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A novel, high conductance channel of mitochondria linked to apoptosis in mammalian cells and Bax expression in yeast

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During apoptosis, proapoptotic factors are released from mitochondria by as yet undefined mechanisms. Patch-clamping of mitochondria and proteoliposomes formed from mitochondrial outer membranes of mammalian (FL5.12) cells has uncovered a novel ion channel whose activity correlates with onset of apoptosis. The pore diameter inferred from the largest conductance state of this channel is ~4 nm, sufficient to allow diffusion of cytochrome c and even larger proteins. The activity of the channel is affected by Bcl-2 family proteins in a manner consistent with their pro- or antiapoptotic properties. Thus, the channel activity correlates with presence of proapoptotic Bax in the mitochondrial outer membrane and is absent in mitochondria from cells overexpressing antiapoptotic Bcl-2. Also, a similar channel activity is found in mitochondrial outer membranes of yeast expressing human Bax. These findings implicate this channel, named mitochondrial apoptosis–induced channel, as a candidate for the outer-membrane pore through which cytochrome c and possibly other factors exit mitochondria during apoptosis.

Introduction

Apoptosis is integral to such diverse cellular processes as tissue remodeling and organogenesis, as well as chemotherapy-induced tumor regression. It is a phenomenon fundamental to higher eukaryotes and essential to mechanisms controlling tissue homeostasis. A key early event in the apoptotic cascade in many cell types is the release of cytochrome c and other proteins from mitochondria (Liu et al., 1996; Kluck et al., 1997; Wei et al., 2001). Once in the cytoplasm, cytochrome c and procaspase 9 bind the cytoplasmic protein apaf-1 and form apoptosomes that promote caspase activation and destruction of the cell (Liu et al., 1996).

The mechanism by which proapoptotic factors are released from mitochondria early in apoptosis is unknown. It has been speculated that a permeability transition of the inner membrane causes swelling of the matrix space. As the inner membrane has a much greater surface area than the outer membrane, the ensuing swelling would rupture the outer membrane and spill cytochrome c into the cytoplasm. However, cytochrome c release has been shown to occur in the absence of a loss of outer membrane integrity in some cell types, suggesting instead a more selective mechanism, e.g., formation of a pore in the outer membrane (Antonsson et al., 1997; Brenner and Kroemer, 2000; Saito et al., 2000; Martinou and Green, 2001; Shimizu et al., 2001). Importantly, the existence of such a channel has not yet been demonstrated by independent criteria, e.g., electrophysiological characteristics.

There is considerable evidence that members of the Bcl-2 protein family exert their pro- and antiapoptotic effects by regulating the release of cytochrome c from mitochondria (Antonsson et al., 1997; Kluck et al., 1997; Yang et al., 1997; Brenner and Kroemer, 2000; Kroemer and Reed, 2000; Saito et al., 2000; Martinou and Green, 2001; Shimizu et al., 2001; Wei et al., 2001). Interestingly, some proteins in the Bcl-2 family have structures similar to pore-

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forming toxins and can create ion channels in artificial membranes (Antonsson et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). Furthermore, Bax can induce transport of cytochrome c in liposomes (Saito et al., 2000). However, there is as yet no direct evidence for a Bax-induced pore or channel in the mitochondrial outer membrane that would correspond to the pathway for release of proapoptotic mitochondrial proteins like cytochrome c.

Hematopoietic FL5.12 cells are mammalian cells that enter apoptosis after withdrawal of the growth factor interleukin-3 (IL-3)* (Oltvai et al., 1993; Gross et al., 1998). Bax oligomerizes and translocates into mitochondria within twelve hours after IL-3 withdrawal. These events immediately precede release of proapoptotic factors, e.g., cytochrome c (Gross et al., 1998), which in turn is prevented by the antiapoptotic protein Bcl-2 (Goping et al., 1998; Gross et al., 2000). In this study, patch clamp techniques are used to directly demonstrate that a novel ion channel activity occurs both in the outer membrane of mammalian mitochondria early in apoptosis, and in the mitochondria of yeast expressing human Bax. Electrophysiological characterization of this high-conductance channel and comparison with other known mitochondrial channels, as well as purified Bax channels, are included.

Results and discussion

Bax content of mitochondrial outer membranes

Hematopoietic FL5.12 cells enter apoptosis upon withdrawal of IL-3 by a process that has been well characterized (Bojes et al., 1997; Gross et al., 1998). The translocation of Bax from the cytoplasm to the mitochondrial outer membrane is an early event in this cascade. As shown in Fig. 1 A, outer membranes of mitochondria isolated 12 h after IL-3 withdrawal contained significantly more Bax (5–10-fold increase when normalized to voltage-dependent anion-selective channel [VDAC] levels) than the same membranes before IL-3 withdrawal, as expected (Gross et al., 1998).

Proteoliposomes were formed by fusion of purified mitochondrial outer membranes with liposomes in the presence of cytochrome c and washed with high salt to remove bound cytochrome c. Proteoliposomes prepared from outer membranes of mitochondria isolated 12 h after IL-3 withdrawal contained much less cytochrome c than proteoliposomes prepared from membranes of control mitochondria (Fig. 1 B). The failure of proteoliposomes with high Bax content to retain cytochrome c is consistent with (but certainly not proof of) increased permeability toward this 12-kD protein. Without characterization of specificity, these preparations were examined for the presence of a novel ion channel activity (see below).

Ion conductance of the native mitochondrial outer membrane

The ion permeability of outer membranes was determined by directly patch-clamping isolated mitochondria with microelectrodes (Kinnally et al., 1987; Lohret et al., 1997). The average conductance of outer-membrane patches of mitochondria isolated from FL5.12 cells 12 h after IL-3 withdrawal was significantly greater than that of patches from mitochondria of control cells (13.3 ± 2.5 nS versus 6.3 ± 3.6 nS; analysis limited to patches showing obvious channel activity). This increase in ion permeability, coincident with release of proapoptotic proteins from mitochondria, suggests the formation of novel pores in the outer membrane. Interestingly, the outer membrane patches excised from mitochondria were slightly cation-selective under both conditions (P_{K+}/P_{Cl-} of ~2; eight independent patches). Current recordings obtained by direct patch-clamping of mitochondria from FL5.12 cells could not resolve individual channel openings or closings, presumably due to the high density of ion channels in the native outer membrane, as previously encountered with mouse-liver mitochondria (Kinnally et al., 1987).

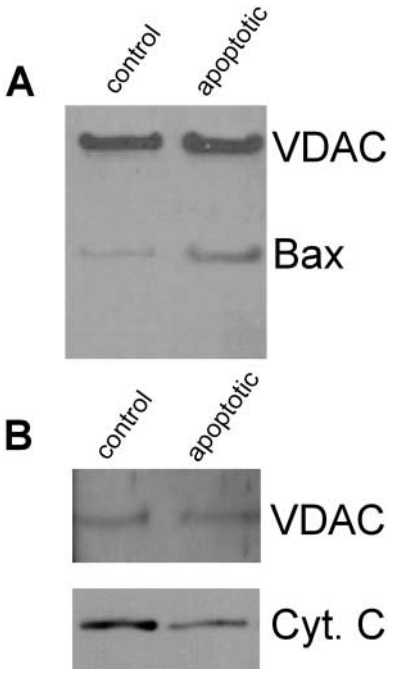

Figure 1. **Proteoliposomes enriched with Bax fail to retain cytochrome c.** (A) Immunoblots show Bax (but not VDAC) levels are higher (5–10-fold) in outer membranes purified from mitochondria isolated from FL5.12 cells 12 h after IL-3 withdrawal (i.e., apoptotic cells) than from normal cells (i.e., control). Silver-stained gels and Western blots with antibodies against cytochrome oxidase subunit IV indicate equivalent content of overall protein and low contamination by inner membranes for both preparations (data not shown). (B) Immunoblots show that less cytochrome c is present in salt-washed proteoliposomes made from mitochondrial outer membranes of apoptotic cells than of normal cells (control).

Characterization of an apoptosis-induced channel

To characterize single-channel events in outer membranes, they were diluted with additional lipid to form proteoliposomes. These proteoliposomes were prepared by the same methods as those used in the cytochrome c trapping experiments, except that cytochrome c was omitted from the preparation. A novel ion channel activity was readily detected by patch-clamping the proteoliposomes derived from apoptotic mitochondria. Current traces from these patches exhibit numerous deflections corresponding to opening and closure of

*Abbreviations used in this paper: IL-3, interleukin-3; MAC, mitochondrial apoptosis–induced channel; TOM, translocase outer membrane; VDAC, voltage dependent anion-selective channel.

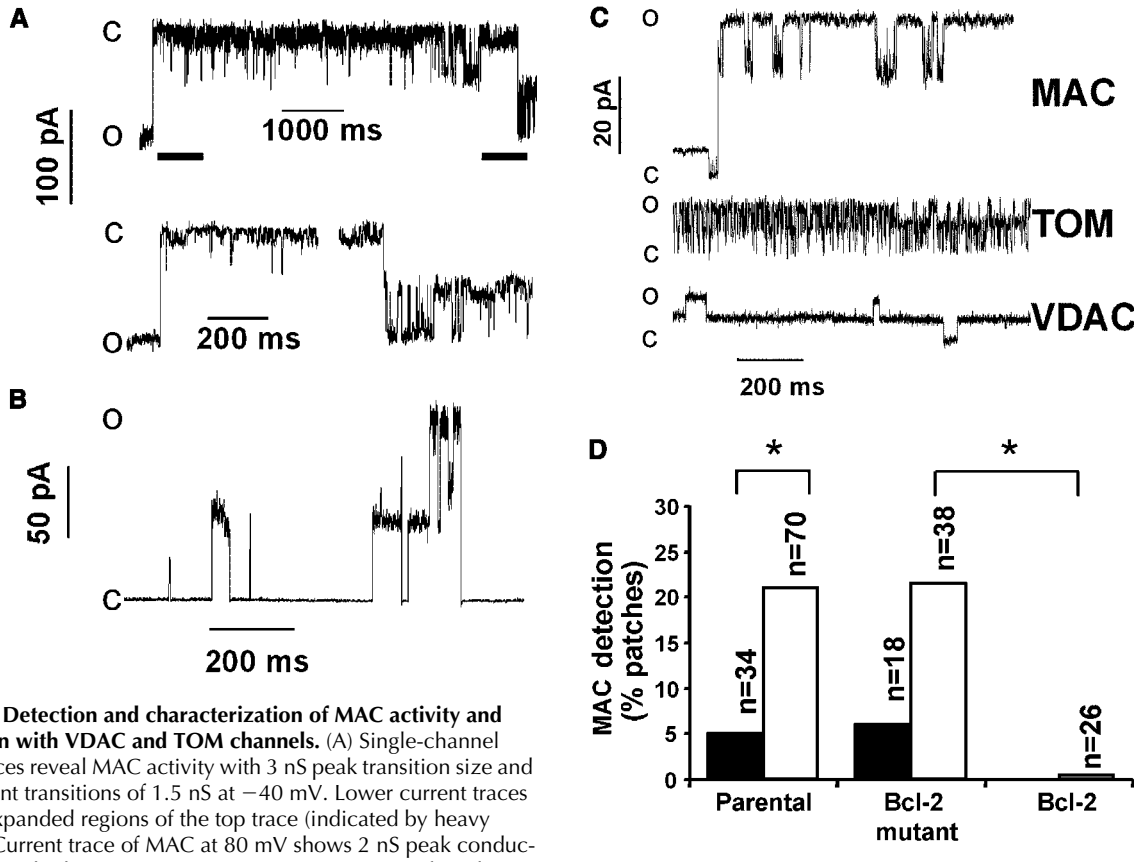

Figure 2. **Detection and characterization of MAC activity and comparison with VDAC and TOM channels.** (A) Single-channel current traces reveal MAC activity with 3 nS peak transition size and predominant transitions of 1.5 nS at −40 mV. Lower current traces are time-expanded regions of the top trace (indicated by heavy lines). (B) Current trace of MAC at 80 mV shows 2 nS peak conductance with multiple 1 nS transitions. (C) Current traces of single channels at 20 mV show MAC character is distinct from TOM channel and VDAC. O and C indicate open and closed conductance levels. Patching media was symmetrical 150 mM KCl, 5 mM Hepes-koh/ pH 7.4. Current traces, low pass filtered at 2 kHz (5 kHz sampling), were obtained using patch-clamp procedures as described in Materials and methods. (D) Histograms show the frequency of detecting MAC in independent patches (n) from proteoliposomes prepared with outer membranes purified from mitochondria isolated before (closed, +IL-3 control) or 12 h after IL-3 withdrawal (open, −IL-3 apoptotic) from FL5.12 cells that were parental or expressing incompetent (Bcl-2 mutant) or functional Bcl-2 (Bcl-2). Statistical difference ($P < 0.05$) between pairs was determined by Fischer's exact statistical test (Fisher, 1935) and indicated by asterisks.

the channel at constant voltage (Fig. 2). Analysis of many traces indicates that the channel has multiple conductance levels with peak, single-channel openings of 2.5 ± 0.6 nS. This maximum ion conductance corresponds to a pore diameter of 4.0 ± 0.5 nm, assuming a pore length of 7 nm, using the method of Hille (1992). Conductance changes of this magnitude have not previously been described for channels in the mitochondrial outer membrane. Although common, these large current transitions occurred at a lower frequency (less than one per minute) than the smaller transitions of 1–1.5 nS (several per second) (Fig. 2, A–C).

This novel, large-conductance ion channel has been designated a mitochondrial apoptosis–induced channel (MAC). The single-channel behavior of MAC is substantially different from the known behavior of VDAC (Colombini et al., 1996) and the protein import channel (translocase outer membrane [TOM]) (Thieffry et al., 1992; Lohret et al., 1997; Hill et al., 1998; Künkele et al., 1998; Ahting et al., 2001) in terms of peak conductance, predominant transition sizes, and frequency and duration of transitions (Fig. 2 C and Table I). Also, none of the properties of the other two outer-membrane ion channels (VDAC and TOM) were noticeably modified 12 h after IL-3 withdrawal. Unlike VDAC and the TOM channel, MAC is not voltage-dependent, i.e., its open probabilities do not vary systematically with voltage. Like the TOM channel, MAC is slightly cation-selective with a permeability ratio for K^+/Cl^- of three. The cation selectivity is consistent with the possibility that MAC might provide a permeability pathway for cytochrome c, which carries a net positive charge at physiological pH.

MAC activity is induced by apoptosis and inhibited by Bcl-2

There is a fourfold increase in the detection of MAC in proteoliposomes prepared with mitochondrial outer membranes purified 12 h after IL-3 withdrawal compared with the control ($P < 0.05$). Normal cultures of FL5.12 cells grown in the presence of IL-3 routinely have ~7% apoptotic cells as indicated by annexin-V staining (unpublished data; Bojes et al., 1997). This background of apoptotic cells could account for the low basal level of detection of MAC (Fig. 2 D).

The electrophysiological experiments were repeated on clones of FL5.12 cells overexpressing Bcl-2, an antiapoptotic protein that prevents release of cytochrome c from mitochondria (Yang et al., 1997). MAC was not detected in cells overexpressing wild-type Bcl-2 after IL-3 withdrawal, but expression of a mu-

Table I. **Comparison of mitochondrial outer membrane and Bax channels**[a]

	mMAC	hMAC	hBax	TOM	VDAC
Peak conductance (nS)	2.5 ± 0.6	2.2 ± 0.3	0.1–2.0	0.71 ± 0.06	0.68 ± 0.09
Transition size (nS)	1.4 ± 0.4	1.5 ± 0.2	0.1–0.5	0.34 ± 0.03	0.36 ± 0.04
Ion selectivity	Cation	Cation	Cation	Cation	Anion
P_K/P_{Cl}	3.0 ± 0.9	4.7 ± 1.3	6.8 ± 1.0	3.6 ± 0.8	0.7 ± 0.1
Voltage dependent	No	No	No	Yes	Yes
Pore size[b] (nm)	4.0 ± 0.5	3.8 ± 0.2	0.8–3.8	2.3 ± 0.1	2.1 ± 0.05

mMAC, mammalian MAC of apoptotic FL5.12 cells; hMAC, MAC of yeast expressing human Bax; hBax, recombinant Bax.
[a]Single channel characteristics for each channel type were determined under the same conditions as indicated in Materials and methods. Characteristics of TOM and VDAC agree with previously published behavior.
[b]Calculated from peak transition size assuming 7 nm pore length (Hille, 1992).

tant Bcl-2 (which does not inhibit apoptosis) resulted in levels of MAC detection similar to those of the parental clones. Thus, effects of Bcl-2 on MAC activity parallel its effects on apoptosis. Elevated detection of this channel early in apoptosis and its suppression by functional Bcl-2 supports a role for this channel in release of proapoptotic factors from mitochondria.

It should be noted, as an aside, that no novel channels were detected in the mitochondrial outer membranes of cells overexpressing wild-type Bcl-2. A similar result was obtained previously with mitochondria isolated from a breast epithelial cell line overexpressing Bcl-2 (Murphy et al., 2001), despite the fact that Bcl-2 forms ion channels in artificial bilayers (Schendel et al., 1997; Schlesinger et al., 1997). However, expression of Bcl-2 in breast epithelial cells did affect an inner membrane channel, preventing calcium-activation of the multiple conductance channel, which may represent the mitochondrial permeability transition pore (Murphy et al., 2001).

MAC activity is similar but distinct from pure Bax

Oligomeric recombinant Bax (human) spontaneously inserts and forms high-conductance ion channels in artificial membranes, and causes release of trapped cytochrome c (Antonsson et al., 1997, 2000; Lewis et al., 1998; Saito et al., 2000).

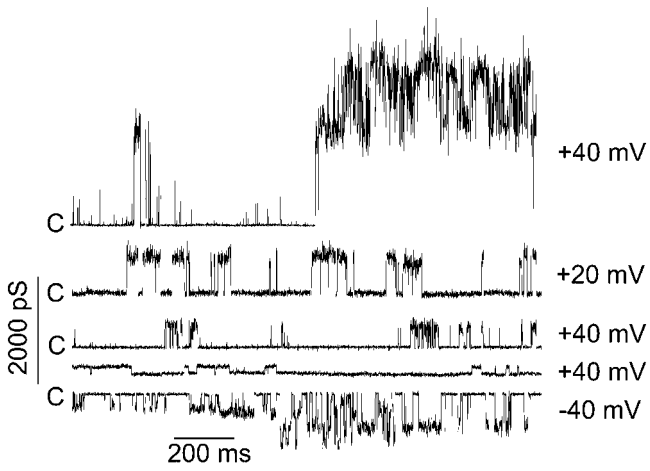

Figure 3. **Bax forms high conductance channels with multiple conductance levels.** Current traces show hBax channel activity at various voltages had transitions ranging from 0.15–2.0 nS. Purified oligomeric hBax (34 ng/μl in the pipette tip) self-inserted into patches excised from liposomes originally formed without protein. Other conditions are as in Figs. 1 and 2.

Comparison of MAC and Bax ion channel activities suggests several similarities (Table I). Like MAC, Bax channels display multiple conductance levels (Fig. 3) and a slight cation selectivity, but their typical peak current transitions (~0.5 nS) are much smaller than that displayed by MAC (~2.5 nS). If MAC is a Bax channel, this important functional difference could relate to specific aspects of in vivo insertion and assembly in the native mitochondrial outer membrane, to post-translational modification (Zha et al., 2000), or to involvement of additional mitochondrial components (Priault et al., 1999a,b; Shimizu et al., 2000, 2001; Antonsson et al., 2001).

MAC activity is induced by Bax expression in yeast

To independently confirm the correlation between MAC activity and the presence of Bax in mitochondrial membranes, proteoliposome experiments were repeated using yeast strains engineered to express human Bax. Although yeast do not normally possess components of the apoptotic cascade found in higher eukaryotes, they die when forced to express hBax, and can be rescued by coexpression of Bcl-2 (Priault et al., 1999a; Gross et al., 2000). Mitochondrial outer and inner membranes were purified from yeast grown either in the presence of doxycyclin to repress hBax expression, or 16 h after doxycyclin withdrawal to induce expression of hBax. To simplify the subsequent single-channel analysis, the yeast strain lacked VDAC (Fig. 4 B).

Expressed human Bax was exclusively localized to the mitochondrial outer membranes (Fig. 4 A), and the same membranes, incorporated in proteoliposomes, displayed an ion channel activity virtually identical (in terms of peak and predominant conductance transitions, and ion selectivity) to MAC from apoptotic mammalian cells (Fig. 4 C and Table I). Inner membrane contamination of the outer membrane preparations was assessed by immunoblotting against the inner membrane protein, subunit IV of the ATP synthase, and was comparable for both types of preparations (unpublished data). MAC activity was never observed in proteoliposomes containing mitochondrial outer membranes devoid of Bax (from wild-type yeast), and rarely detected in the repressed control. In contrast, 40% of the patches showed activity consistent with MAC when Bax expression was induced. Hence, this induction resulted in a >10-fold increase in MAC detection and in Bax content of the outer membranes compared with the repressed levels (Fig. 4). Thus, MAC activity is induced in the mitochondrial outer membrane by the presence of Bax in the absence of VDAC.

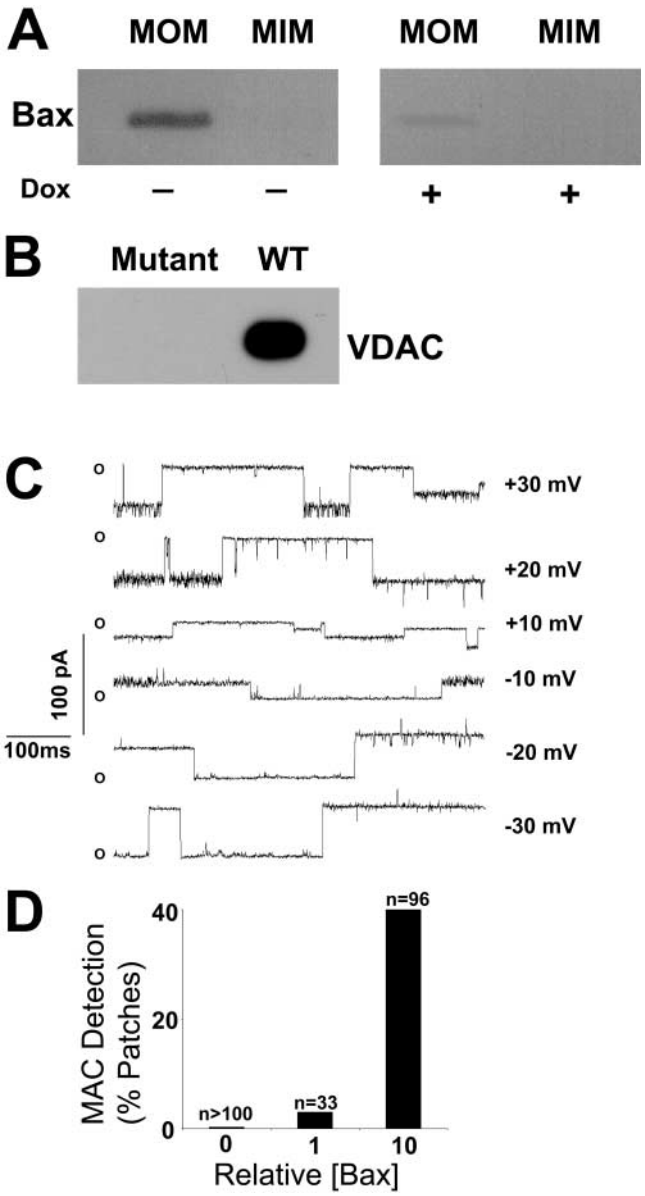

Figure 4. **MAC activity induced by Bax expression in yeast.** VDAC-less yeast carrying a human Bax plasmid were grown either in the presence of doxycyclin to repress expression or in its absence for 16 h to enable expression. (A) Immunoblot shows Bax localized to mitochondrial outer (MOM) and not inner (MIM) membranes. Densitometry (Unscanit program) indicated Bax levels were ~10-fold higher in outer membranes of yeast grown without rather than with doxycyclin (Dox). (B) Immunoblot of mitochondria (15 μg) confirms that this strain (Mutant) is VDAC-deficient compared with the wild-type strain (WT). (C) Current traces of MAC were recorded from patches excised from proteoliposomes containing mitochondrial outer membranes purified from yeast expressing human Bax. (D) Histograms show the frequency of detecting MAC in independent patches (n) from proteoliposomes prepared with outer membranes purified from mitochondria with different Bax content. Wild-type yeast do not express Bax (0 level). The Bax levels of Western blot shown in A were normalized to outer membranes of DBY747/b5 yeast grown in the presence of doxycyclin. MAC detection was statistically different ($P < 0.001$) before and after induction of Bax expression. Other conditions are as in Figs. 1 and 2.

Conclusions and future prospects

This is the first demonstration using electrophysiological methods of a novel ion channel activity in the outer membrane of mitochondria associated with onset of apoptosis. This channel, named MAC, is the first whose pore size, inferred from single-channel conductance, is sufficient to allow proteins as large as or larger than cytochrome c to diffuse through the outer membrane. This postulated function is consistent with the inability of MAC-containing proteoliposomes to trap cytochrome c.

Bax clearly plays an essential role in MAC activation, forming the channel either by itself or in association with one or more outer-membrane components common to mammalian and yeast mitochondria. Contrary to some reports published previously (Shimizu et al., 2000, 2001), but in support of others (Priault et al., 1999a,b; Antonsson et al., 2001), this component is not VDAC, since VDAC was absent from the yeast strain in which MAC activity was induced by hBax expression. Although antibodies against hBax had no effect on MAC activity, preliminary experiments suggest cytochrome c reduces the ion conductance of this channel, but not of VDAC or TOM (unpublished data). If Bax alone forms the MAC channel, the observed differences in electrophysiological properties between MAC and channels induced by recombinant Bax in synthetic membranes likely arise from fundamental differences in Bax insertion, assembly, and/or regulation. The antiapoptotic role of Bcl-2 may relate to its ability (as demonstrated above) to suppress the activity of MAC, perhaps by associating with Bax in a manner that inhibits Bax oligomerization (Gross et al., 1998). Extension of the electrophysiological approaches described in this report should lead to a fuller understanding of the molecular mechanisms underlying the formation of this newly discovered channel; its regulation by apoptosis-associated proteins (e.g., Bcl-2 family members, caspases, and cytochrome c); and, ultimately, its role in apoptosis.

Materials and methods

Cells and growth conditions

Parental FL5.12 cells were cultured as described previously (Gross et al., 1998) in Iscove's modified Eagle media, 10% fetal bovine serum, 10% WEHI-3B supplement (filtered supernatant of WEHI-3B cells secreting IL-3). Fl5.12 clones overexpressing Bcl-2 or Bcl-2 mutant (G145E substitution) were passed in this media plus geneticin (1 mg/ml) (Yin et al., 1994). Cultures were kept below 1.5 million cells/ml. Cells were washed three times in media with (control) or without (apoptotic) IL-3 12 h before the isolation of mitochondria. The DBY747/b5 (mat α, his 3, leu2, trp1, ura3, por1::URA3) strain of yeast carrying the pCM184-human Bax plasmid was grown in YPLac media either in the presence or absence of doxycyclin (10 μg/ml) for 16 h at 30°C (Priault et al., 1999b).

Isolation of mitochondria and preparation of proteoliposomes

Mitochondria were isolated from 2–15 g of FL5.12 cells as described previously (Campo et al., 1992) for outer membrane preparations. Smaller preparations of mitochondria were isolated from ~15–20 million cells as described previously (Murphy et al., 2001). Yeast mitochondria were prepared by a modification of Daum et al. (1982) as described previously (Lohret et al., 1997). Mitochondrial outer membranes were stripped from inner membranes by French pressing isolated mitochondria using modifications of the method of Decker and Greenawalt (1977). Outer membranes were separated from inner membranes as described by Lohret et al., (1997) and Mannella (1982). Proteoliposomes were formed by a modification of the method of Criado and Keller (1987) (Lohret et al., 1997). Briefly, small liposomes were formed by sonication of lipid (type IV-S soybean L-α-phos-

phatidylcholine; Sigma-Aldrich) in water. Mitochondrial outer membranes (30–35 μg protein) and small liposomes (600 μg lipid) with or without cytochrome c (30 μg) were mixed with 5 mM Hepes, pH 7.4 (50 μl volume), and dotted on a glass slide. Samples were dehydrated (~3 h) and rehydrated overnight with 150 mM KCl, 5 mM Hepes, pH 7.4, at 4°C. For trapping experiments, proteoliposomes were washed in 0.5 M NaCl, 5 mM Hepes, pH 7.4, to remove bound cytochrome c and pelleted at 45,000 rpm for 2 h in a TLA rotor (Beckman Coulter ultracentrifuge). Pellets were resuspended in 10 mM Tris, pH 7.4, and prepared for electrophoresis.

Bax protein

Human Bax-α (hBax) lacking 20 amino acids at the COOH end was expressed as a glutathione *S*-transferase fusion protein in *Escherichia coli* and purified as described previously (Lewis et al., 1998).

Immunoblotting

Proteins were separated by SDS-PAGE and electrotransferred onto PVDF membranes. Indirect immunodetection employed chemiluminescence (Amersham Pharmacia Biotech) using HRP-coupled secondary antibodies. Membrane proteins (0.5–6 μg/lane) were probed with primary polyclonal antibodies against hBax (Santa Cruz Biotechnology, Inc.; 1:5,000), mammalian VDAC (Stanley et al., 1995), 1:500–5000), or yeast VDAC antibodies (Molecular Probes, 1:5000) and a secondary anti–rabbit antibody (Jackson ImmunoResearch Laboratories; 1:5,000). Cytochrome c (≥25 ng) was detected with a monoclonal antibody (Research Diagnostics; 1:2,500) and a secondary anti–mouse antibody (Jackson ImmunoResearch Laboratories; 1:2,500).

Patch-clamping analysis

Patch-clamp procedures and analysis used were described previously (Lohret et al., 1997). Briefly, membrane patches were excised from proteoliposomes after formation of a giga-seal using micropipettes with ~0.4-μm diameter tips and resistances of 10–20 MΩ at room temperature. Unless otherwise stated, the solution was symmetrical 150 mM KCl, 5 mM Hepes, pH 7.4. Ion selectivity was determined from the reversal potential after perfusion of the bath with 30 mM KCl, 56 mM sucrose, 184 mM mannitol, 5 mM Hepes, pH 7.4. Voltage clamp was performed with the excised configuration of the patch-clamp technique (Hamill et al., 1981) using a Dagan 3900 patch clamp amplifier in the inside-out mode. Voltages are reported as bath potentials.

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