harvested with trypsin, and mitochondria were isolated as described previously (25). As indicated, apoptosis was induced with 1 μM staurosporine 16 h before harvesting. FL5.12 cells were grown in Iscove’s modified Eagle’s medium with 10% fetal bovine serum and 10% WEHI-3B supplement (26), and mitochondria were isolated as described previously (13).

Recombinant Proteins—N-terminally His-tagged, full-length mouse BID protein and t-Bid were prepared using the cysteine-less clone, p22BID30S126S, as described previously (27, 28). Monomeric human BAX, truncated for 20 amino acids at the C terminus (rBaxΔC), was expressed in Escherichia coli and purified as described previously (14, 29, 30).

Immunoblotting—Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and detected by ECL (GE Healthcare). The primary antibodies were against Bax (N-20, Santa Cruz Biotechnology), Bak (anti-N terminus, Millipore Upstate Biotechnology, Inc.), and VDAC1 (31HL, Calbiochem). The secondary antibodies were horse-radish peroxidase-coupled goat anti-rabbit and anti-mouse (Sigma). When stated, pixel densities were quantified by densitometry (Scion Imaging, National Institutes of Health).

Cytochrome c Release Assays—Isolated mitochondria (0.2 mg/ml) were incubated with t-Bid (0.02–1 μM), Bid (1.6 μM), or an equivalent amount of vehicle (150 mM KCl, 20 mM HEPES, pH 7, 30% glycerol, 2% octyl glucoside) for 5–30 min at room temperature in 70 mM sucrose, 230 mM mannitol, 1 mM EDTA, 2.3.3 (Strathclyde Electrophysiological Software; courtesy of J. Dempster, University of Strathclyde, UK) were used for current analysis. Sample rate was 5 kHz with 2-kHz filtration. When necessary, simple perfusion of the chamber often resulted in excision of patches. Permeability ratios were calculated from the reversal potential in the presence of a 150:30 mM KCl gradient as described previously (32). Dibucaine (Sigma D0638) and cytochrome c (Sigma C-7752) were introduced and removed by perfusion of the bath (0.5 ml volume) with 3–5 ml of patching solution.

The channel activity of monomeric human Bax, truncated for 20 amino acids at the C terminus (BaxΔC20), activated by t-Bid (14, 29, 30) was characterized in liposomes devoid of other proteins. Micropipette tips were filled with media containing 380 ng/μl monomeric BaxΔC plus 35 ng/μl t-Bid and then backfilled with patching media. Hence, the actual Bax concentration was lower than that loaded in the tips. Seals were formed with these micropipettes on giant liposomes prepared as described previously (32, 33).

Mathematical Analysis—The number of helices forming MAC was estimated assuming α-helices had a diameter (D) of 1.2 nm (34) and aligned on center perpendicular to the membrane. The resulting polygon had a variable number (n) of vertices, where the center of each helix was located at a vertex of the polygon. The area (A) was approximated by a circle whose area was calculated by Equation 1 as more helical staves were added to the polygon in an approach similar to Sanson et al. (35),

\[
A = \pi D^2 \left( \frac{1}{1 - 2 \cos^2 \left( \frac{2\pi}{n} \right)} - \frac{1}{2} \right)^2
\]

(Eq. 1)

The area was then corrected by adding n times the triangular area (2A_\text{r}) between staves in the pore. A_\text{r} is defined by subtracting the area of the two arcs A_\text{r} (Equation 2) and A_\text{p} (Equation 3) from the area of the right triangle A_\text{p} (Equation 4) that is defined by the base r and height R + r in Equation 5 (see supplemental Fig. 1S).

\[
A_\text{r} = \frac{(n - 2)}{2n} \pi r^2 = \pi r^2(n - 2)/4n
\]

(Eq. 2)

\[
A_\text{p} = \frac{1}{2} \pi R^2
\]

(Eq. 3)

\[
A_\text{p} = \frac{1}{2} \text{base} \cdot \text{height} = \frac{1}{2} \sqrt{(r + R)^2 - r^2} = \frac{1}{2} (r + R) \cos(\pi/n)
\]

(Eq. 4)

The conductance of at least 1.5 nS was seen within 30 min of seal development; this typically occurred when t-Bid was included in the micropipette tip. This channel activity is referred to as MAC in mitochondria isolated from Parental cells, MAC-Bak from Bax KO cells, and MAC-Bax from Bak KO cells. The permeability of untreated mitochondria is also reflected in the seal resistances that were not found to be significantly different for the various cell lines (n = 10–23 independent patches for each cell line).

Levels of t-Bid used in the patch pipettes for these experiments were 20 nm for Parental and Bax KO cells, 250 nm for Bak KO cells, and 1 μM for DKO cells unless otherwise indicated. pClamp version 8 (Axon Instruments) and WinEDR version 2.3.3 (Strathclyde Electrophysiological Software; courtesy of J. Dempster, University of Strathclyde, UK) were used for current analysis. Sample rate was 5 kHz with 2-kHz filtration.
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\[ A_c = A_\Delta - (A_r + A_\varphi) = \]
\[ \frac{1}{2}r \sqrt{(r + R)^2 - r^2} - \left( \frac{1}{2n} \pi R^2 + \frac{n - 2}{4n} \pi r^2 \right) \quad (\text{Eq. 5}) \]

where we define \( R \) in Equation 6 as,
\[ R = (\sin(\pi/n))^{-1} - 1 \quad (\text{Eq. 6}) \]

To determine the relationship between the number \( n \) of \( \alpha \)-helices and the area, these corrected data were best fit (correlation coefficient of 0.9991) in Fig. 7A by the polynomial Equation 7,
\[ \text{area} = 7 \times 10^{-12} n^2 + 9 \times 10^{-11} n - 4 \times 10^{-10} \quad (\text{Eq. 7}) \]

The cross-sectional pore area was converted into predicted conductance that was corrected for access resistance using the method of Hille (36) through Equation 8.
\[ \rho_{\text{pore}} = (L + (\pi a)/2)(\rho_{\text{w1}}/\pi a^2) \quad (\text{Eq. 8}) \]

where \( \rho_{\text{pore}} \) and \( \rho_{\text{w1}} \) are the resistivity of the pore and a solution of 0.15 M KCl; \( L \) is pore length (5.5 nm corresponds to the thickness of the outer membrane (37)), and \( a \) is the radius of the pore. This equation established the relationship between the conductance and number of \( \alpha \)-helices forming the MAC pore (Fig. 7B).

MAC formation occurred in incremental steps of conductance (transition size) often of \( \sim 300 \) pS. These same equations were used to estimate the number of \( \alpha \)-helices inserted into the barrel of the MAC pore to generate the observed transitions. For example, an initial transition of \( \sim 300 \) pS corresponds to insertion of 6–8 helices. However, the relationship between the transition size and the number of \( \alpha \)-helices inserted into the barrel is not linear, i.e. transitions of \( \sim 300 \) pS into moderately larger 1–3-nS channels likely correspond to the insertion of 1 \( \alpha \)-helix to the pore (Fig. 7D). The predicted transition sizes generated by insertions of 1, 2, 4, or 6 \( \alpha \)-helices into pores of varying conductance (Fig. 7D) were calculated using the method of Hille (36) and corrected for access resistance (Equation 8), where the radius \( (a) \) was calculated from the area in Equation 7. Analyses were done using Excel and Prism 4 from GraphPad Software.

RESULTS

The presence of either Bax or Bak is sufficient to allow initiation of the mitochondria-dependent death program, although the absence of both proteins is necessary to inhibit cytochrome \( c \) release (38, 39). MEF lines deficient in either or both Bax and Bak were used to define the roles of these Bcl-2 family proteins in MAC formation and cytochrome \( c \) release. These cell lines include the Parental (Bax\( ^{+/+} \)/Bak\( ^{+/+} \)), the single knockouts Bax KO (Bax\( ^{-/-} \)/Bak\( ^{+/+} \)) and Bak KO (Bax\( ^{+/+} \)/Bak\( ^{-/-} \)), as well as the double knock-out DKO (Bax\( ^{-/-} \)/Bak\( ^{-/-} \)). The presence of Bax and Bak in cell extracts of each MEF line was assessed in the Western blots of Fig. 1A. The mitochondrial amounts of Bax and Bak for these cell lines treated without (control) or with (apoptotic) 1 \( \mu \)M staurosporine are shown in the histograms of Fig. 1B relative to VDAC, which is constitutively expressed in the outer membrane. Although the relative levels of Bak remained stable, Bax levels in mitochondria increased almost 2–4-fold after induction of apoptosis.

MAC Forms in the Mitochondrial Outer Membrane of Apoptotic MEF Cells Expressing Bax and/or Bak—We previously found MAC could form in Bax KO but not DKO mitochondria (8); these studies were expanded to include Bak KO cells. Activity of the mitochondrial outer membrane channel, MAC, is detected as increased conductance when mitochondria of apoptotic cells are patch-clamped. This increase in membrane permeability ranged from 3 to 5 nS and was statistically significant \((p = 0.0001; n = 20–23 \text{ patches})\) for mitochondria from Parental, Bak KO, and Bak KO lines after induction of apoptosis by staurosporine (Fig. 1C). MAC was scored “present” if the increase in conductance of the outer membrane was at least 1.5 nS. These data indicate MAC was formed by 16 h after induction of apoptosis in these cells. In contrast, the conductances of mitochondrial patches from untreated cells and apoptotic DKO cells were essentially identical \((p = 0.8; n = 20–23 \text{ patches})\), indicating that MAC formation requires expression of either Bax or Bak (Fig. 1C).

MAC is the outer membrane channel associated with cytochrome \( c \) release from mitochondria to the cytosol during intrinsic apoptosis. The fraction of cytochrome \( c \) released to the cytosol was determined for the four MEF lines following treatment with staurosporine. As shown in Fig. 1D, cytochrome \( c \) was released to the cytosol in Parental, Bax KO, and Bak KO cells, but not DKO cells at the time MAC was detected during staurosporine treatment. These results show that expression of either Bax or Bak is necessary for cytochrome \( c \) release and MAC formation. Furthermore, these findings indicate that Bax and Bak are functionally redundant with respect to both processes.

The MAC activity of MEF mitochondria showed electrophysiological properties similar to those reported previously for mitochondria from other apoptotic cells. As previously found, the peak conductances were large and variable (in the range 1.5–5 nS), showed no obvious dependence on voltage (±50 mV), and were slightly cation-selective (8, 10, 13). To further explore the possibility that MAC activity of mitochondria containing only Bax is different from that of mitochondria containing only Bak, a system triggered by t-Bid was established in which MAC formation could be monitored in real time while cytochrome \( c \) is released.

Cytochrome \( c \) Release Induced by t-Bid Is Dose-dependent and Requires Bax or Bak Expression—The BH3-only protein Bid is cleared to form activated t-Bid during apoptosis and triggers the release of cytochrome \( c \) from mitochondria of cells that express Bax and/or Bak (29, 40, 41). Mitochondria from the various MEF cell lines were incubated with different concentrations of t-Bid for up to 30 min, and the amounts of cytochrome \( c \) released were measured (Fig. 2). Although uncleaved Bid or vehicle alone had no effect, t-Bid induced a dose-dependent release of cytochrome \( c \) with \( \text{EC}_{50} \) (effective concentration for release of 50% of cytochrome \( c \)) of 7 nM for Parental, 17 nM for Bax KO, and \( \sim 250 \text{ nM} \) for Bak KO mitochondria (Table 1 and Fig. 2A). As expected, t-Bid failed to induce cytochrome \( c \)
The release of cytochrome c from mitochondria. Increasing the dose of t-Bid from 20 nM to 1 μM increased the extent of release after 30 min most dramatically in mitochondria of DKO cells. The kinetics show single MAC-Bax (Fig. 1 and Table 1). The frequency of observing MAC conductances is summarized in the histogram of Fig. 3C as % patches ≥1.5 nS. Patches were scored positive for MAC if they developed a sustained, voltage-independent (±50 mV) increase in conductance of at least 1.5 nS within 30 min of sealed formation. Whenever possible, additional MAC characteristics were also assessed for assignment, including a slight cation selectivity and blockade by either cytochrome c (10) or dibucaine (12), which are known MAC effectors (Table 1 and Fig. 4). Fortunately, MAC activity can be distinguished from that of the outer membrane metabolite channel, VDAC, and the protein import channel TOM by peak conductance, kinetics, and transition sizes (13).

The characteristic of increasing conductance ≥1.5 nS within 30 min that was attributed to MAC development might instead be due to a loss of the seal between the micropipette and the membrane. Consistent with this possibility, there was a background level of patches that developed conductances ≥1.5 nS in controls. In contrast to the parental and single knock-outs, about the same levels of patches with conductances of >1.5 nS was detected in t-Bid-treated mitochondria of DKO cells and parental mitochondria treated with either vehicle or Bid (Fig. 3C). Hence, Bax and/or Bak expression was required to significantly increase the observation of t-Bid-treated patches with 1.5-nS conductances. Because similar background levels were found with Bid in

**FIGURE 1.** MAC forms in the mitochondrial outer membrane of apoptotic cells expressing Bax and/or Bak. A, Western blots show normal expression of Bax (5 μg of protein) and Bak (30 μg of protein) in whole cell extracts of Parental and indicated knock-out MEF cells. β, changes in Bax and Bak protein levels in mitochondria isolated from Parental and indicated knock-out cell lines were estimated by densitometry after immunoblotting. Cells were harvested 16 h after addition of vehicle (–) or 1 μM STS (+) to induce apoptosis. Pixel densities of Bax and Bak bands were normalized to those of VDAC (a constitutive mitochondrial outer membrane protein) in the same gel lanes using the Parental (–) condition as standard. Data are the average of two independent experiments. C, peak conductances of outer membranes were measured by directly patch clamping mitochondria isolated from control and STS-treated MEF cells. The increase in conductance corresponds to the difference between mean conductance of STS-treated and control patches. These permeability increases are consistent with the presence of MAC in mitochondria of apoptotic Parental, Bak KO, and Bak KO cells, but not in the STS-treated DKO cells. Data are mean ± S.E. of at least three different preparations. E and F, current traces show single MAC-Bax (E) and MAC whose peak conductances were −5 nS that were recorded at +10 and −20 mV from mitochondria isolated from Bak KO and parental cells, respectively, after treatment with 1 μM STS for 16 h. Filtration was 2 kHz with 5-kHz sampling. Dotted lines indicate the 0 pA level. Bath solution was 150 mM KCl, 5 mM HEPES, 0.23 mM CaCl2, 1 mM EGTA, pH 7.4.

release in mitochondria from DKO cells. The kinetics show the release of cytochrome c occurs over minutes (Fig. 2B). The release of cytochrome c from mitochondria lacking either Bax or Bak was delayed compared with that of Parental mitochondria. Increasing the dose of t-Bid from 20 nM to 1 μM increased the extent of release after 30 min most dramatically in mitochondria lacking Bak.

**t-Bid Triggers MAC Formation in Mitochondria Containing Bax and/or Bak**—The conditions established for cytochrome c release were applied to patch clamp experiments to monitor MAC formation. t-Bid was included in the micropipette so that this protein could interact with the cytosolic face of the outer membrane patch, and membrane permeability was continuously monitored as current flow. Because of differences in EC50 for cytochrome c release, the t-Bid concentration used to backfill the micropipette tip was 20 nM for mitochondria from Parental and Bak KO cells, 250 nM for Bax KO mitochondria, and 1 μM for DKO mitochondria. MAC formation was detected as increases in patch conductance with time, i.e. increases in current flow, after development of a seal between the membrane patch and the micropipette.

Using this approach, MAC typically formed within 10 min of sealing the micropipette on intact mitochondria of normal Parental, Bax KO, and Bak KO cells in ~65% of the patches if t-Bid, but not if Bid or vehicle, was included in the micropipette (Fig. 3 and Table 1).
parental mitochondria or t-Bid-treated liposomes and DKO mitochondria, it is unlikely that pure t-Bid formed large pores. These findings are consistent with those of Schendel et al. (42) who reported t-Bid alone formed relatively small pores.

**MAC Formation Is Independent of VDAC1 or VDAC3**—These data show that Bax or Bak are needed for MAC formation and raise the question whether the same is true for other mitochondrial outer membrane proteins. The metabolite channel VDAC is constitutively expressed in mitochondria and has several isoforms in mammalian cells (43). Although VDAC2 is known to suppress apoptosis by sequestering Bak under normal conditions (16), the roles of VDAC1 (the most common isoform) and VDAC3 are not fully understood. Baines et al. (23) reported that these proteins are not required for cytochrome c release. We examined MAC formation induced by t-Bid in mitochondria lacking VDAC1 and VDAC3, but expressing both Bax and Bak (Fig. 5). VDAC1−/− VDAC3−/− MEF cells are referred to as VDAC1VDAC3 KO cells.

t-Bid induced a dose-dependent release of cytochrome c from mitochondria of untreated VDAC1VDAC3 KO cells; vehicle and uncleaved Bid did not (Fig. 5B). The EC50 of t-Bid-induced cytochrome c release in untreated VDAC1VDAC3 KO mitochondria was similar to the one measured for untreated Parental MEF mitochondria (compare Fig. 5B with Fig. 2B). This result suggests that t-Bid might not have an indirect apoptotic effect mediated by VDAC1 as proposed by Rostovtseva et al. (44). As with Parental MEF mitochondria, t-Bid induced MAC formation detectable by patch clamping mitochondria of VDAC1VDAC3 KO cells (Fig. 5C). In contrast, vehicle and Bid did not induce MAC formation. MAC activity of VDAC1VDAC3 KO cells was similar to that from other cell types (Table 1). MAC from VDAC1VDAC3 KO cells also displayed the characteristic blockade (decrease in conductance and changes in noise level) induced by cytochrome c as well as the reversible inhibition by dibucaine (Fig. 5, D–F) (10, 12). These data indicate that neither VDAC1 nor VDAC3 has a role in t-Bid-induced MAC formation and cytochrome c release.

**MAC Formation Can Be Monitored in Real Time**—Continuous current measurement can be used to follow the increase in permeability of the outer membrane due to formation of the MAC pore in mitochondria from various MEF cell lines (Fig. 6, A–E). In fact, analysis of 123 conductance levels recorded from mitochondria from the four MAC-competent cell types showed considerable heterogeneity. These are Parental, Bak KO, Bak KO, and VDAC1VDAC3 KO cells. The time-dependent increases in permeability, i.e. patch conductance, were similar for MAC of all these cell types, with frequent occurrences of ~300 pS incremental steps over a time course of 5 or 10 min. Typically, the first transitions from base-line conductance (after seal formation) were 280 ± 74 pS (n = 12 patches). Larger steps, e.g. 600 or 1000 pS, were also observed (Fig. 6F). Similar stepwise increases in permeability were obtained with recombinant BAX lacking the C-terminal helix-9, which like wild-type Bax, is capable of forming high conductance channels in protein-free liposomes after activation by t-Bid (7, 8, 29, 45, 46) (Fig. 6F). These results also suggest that helix 9 of Bak is not involved in MAC formation.

**TABLE 1**

| Electrophyiologys of MAC activity induced by t-Bid | Parental (MAC) | Bax KO (MAC-Bax) | Bak KO (MAC-Bax) | VDAC1VDAC3 KO (MAC) | FLS12 (MAC)* |
|-----------------------------------------------|----------------|-----------------|-----------------|-----------------|--------|
| t-Bid (EC50 [nM]) and cytochrome c release    | 7              | 17              | 247             | 20              | NA     |
| t-Bid (micropipette [nM])                     | 20             | 20              | 250             | 1000            | NA     |
| MAC assembly frequency (% patches)           | 64 (n = 11)    | 68 (n = 12)    | 67 (n = 12)    | 75 (n = 12)    | NA     |
| Peak conductance (nS)                        | 3.5 ± 1.8 (n = 7) | 3.1 ± 2.0 (n = 8) | 3.0 ± 1.9 (n = 8) | 3.5 ± 1.5 (n = 9) | 25–45 (n = 57) |
| Ion selectivity (pNa/pK+)                     | 3.1 ± 1.4 (n = 5) | 3.7 (n = 1)     | 3.4 ± 1.2 (n = 4) | 3.4 ± 1.5 (n = 7) | 3.0 ± 0.9 |
| Voltage-dependent (+50 mV)                   | No             | No              | No              | No              | No     |
| No. of MAC assembled in 10 min               | 5 of 7         | 6 of 8          | 5 of 8          | 9 of 9          | NA     |
| Predicted diameter (nm)**                    | 6              | 5.6             | 5.5             | 6               | 4–7    |
| Predicted no. of helices forming pore**      | 18             | 17              | 17              | 18              | 16–21  |

* Data were included for comparison with MAC recorded from mitochonidria and proteoliposomes (8, 10, 13).

* n = number of independent determinations.

* See under "Experimental Procedures"; data are based on peak conductance assuming 1.2-nm helices are inserted normal to the membrane to form circular pores.
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MAC Can Be Modeled as a Barrel and Stave Pore—To determine whether the observed sequential increases in mitochondrial permeability could be explained in terms of a simple physical mechanism, MAC assembly was modeled as addition of staves to a barrel-like pore. In this model, MAC formation involves the addition of α-helices to the pore during oligomerization of Bax and/or Bak, which are presumed to be the constituents of MAC (not merely its organizing factors). Sequential incorporation of α-helices would increase the circumference, and hence the cross-sectional area, of developing pores in a predictable manner if the helices were isotropically inserted normal to the membrane plane. Under these assumptions, the predicted dependence of pore area on the number of Bax/Bak-derived α-helices can be calculated as shown in Fig. 7A. The supplemental Table 1S shows the expected maximal radius and conductance of circular pores formed by 3–25 α-helices, assuming the diameter of a single α-helix is 1.2 nm (34). Note that the observed conductances would be less than predicted here if the helices were tilted or if the shape of the pore was more slit-like than circular.

The cross-sectional area of a large pore can be estimated from its measured conductance after correcting for access resistance using the approach of Hille (36). The minimum number of transmembrane helices needed to form a pore with known conductance can be predicted from this area. Fig. 7B shows the relationship between the predicted number of helices in the pore and the various conductances observed during MAC assembly in current traces like those of Fig. 6. Hence, an estimate of the minimum number of α-helical “staves” forming a pore can be derived from the area calculated from its conductance. This relationship was used to estimate the minimum number of transmembrane helices in MAC observed in several cell types using various apoptotic inducers like STS-treated MEFs as well as yeast expressing human BAX and recombinant BAXΔC in protein-free liposomes (Fig. 7C). In general, under the assumption of untilted transmembrane helices forming pores with circular profiles, mature (cytochrome c transport competent) MAC is estimated to contain a minimum of 15–20 α-helices.

How well does the model predict the observed MAC-associated conductances? A minimal pore formed by three transmembrane helices is predicted to have a conductance of less than 20 pS, which is too small to be reliably detected in these

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**FIGURE 3.** MAC formation induced by t-Bid in normal mitochondria requires Bax or Bak expression. Isolated mitochondria were directly patch-clamped to detect MAC activity, using micropipettes whose tips contained t-Bid, Bid, or vehicle alone. A, current traces recorded from membrane patches clamped at +40 mV or −40 mV show formation of MAC in less than 10 min for mitochondria isolated from Parental cells when the micropipette contained 20 nM t-Bid, but not Bid or vehicle alone. In similar experiments, MAC activity was not detected in mitochondria isolated from DKO cells or protein-free liposomes exposed to 1 or 2.3 μM t-Bid, respectively. B, current traces recorded at 40 mV and displayed at higher time resolution than A show channel activity induced by t-Bid in mitochondria from Parental cells (labeled MAC), Bax KO cells (labeled MAC-Bak), Bak KO cells (labeled MAC-Bax), and DKO cells. Peak conductances in these traces were 3.2, 3.7, 4.5, and 0.5 nS, respectively. C, histogram shows the frequency of detecting patches with conductances ≥1.5 nS MAC formation in mitochondria from the four MEF cell lines and in liposomes (negative control) when vehicle, t-Bid, or Bid were included in the micropipette. n corresponds to the number of independent patches. Other conditions are as in Fig. 1.
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FIGURE 4. Dibucaine and cytochrome c regulate MAC activity induced by t-Bid. MAC activity was induced by patch clamping isolated mitochondria with micropipettes containing t-Bid. A, current trace of MAC-Bax shows a decrease in conductance from 2.4 to 0.62 nS after perfusion of the bath with media containing 100 μM of the MAC inhibitor dibucaine. B and C, current traces of MAC-Bax (B) and MAC-Cyt c (C) show decreases in conductance from 3.6 to 0.95 nS in B and from 1.9 to 0.9 nS in C after perfusion with media containing physiological levels (100 μM) cytochrome c (Cyt c).

experiments (supplemental Table 1S). The reliably detected transitions from base-line conductance (after seal formation) were, as noted above, typically 280 pS. A pore of this size is predicted to contain a minimum of six transmembrane helices (Fig. 7E and supplemental Table 1S). The pore of MAC then could enlarge by addition of one α-helix that would account for an increase in conductance of ~200 pS (pathway 1) or by addition of two α-helices at a time (~300 pS, pathway 2). Once MAC reaches a conductance of ~1 nS, the transitions observed remain ~300 pS, which is consistent with further assembly proceeding with insertion of single helices to the MAC pore (pathway 3).

Assuming each Bax or Bak monomer contributes two helices to the pore, this result suggests that the earliest detectable MAC pore is composed of a homo- or heterotrimer of Bax and/or Bak. However, the diameter corresponding to the ~280 pS conductance of this earliest detectable pore (~1 nm) is too small to transport cytochrome c. The mature pore would be competent to transport cytochrome c and, hence, would have a diameter greater than that of cytochrome c (>3 nm); the minimum conductance of such a pore corresponds to ~1.5 nS. To reach this size, the pore complex would need to contain a minimum of ~6 Bax or Bak monomers, each contributing two helical staves to the pore "barrel." The larger stable conductances observed (in the range 3–5 nS) would correspond to pores composed of 9–12 Bax or Bak molecules (supplemental Table 1S), again assuming two helices were contributed to the pore structure.

Consistent with these predictions, other laboratories have previously identified complexes containing 9–13 monomers of Bax using biochemical rather than the biophysical approaches used here (14, 47).

Although the transition sizes are heterogeneous, the electrophysiological data show incremental increases in mitochondrial outer membrane conductance under all conditions examined. This finding argues strongly against fully formed MAC spontaneously opening in or directly inserting into the mitochondrial outer membrane. Instead, the data argue for a gradual, stepwise assembly of the MAC pore and that the mechanism of stave addition may vary for early and late stages of pore formation, i.e. the conductance step of ~300 pS (Fig. 7D) observed at both early and late stages of MAC formation might correspond to addition of two transmembrane helices to a small pore (6–8 helices) but only 1 helix to a large pore (14–15 helices, see supplemental Table 1S).

DISCUSSION

Formation of MAC, the channel that permeabilizes the mitochondrial outer membrane to cytochrome c and so commits the cell to intrinsic apoptosis, was visualized in real time by patch clamping isolated mitochondria. This pore often grew in a stepwise manner in under 10 min to a diameter that was sufficiently large to allow passage of cytochrome c. Its formation required the presence of either of the pro-apoptotic proteins Bax or Bak in the mitochondrial outer membrane. In contrast, MAC formation was insensitive to the presence or absence of VDAC1 and VDAC3, which are pores constitutively found in the outer membrane. This model further predicted that the pore of functional MAC is minimally a hexamer of Bax and/or Bak assuming each monomer contributed two transmembrane helices. Stepwise assembly of the MAC pore by Bax/Bak oligomerization would be consistent with the known tendency of Bax and Bak to form dimers, tetramers, and higher order oligomers (14, 18, 21, 45, 48).

Although Bax and Bak have multiple transmembrane domains, helices 5 and 6 of Bax are amphipathic. This characteristic makes them good candidates to form, at least in part, the pore of MAC (18). Bax lacking helices 5 and 6 do not release cytochrome c (19, 20). Given the structural similarities between Bax and Bak, the same helices could underlie pore formation in MAC by either protein (21). These findings support the notion that each monomer contributes two helices to the MAC pore.

The observed incremental increases in permeability measured in patch clamp experiments presumably correspond to MAC assembly. Such stepwise increases in conductance argue against MAC being a large lipidic pore. The conductance transitions associated with insertion of ceramide molecules into phospholipid bilayers are ~600 pS (4 nS in 1 M salt), which are...
much larger than those associated with MAC assembly (49).
Ceramide channels in mitochondria typically have a molecular mass cutoff of 60 kDa, which has been modeled to contain 40 staves or columns and have a pore size of \( \frac{6}{11011} \) nm. However, conductances of up to 30 nS (200 nS in 1M KCl) have been reported (50–52), which would correspond to diameters \( \frac{14}{11022} \) nm using the method of Hille (36). These higher conductances and inferred pore diameters are much larger than those usually observed with MAC (3–5 nS; Fig. 7 and Table 1).

Along similar lines, recombinant BAX is capable of forming gargantuan “lipidic” pores that allow free diffusion of up to 2-MDa molecules in artificial systems; this observation is consistent with a massive reorganization of the bilayer (53, 54). It may be possible that the smaller channel activities formed by Bax and recorded in electrophysiological studies (Figs. 6 and 7) might coalesce into such lipidic pores. However, the MAC pore formed in the native mitochondrial outer membrane does not display such large conductances. The size seems to stabilize at 3–5 nS, which corresponds to diameters estimated at 5 or 6 nm (Fig. 7C and Table 1), suggesting that some of the more extreme in vitro behaviors of Bax and Bak are not realized in the native mitochondrial outer membrane.

**FIGURE 5.** **VDAC1 and VDAC3 are not essential for MAC formation.** A, Western blots show expression of Bax, Bak, and VDAC1 in mitochondria of VDAC1/VDAC3 KO MEF cells (lane 1; 1 µg) and FL5.12 cells (lane 2; 2 µg). B, release of cytochrome c (Cyt c) from mitochondria isolated from VDAC1/VDAC3 KO MEF cells after incubation with t-Bid, Bid, or vehicle, determined by ELISA and expressed relative to release by alamethicin (80 µg/ml). C, histogram shows the detection frequency of patches with conductances \( \geq 1.5 \) nS in mitochondria isolated from VDAC1/VDAC3 KO cells patch-clamped with micropipettes backfilled with t-Bid (1 µM), Bid (1.6 µM), or vehicle. D, current traces show dibucaine reversibly inhibited MAC induced by t-Bid in VDAC1/VDAC3 KO mitochondria. The downward deflection in the top current trace shows MAC assembled ~5 min after seal formation. Middle and lower current traces show the closing effect of adding (upward deflection) and opening effect (downward deflection) of washing out 200 µM dibucaine from the bath. E, current traces show MAC activity in membrane patches clamped at +20 mV in the absence (upper, 2.6 nS) and presence of 100 µM cytochrome c (lower panel, +Cyt c; 2.1 nS). Immediately after perfusion, the conductance of MAC decreased ~20%, although the noise of the current trace increased, which is consistent with the type 1 effect of cytochrome c (10). F, current trace from VDAC1/VDAC3 KO mitochondria patch-clamped at 20 mV shows a large decrease in MAC conductance from 4.2 to 1.0 nS after perfusion with 100 µM cytochrome c (+Cyt c) illustrating a type 2 effect (10). Other conditions were the same as in experiments of Figs. 1 and 3.
Here we directly examined the kinetics of the key step of intrinsic apoptosis, i.e., the permeabilization of the outer membrane to cytochrome c as a result of MAC formation. We found that MAC typically formed in 64–75% of the membrane patches from parental, VDAC1VDAC3, Bax, and Bak KO cells in about 10 min (Table 1 and Figs. 3 and 5). In a recent elegant study of recombinant BAX assembly in liposomes, Lovell et al. (55) reported pore formation using fluorescence techniques in a time frame similar to that observed here in native membranes with endogenous Bax.

Biochemical studies have shown Bax forms oligomers that are functional and release cytochrome c and that Bcl-2 prevents Bax oligomerization. Antonsson et al. (14) report Bax forms high molecular mass complexes of 96 and 260 kDa in the mitochondrial outer membrane during apoptosis. Because the molecular mass of Bax is 20 kDa, these complexes may contain as many as ~4 and ~10 molecules of Bax. These finding are consistent with those predicted here by modeling, which suggested 9–12 monomers of Bax or Bak would be needed to form stable MAC (supplemental Table 1S).

More recently, Valentijn et al. (47, 56) also reported Bax forms 200-kDa complexes putatively containing 9–10 monomers during anoikis. However, they suggest that these complexes are inactive (i.e., incompetent for cytochrome c release) because of a limited accessibility of Bax N terminus to monoclonal Bax N terminus antibodies in these complexes during blue native-PAGE experiments. The Bax N terminus is thought to be accessible only after activation (57). The authors thus conclude that at least a separate step is needed after oligomerization to convert these Bax oligomers into molecular structures competent for cytochrome c release (47). In this last case, our data suggest that this extra step would correspond to a gradual activation of Bax.
monomers or dimers already present in these high molecular weight complexes (Fig. 6 and Fig. 7).

MAC has an immense pore that is slightly cation-selective and not voltage-dependent. All MACs function by providing cytochrome c with a transport pathway across the outer membrane. These basic characteristics are independent of Bax and Bak content for MAC. Despite the fundamental similarities in the channel behavior of MAC-Bax and MAC-Bak, these channels differ in several important respects. The concentration of t-Bid needed to induce equivalent levels of MAC activity is much greater in Bak-deficient than in Bax-deficient MEF cells, the assembly of MAC channels is slower in the former cells, and the amount of cytochrome c released by t-Bid treatment is less (Fig. 1D). These results are consistent with those of Wei et al. (58) who report that recombinant t-Bid was much more effective in triggering cytochrome c release in Bax KO mitochondria by comparison with Bak KO mitochondria. These differences might be explained, in part, by Bax having a lower affinity for t-Bid than Bak. But there may be another explanation. There may be higher endogenous levels of Bak than Bax in mitochondria from control (nonapoptotic) cells. Recall that Bak is constitutively expressed on mitochondria, whereas most Bax is normally found in the cytosol and only moves to mitochondria after truncation of Bid. In the MAC induction experiments, mitochondria were isolated from normal (nonapoptotic) cells and then treated with t-Bid. The amount of Bax bound to or resident in the mitochondrial outer membrane under these conditions would be low and could limit MAC formation in the Bak-deficient cells. Thus, even if the affinities of Bax and Bak for t-Bid were similar, very high, Bax-saturating levels of t-Bid might be needed to generate detectable MAC formation in the Bak-deficient mitochondria. Limiting levels of Bax in these mitochondria might also be responsible for the slower pore formation times and inefficient cytochrome c release observed in these cells.

Here we monitored in real time the assembly of the cytochrome c release channel MAC, which represents the commitment step of apoptosis. The data were consistent with formation of a barrel and stave pore that finally contained many Bax and/or Bak molecules. Functional MAC channels form within minutes of exposure of mitochondria containing Bax and/or Bak to nanomolar levels of t-Bid. Hence, these studies reveal how the delicate control of MAC formation by Bcl-2 family pro-
tein regulates the switch that directs cells to either survival or death pathways.

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