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Review

Regulation of the mitochondrial apoptosis-induced channel, MAC, by BCL-2 family proteins

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Abstract

Programmed cell death or apoptosis is central to many physiological processes and pathological conditions such as organogenesis, tissue homeostasis, cancer, and neurodegenerative diseases. Bcl-2 family proteins tightly control this cell death program by regulating the permeabilization of the mitochondrial outer membrane and, hence, the release of cytochrome *c* and other pro-apoptotic factors. Control of the formation of the mitochondrial apoptosis-induced channel, or MAC, is central to the regulation of apoptosis by Bcl-2 family proteins. MAC is detected early in apoptosis by patch clamping the mitochondrial outer membrane. The focus of this review is on the regulation of MAC activity by Bcl-2 family proteins. The role of MAC as the putative cytochrome *c* release channel during early apoptosis and insights concerning its molecular composition are also discussed.

Keywords: MAC; Mitochondrial apoptosis-induced channel; Apoptosis; Patch clamp; Bcl-2; Bax

1. Introduction

Apoptosis is a phenomenon fundamental to higher eukaryotes that is integral to such diverse cellular processes as tissue homeostasis and organogenesis. A large variety of different stimuli can trigger apoptosis, which is a cell death program (recently reviewed in [1–5]). Two major signaling pathways leading to cell death by apoptosis have been identified and are the extrinsic pathway (or the death receptor pathway) and the intrinsic pathway (or mitochondrial pathway). The extrinsic pathway involves the activation of receptors in the plasma membrane through the binding of ligands such as the Fas/CD95, TNFα, and TRAIL [6]. This process leads to the activation by proteolysis of the initiator pro-caspases 8 and/or 10 which in turn activate the executioner caspases 3 and/or 7. The executioner caspases are responsible for the appearance of late apoptosis markers such as DNA fragmentation, phosphatidylserine exposure in the outer leaflet of the plasma membrane, and blebbing of the same membrane.

The molecular mechanisms underlying activation of the intrinsic pathway remain unclear. However, it is known that mitochondria play a pivotal role in the response of a wide variety of cells to various apoptotic signals that activate this pathway. Mitochondria release a number of factors from their intermembrane space, like cytochrome *c*, Smac/Diablo, and AIF, which promote and amplify the apoptotic cascade from the formation and activation of the apoptosomes to the final destruction of the cell [1–3,7]. The Bcl-2 family of proteins is a key regulator of the mitochondrial response to apoptotic signals in the intrinsic pathway and contains both pro- and anti-apoptotic members. Many of these proteins localize to mitochondria and finely control the process of apoptosis through regulation of the release of mitochondrial mediators of the apoptotic program (recently reviewed in

Abbreviations: PTP, permeability transition pore; MAC, mitochondrial apoptosis-induced channel; IL-3, interleukin-3; VDAC, voltage dependent anion-selective channel; ANT, adenine nucleotide translocator; TOM, translocase of the outer membrane

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[1–5]). The extrinsic and intrinsic pathways initially appeared to be independent. However, it is now clear that a crosstalk exists between the two pathways that is mediated by the 'BH3 domain only' proteins, e.g., Bid [8].

The mechanisms responsible for the release of mitochondrial mediators of cell death are still a subject of lively discussion. It was first hypothesized that the opening of the permeability transition pore (PTP) of the inner membrane would cause swelling of the matrix space, which ruptures the outer membrane, and spills cytochrome *c* and other pro-apoptotic proteins into the cytosol [9–11]. However, it was recently shown that sustained PTP opening is primarily involved in necrosis and ischemia–reperfusion [12–14]. Remarkably, cyclophilin-D deficient cells died normally in response to apoptotic stimuli known to activate both the extrinsic and intrinsic pathways, but showed resistance to necrotic cell death induced by reactive oxygen species and Ca^{2+} overload [12]. Furthermore, cytochrome *c* release can occur in the absence of mitochondrial depolarization and without loss of outer membrane integrity. These observations suggest that, instead of rupturing, a more selective mechanism of permeabilization may be operating, like the formation of a pore in the outer membrane, i.e., the Mitochondrial Apoptosis-induced Channel, MAC [7,15–18]. MAC activity is exquisitely regulated by Bcl-2 family proteins and can initiate release of apoptotic mediators from mitochondria to commit the cell to die [15,18–20]. Nevertheless, MAC and PTP transient opening may act alone or in combination, depending on cell type and death stimulus, to re-localize Bax to the mitochondria, remodel the cristae, and maximize cytochrome *c* release to amplify the death signal [17,21].

The focus of this review is on how Bcl-2 family proteins regulate the permeability of the mitochondrial outer membrane through the formation of MAC and on recent evidence showing that the pro-apoptotic protein Bax is a component of this channel.

2. MAC is the putative cytochrome *c* release channel

MAC was discovered by directly patch clamping mitochondria isolated from FL5.12 cells in which apoptosis was induced by withdrawal of interleukin-3 (IL-3), a stimulus known to activate the intrinsic pathway [15,22,23]. In these studies, the mean conductance of mitochondrial patches from apoptotic cells was greater than that of mitochondrial patches from control cells [15]. This observed increase in permeability suggested the appearance of a novel channel in the outer membrane during apoptosis.

MAC formation is an early event of the intrinsic pathway and is temporally associated with cytochrome *c* release in FL5.12 cells. Loss of lipid asymmetry and integrity in the plasma membrane occurs at ~36 h after induction of apoptosis in FL5.12 cells. In contrast, cytochrome *c* release and MAC formation are complete by 12 h [18]. Recently, MAC has also been detected in isolated rat liver mitochondria when liver apoptosis is induced in vivo [24]. However, MAC was only observed in populations of mitochondria in which cytochrome *c* and Smac/Diablo were already released, suggesting MAC forms at a later stage of apoptosis. Note, however, that the lipopolysaccharide+D-galactosamine treatment used in this study preferentially activates the extrinsic pathway of apoptosis [25]. Thus, differences observed in the onset of these events could be because the molecular mechanisms essential to mitochondrial permeabilization may be different in the extrinsic and intrinsic pathways [21,26].

The single channel behavior of MAC is easily distinguishable from the translocase of the outer membrane (TOM) and the voltage dependent anion-selective channel (VDAC), two constitutive channels of the mitochondrial outer membrane [15,18,20,27,28]. Both VDAC and TOM channels are voltage dependent and have a mean peak conductance of about 700 pS [15,18]. In contrast, MAC is a voltage-independent channel, with a slight cation-selectivity and a variable but high peak conductance (from 1.5 to greater than 9 nS) (Fig. 1A and [15,18]). As shown in Table 1, the mean peak conductance of MAC from apoptotic FL5.12 cells and HeLa cells are 4.5 nS and 3.3 nS, respectively [15,18,20]. Importantly, conductance can be used to estimate the pore size if the channel is large and water filled. The pore diameter of MAC with a conductance of ~4.5 nS, assuming a pore length of 5.5 nm [29], has been estimated to be ~5 nm using the method of Hille [30]. The estimate of pore diameter increases to more than 7 nm if access resistance is included. Therefore, the estimated pore diameter of MAC is much larger than the diameter of cytochrome *c*, which is ~3 nm. MAC has a variable conductance and there may be several reasons for the heterogeneity of MAC's single channel conductance. There may be an asynchronous response of individual mitochondria within a cell to an apoptotic signal [31,32]. Thus, MACs with smaller conductances may reflect channels found in mitochondria earlier in their individual response. Alternatively, the heterogeneity may reflect MAC of different compositions and/or the inherent properties of MAC components, and this last hypothesis will be discussed in more detail in the Section 5 of this review.

Several observations favor the designation of MAC as the cytochrome *c* release channel during the early phase of intrinsic apoptosis. The estimated pore size of MAC is sufficient to allow passage of ~3 nm diameter cytochrome *c* (Table 1). In addition, proteoliposomes containing mitochondrial outer membranes from apoptotic FL5.12 cells fail to retain cytochrome *c* compared with those isolated from the control [15]. Finally, physiological concentrations of cytochrome *c* modify the current flow through MAC in a manner consistent with its entrance into the pore. Indeed, the blocking effects of cytochrome *c* on

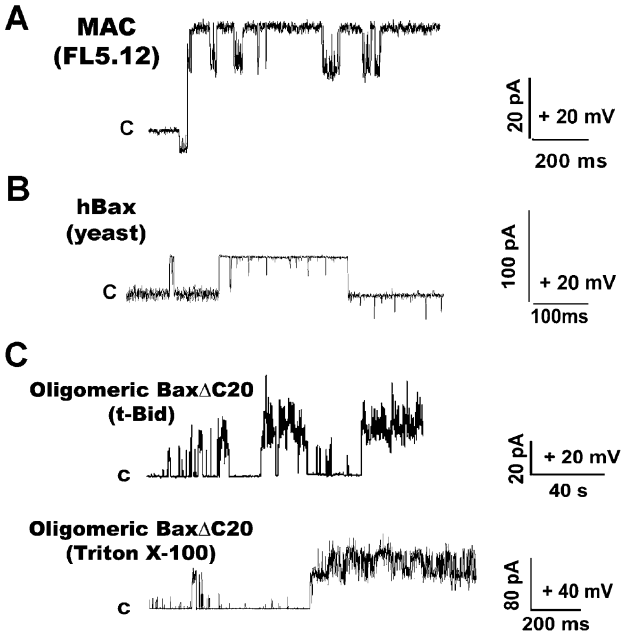

Fig. 1. A comparison of MAC and two oligomeric Bax channel activities. (A) A typical current trace is shown of a single MAC in a patch excised from a proteoliposome containing mitochondrial outer membranes of IL-3 starved FL5.12 cells at +20 mV. (B) A typical current trace is shown at +20 mV of a single hBax channel in proteoliposomes containing mitochondrial outer membranes of VDAC-less yeast expressing a c-*myc*-tagged human Bax protein. (C) Current traces at +20 and +40 mV are shown of the channel activities recorded when oligomeric, recombinant, human Bax truncated for 20 AA at the C-terminus (Oligomeric BaxΔC20) is included in the micropipette tip and seals were formed on giant liposomes containing no proteins. Oligomerization of Bax was induced by preincubation of the corresponding monomeric form of Bax with Triton X-100 or the 'BH3 only domain' protein t-Bid as indicated. All current traces were obtained by patch clamping and are shown at 2 kHz filtration with 5 kHz sampling. Patching media was symmetrical 150 mM KCl, 5 mM HEPES–KOH/ pH 7.4. Giant liposomes containing azolectin with or without outer membranes were formed by dehydration-rehydration as previously described [28]. Parts of this figure were modified from refs. [15,20].

the current flow through MAC are similar to those of other molecules on their translocating channels, e.g., ATP on VDAC and DNA on hemolysin channels [18,33,34].

3. General characteristics of Bcl-2 family proteins

The proteins of the Bcl-2 family possess either pro- or anti-apoptotic activity. Although their overall amino acid sequence homology is fairly low, the proteins contain up to four highly conserved domains, referred to as the Bcl-2 homology domains or BH domains. The BH domains are essential for interactions between the proteins and for their activities [35–38].

The pro-apoptotic subfamily can be divided into two groups: (i) the multi-domain proteins, including Bax, Bak, and Bok; and (ii) the 'BH3 domain only' proteins, including e.g., Bid, Bad, and Bim. Multi-domain proteins contain the BH1-3 domains while 'BH3 domain only' proteins are homologous in only the BH3 domain which represents the so called death domain [1–5].

Despite their structural similarities, the subcellular localization of these pro-apoptotic members is different. For example, while Bak is inserted into the mitochondrial outer membrane in normal cells, Bax is found predominantly in the cytosol as soluble monomers or, to a lesser extent, loosely associated with the mitochondria [39,40]. Upon apoptotic stimulation, Bax undergoes conformational changes accompanied by a translocation of the protein from the cytosol to the mitochondria. Bax is then inserted and finally oligomerized in the mitochondrial outer membrane [23,41–43]. Similar to Bax, Bak activation involves conformational changes and subsequent oligomerization in the outer membrane [44–46]. Finally, the fact that both Bax and Bak need to be inactivated to protect cells from different apoptotic stimuli suggests that these two proteins may be functionally redundant [16].

The 'BH3 domain only' proteins appear to function mainly through the activation of the pro-apoptotic multi-domain proteins. The best studied member of this subfamily is Bid, which is essential to the crosstalk signaling between the extrinsic and intrinsic signaling pathways of apoptosis [6,16,39]. In fact, caspase 8 is activated through the extrinsic pathway and cleaves Bid. This cleavage generates a C-terminal fragment (t-Bid). Finally, t-Bid interacts with Bax and Bak to trigger their activation, thereby facilitating the release of pro-apoptotic factors from the intermembrane space of mitochondria.

The anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, contain all four conserved BH domains (BH1-4) but their intracellular localization varies. Bcl-2 is found exclusively in intracellular membranes, including the mitochondrial

Table 1
Comparison of the electrophysiological properties of MAC and Bax channels measured by patch clamp techniques[a]

	MAC[b] (FL5.12)	MAC[c] (HeLa)	hBax[d] (yeast)	Oligomeric BaxΔC20[e]
Peak conductance (nS)	4.5±2.4	3.3±1.3	3.4±1.0	5.0±3.0
Selectivity	Sl. cationic	Sl. cationic	Sl. cationic	Sl. cationic
P_K/P_{Cl}	3.0±0.9	n.d.	4.7±1.3	6.8±1.0
Voltage dependent	No	No	No	No
Pore size (nm)[f]	4.9±1.4	4.2±0.8	4.3±0.7	5.1±1.7

n.d.: not determined.
sl.: slight.
[a] All these measurements were done in a buffer containing 0.15 M KCl and azolectin was used as artificial lipid.
[b] MAC of IL-3 starved mouse FL5.12 cells [15,18].
[c] MAC of staurosporine-treated HeLa cells [20].
[d] hBax channels of yeast expressing c-*myc*-tagged human Bax [15,60].
[e] Recombinant oligomeric BaxΔC20 channels in artificial membranes [15,20].
[f] Calculated from peak conductance using the method of Hille [30].

outer membrane, the endoplasmic reticulum, and the nuclear envelope [47]. In contrast, Bcl-x_L is found both in intracellular membranes and as a soluble form in the cytosol. This soluble form of Bcl-x_L translocates to mitochondria during apoptosis [48]. Bcl-2 and Bcl-x_L heterodimerize with Bax and their overexpression protects cells from apoptosis induced by different stimuli. These findings indicate these two anti-apoptotic proteins are functionally redundant [49]. Notably, Bcl-2 overexpression inhibits both Bax activation and oligomerization [42,50].

4. Recombinant proteins and heterologous expression in yeast as models to study the channel-forming activities of the Bcl-2 family proteins

The development of model systems often provides a key to understanding complex systems as they are not encumbered by the intricacies of the cell. Model systems can also reveal unexpected properties relevant to signaling pathways, such as the intrinsic apoptotic pathway. The two core model systems developed to study the function of the Bcl-2 family proteins are the in vitro recombinant protein system and the heterologous expression of these proteins in the yeast *Saccharomyces cerevisiae*.

4.1. Channel-forming activities of recombinant Bcl-2 family proteins in vitro

Bcl-x_L deprived of its hydrophobic C-terminus was the first Bcl-2 family member for which a 3-dimensional structure was determined [51]. Two amphipathic α-helices, α5 and α6, form a structure resembling that found in bacterial toxins capable of pore formation. This observation led to the hypothesis that Bcl-2 family proteins could form channels, which are able to make membranes permeable to ions and/or proteins. This hypothesis was further examined by several groups using purely synthetic systems whereby the channel forming activities of recombinant Bcl-2 family proteins were determined in planar bilayers. The principal results of these investigations are presented in Tables 2 and 3 [52–56].

Surprisingly, both anti-apoptotic and pro-apoptotic proteins have channel-forming activity in artificial lipid membranes. Typically, the channels formed are slightly cation-selective and voltage independent. The activity of all these channels is enhanced at low pH, which is consistent with the proposal that these proteins are structural and functional homologs of bacterial toxins [57]. It should be noted, however, that the anti-apoptotic members of the Bcl-2 family proteins and t-Bid only form large-conductance channels when they are assayed or pre-inserted in the artificial membranes at low pH (Table 2 and [52,55]). Furthermore, no channel activity has been detected that can be attributed to Bcl-2 in mitochondria of cells overexpressing this protein [15,58]. These observations have raised questions about whether or not the anti-apoptotic and 'BH3 domain only' members of the Bcl-2 family have channel-forming activity under physiological conditions.

Table 2
Electrophysiological characteristics of some recombinant anti-apoptotic members of the Bcl-2 family proteins in planar bilayers[a]

	Bcl-2ΔC21[b] (aa 1–218) 40 nM	His6-Bcl-2ΔC21[c] (aa 1–218) 2 μM	Bcl-xLΔ-His6[d] (aa 1–209) 130 nM–3 μM
Peak conductance pH ~7 (nS)	2.1	0.03	0.42
Selectivity	Slightly cationic	cationic	cationic
Voltage dependent	No	No	No
Transition size (nS)/substates	0.08 to 1.9 3 substates	0.005 to 0.027 3 substates	0.12–0.27 3 substates
pH dependence	Increased insertion and activity at pH 4	Increased insertion and activity at pH 4	Increased insertion and activity at pH 4
Lipids used	10–20% PC, 80–90% anionic lipids	20% diphy-PC, 80% diphy-PS	40% PS, 60% PC
Pore size (nm)[e]	3.9	0.4	1.7

[a] All conductances and transition sizes were normalized to buffer containing 0.15M KCl. Note that these recombinant proteins were used as monomers during these experiments.
[b] From [52].
[c] From [56].
[d] From [54].
[e] Calculated from peak conductance using the method of Hille [30].

In contrast, Bax has complex channel-forming activity. Monomers of Bax were originally shown to form channels that are quite large (from a few pS to 1.5 nS) [52,53]. However, even this large peak conductance corresponds to a pore that is undoubtedly too narrow to allow passage of small proteins like cytochrome *c* (Table 3). Bax channels are heterogeneous in size, poorly selective, and their sometimes slight voltage dependence is a matter of debate [52,53]. Most importantly, Antonsson's group showed that the oligomerization of Bax before its insertion into planar bilayers allows for the formation of non-selective, voltage-independent channels that show a gradual increase in conductance from 0.09 to 5.4 nS [59]. The estimated diameter of the 5.4 nS conductance Bax is 5.5 nm, which is theoretically consistent with the passage of ~3 nm cytochrome *c*. This discovery was recently confirmed in a study in which the channel-forming activity of oligomeric Bax was monitored by patch clamp techniques (see Table 1, Fig. 1C and [20]). Importantly, physiological concentrations

Table 3
Electrophysiological characteristics of some recombinant pro-apoptotic members of the Bcl-2 family proteins in planar bilayers[a]

	BaxΔC19[b] (aa 1–173) 1 nM	BaxΔC20[c,d] (aa 1–172) 20 nM	t-Bid (BidΔN55)[e] (aa 56–195) 2–4 μM
Peak conductance at pH ~7 (nS)	1.50	1.60	0.27
Selectivity	Slightly anionic	Slightly cationic	n.d.
Voltage dependent	No	Yes	No
Transition size (nS)/substates	0.022–1.0 4 substates	0.026–0.25 4 substates	0.012–0.20 3 substates
pH dependence	Increased insertion and activity at pH 4	Eight times more active at pH 4	n.d.
Lipids used	10–20% PC, 80–90% anionic lipids	60% diphy-PC, 40% PS	50% POPE, 50% POPG
Pore size (nm)[f]	3.2	n.d.	1.4

n.d.: not determined.
[a] All conductances and transition sizes were normalized to buffer containing 0.15M KCl, except in BaxΔC20. Note that this recombinant proteins were used as monomers during these experiments.
[b] From [52].
[c] From [53].
[d] The buffer contains 0.125 M NaCl.
[e] From [55].
[f] Calculated from peak conductance using the method of Hille [30].

of cytochrome *c* modify the current flow through these oligomeric Bax channels in a manner consistent with its entrance into the pore, as was previously reported for MAC (see above Section 2 and [18,20]).

4.2. Lessons from yeast

The budding yeast *Saccharomyces cerevisiae* is a powerful tool to understand the complexities of the function of Bcl-2 family members (reviewed in [60]). Since the first reports showing heterologous expression of Bax induces cell death and cytochrome *c* release in yeast [61,62], investigators have now reached a level where data obtained in yeast provide new information about the function of apoptosis-regulating proteins. Moreover, the genome of *S. cerevisiae* is completely sequenced and contains no homologs of any Bcl-2 family proteins. All together, these observations indicate that yeast is an excellent model system in which to study the molecular mechanisms of permeabilization of the mitochondrial outer membrane during apoptosis.

An electrophysiological investigation of yeast mitochondria expressing human Bax (hBax) was undertaken. A VDAC-less strain was used in order to limit the number of channel activities in the mitochondrial outer membrane [63]. Outer membranes purified from mitochondria of yeast expressing and not expressing hBax were examined. A novel channel activity is present in the hBax-containing mitochondria, and absent in the control mitochondria (Fig. 1B and [15]). This novel channel is detected with a frequency that correlates with the level of hBax expression. This hBax channel activity displays a large peak conductance (3 to 4 nS), several substates, no voltage dependence, and a slight cation-selectivity (Table 1). This channel activity is similar to that of MAC found in mitochondria of apoptotic FL5.12 and HeLa cells (compare Fig. 1A and B; Table 1;[15,20]). The peak conductance indicates a pore size greater than 4 nm, which is theoretically large enough to allow the permeation of ~3 nm cytochrome *c*. Note that, under conditions where Bax induces the release of cytochrome *c*, a cytochrome *c*–GFP fusion protein is not released [64], suggesting that the 40-kDa fusion protein is not transported by this channel in yeast. While speculative, this finding suggests channels composed of just Bax may be unable to transport larger proteins such as Smac/Diablo (48 kDa for the native dimer) and AIF (65 kDa) that are normally released during apoptosis.

5. Regulation of MAC by the pro-apoptotic multi-domain proteins

5.1. Role of bax

Many reports indicate that the pro-apoptotic multi-domain Bcl-2 family proteins, especially Bax and Bak, are directly involved in the release of cytochrome *c* from mitochondria [1–5]. Although the complete molecular identity of MAC is yet to be determined, there is strong evidence indicating Bax plays a crucial role in MAC channel formation. First, MAC and recombinant oligomeric Bax channels have similar channel activities, including a comparable distribution of conductances above 1.5 nS, a slight cation-selectivity, and the effects of cytochrome *c* on MAC activity are consistent with the permeation of this heme protein (Fig. 1A and C, Table 1, and [20]). Dibucaine and propranolol inhibit cytochrome *c* release in isolated rat liver mitochondria mediated by recombinant oligomeric Bax. Importantly, both pharmacological agents inhibit MAC activity [65,66]. Furthermore, MAC activity appears when Bax levels increase in mitochondria of apoptotic FL5.12 cells [15]. Finally, MAC is also detected as an increase in outer membrane permeability by patch clamp techniques in staurosporine-treated HeLa cells when Bax-GFP forms clusters in mitochondria and cytochrome *c* is released (Fig. 2 and [20]). While correlative, these lines of evidence support a mechanistic link between Bax translocation and oligomerization, MAC formation, and cytochrome *c* release, which is strengthened by the molecular and immunological studies described below.

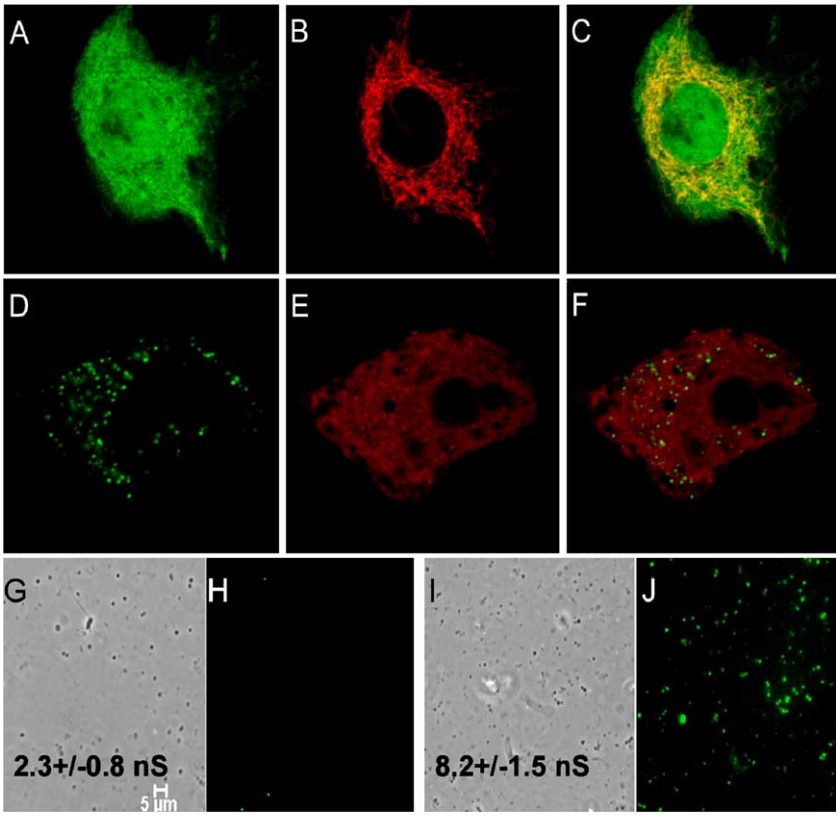

Fig. 2. MAC formation coincides with cytochrome *c* release and GFP-Bax translocation into mitochondria. Confocal images of staurosporine-treated HeLa cells show fluorescence of GFP-Bax in green (A, D) and cytochrome *c* immunofluorescence in red (B, E), and are merged in panels C and F. Diffuse GFP-Bax fluorescence becomes punctate with time indicating Bax forms clusters at the level of the mitochondrial outer membrane during apoptosis. Cytochrome *c* immunofluorescence becomes diffuse with time showing cytochrome *c* release during staurosporine treatment. Phase contrast (G, I) and fluorescence (H, J) images show mitochondria isolated from control (G, H) or apoptotic cells (I, J) at the time the GFP-Bax fluorescence became punctate in the staurosporine-treated cells. The mean (±SE) conductances of patches shown in panels G and I were measured by patch clamping these mitochondria to determine onset of MAC activity. Scale bar is 5 µm for G–J. Parts of this figure were modified from ref. [20].

Bax is a component of MAC of staurosporine-treated HeLa cells because MAC activity is depleted after immunoprecipitation of oligomeric Bax. In this model, MAC activity is present in total mitochondrial lysates and fractions containing oligomeric, but not monomeric, Bax (Fig. 3A and [20]). This is expected as Bax oligomers form in the outer membrane following Bax translocation to mitochondria in response to apoptotic signals at the time that cytochrome *c* is released [17,20,42,50]. However, these findings also indicate MAC activity is associated with Bax oligomers and not monomers. Importantly, MAC activity is depleted from solubilized mitochondrial fractions by Bax antibodies raised against an N-terminal epitope of the protein (Fig. 3B and C; [20]). This epitope is inaccessible in monomeric Bax but becomes exposed following Bax activation. Thus, these antibodies selectively immunoprecipitate oligomeric Bax [67,68]. As expected, these Bax antibodies immunoprecipitated all the Bax in the oligomeric fractions, but none in the fraction containing Bax monomers and only partially cleared the total lysates of Bax (Fig. 3B). The concomitant loss of MAC activity and oligomeric Bax by immunoprecipitation strongly supports the notion that Bax is a component of MAC. Future investigations should expand these studies to include other pro-apoptotic members of the Bcl-2 family proteins, e.g. Bak, in order to determine if other multi-domain pro-apoptotic factors underlie MAC activity.

5.2. Role of bak

Pro-apoptotic multi-domain Bak forms oligomers in the mitochondrial outer membrane and co-immunoprecipitates with Bax during early apoptosis [46]. Previous studies suggest Bax and Bak are functionally redundant with respect to their role in apoptosis (see [1–4] for review). For instance, cytochrome *c* release occurs in Bax and Bak single knockout Mouse Embryonic Fibroblasts (MEF) cells but not in the double Bax/Bak knockout cells during staurosporine treatment [16]. Hence, deletion of both Bax and Bak genes is needed to make these cells resistant to apoptosis.

The same Bax and Bak knockout cell lines were used to evaluate the involvement of Bax and Bak in MAC formation (Fig. 3 D–F and [20]). The mean conductance of mitochondria isolated from control and staurosporine-

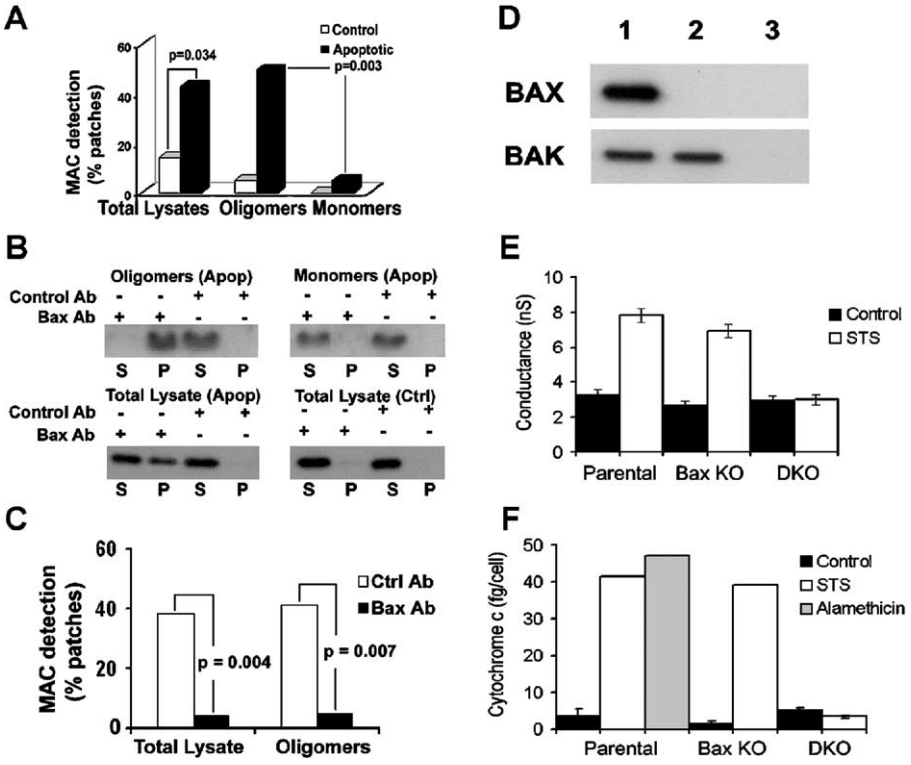

Fig. 3. Are Bax and/or Bak components of MAC? (A) Histograms show the fraction of patches displaying MAC activity after reconstituting total mitochondrial lysates, oligomeric Bax (oligomers) or monomeric Bax (monomers) fractions of control or apoptotic cells in proteoliposomes. $N=20-23$ independent patches for each conditions. P values shown were calculated using the Fisher Exact Statistical test [86]. (B) Total mitochondrial lysates and fractions of HeLa cells containing oligomeric and monomeric Bax were immunoprecipitated with anti-Bax antibodies (Bax Ab) or total rabbit IgG (control Ab). The pellets containing the immunoprecipitated proteins (P) and their respective supernatants (S) were subjected to SDS-PAGE, and the presence of Bax was assessed by Western blot. (C) The supernatants from the immunoprecipitation assays described above and corresponding to immunoprecipitates with anti-Bax antibodies (Bax Ab, closed) or with total rabbit IgG (Control Ab, open) were reconstituted in proteoliposomes. MAC detection frequency was determined by patch clamping. $N=20-23$ independent patches/condition. (D) Western blots show expression of Bax and Bak in three MEF cell lines: Lane 1. Parental, Lane 2. single Bax KO, Lane 3. Bax/Bak double KO (DKO). Total membrane extracts containing 20 μg protein were probed for Bax and Bak, respectively. (E) Mitochondria were isolated from the indicated cell lines that were and were not treated with staurosporine (STS). The mean conductance of the outer membrane was measured by patch clamping isolated mitochondria ($N=20-23$ patches per condition). (F) Cytochrome c release was assayed by ELISA in the supernatants after permeabilization of the cells with digitonin. When indicated, alamethicin (80 μg/mL) was added during digitonin treatment as a positive control for cytochrome c release [66]. Parts of this figure were modified from ref. [20].

treated cells was measured using patch clamp techniques. A significant increase in outer membrane permeability was observed in mitochondria of staurosporine-treated parental and Bax deficient cells compared to those of untreated cells (Fig. 3E). This increase in mean conductance is consistent with the presence of MAC activity in apoptotic mitochondria as was found with mitochondria of other apoptotic cells [15,20]. However, no increase in mean conductance is 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 (Fig. 3F). While alternative interpretations are possible, and considering that Bax is a component of MAC of staurosporine-treated HeLa cells, these data support the notion that Bak may replace Bax as a structural component of MAC in Bax deficient cells. That is, Bax and Bak may be functionally redundant with respect to MAC.

6. Regulation of MAC by Bid

During apoptosis, the 'BH3 domain only protein' Bid is cleaved by caspase 8 to produce a 15.5-kDa C-terminal (t-Bid) and a 6.5-kDa N-terminal fragment. The fragment t-Bid was shown to trigger oligomerization of both Bax and Bak in the mitochondrial outer membrane, which resulted in cytochrome c release [39,45]. It was also reported that a mixture of t-Bid plus the 6.5-kDa N-terminal fragment of Bid was able to trigger recombinant monomeric Bax oligomerization in artificial membranes [59]. This combination also allows the formation of 100–200 pS, voltage-dependent, and cation-selective channels [59]. However, if t-Bid alone is added to recombinant monomeric Bax, voltage-independent and slightly cationic channels with conductances of 1.5 to 10 nS are detected by patch clamp techniques [20]. Moreover, cytochrome c is transported through these t-Bid induced Bax channels, which makes them very similar to MAC [20]. Finally, MAC-like activities

and cytochrome *c* release are observed when recombinant t-Bid is added to isolated mitochondria containing endogenous Bax and Bak (unpublished results), suggesting that t-Bid could allow cytochrome *c* release through the direct activation of MAC.

7. Regulation of MAC by the anti-apoptotic multi-domain proteins

Bcl-2 and Bcl-x_L are the best studied members of the sub-family of anti-apoptotic proteins in the Bcl-2 family [1–5]. MAC was never detected in IL-3 starved FL5.12 cells that overexpress Bcl-2 [15]. Moreover, overexpression of Bcl-x_L abolishes MAC-like channel detection in yeast overexpressing hBax [15]. Together, these results suggest that these two anti-apoptotic proteins are able to inhibit MAC formation and the molecular mechanisms of this inhibition remain to be determined. Note that no new channel activities are detected when Bcl-2 is overexpressed in FL5.12 or MDA-231 cells, indicating this protein does not form channels in native mitochondrial membranes [15,58]. However, recombinant Bcl-x_L forms or activates small channels in yeast mitochondria, which have a size comparable to those first described in artificial membranes [54,69]. Finally, channels whose conductance is between 0.75 and 1 nS are detected in isolated yeast mitochondria or mitochondria from squid giant synapse after addition of a caspase cleaved recombinant Bcl-x_L (ΔN-Bcl-x_L) [69]. These channels have conductances and other properties similar to mitochondrial channels detected in giant squid synapses during early stages of hypoxia-mediated apoptosis, when Bcl-x_L is cleaved by caspases [70]. Nevertheless, the pore size appears to be too small to allow for cytochrome *c* transport through the mitochondrial outer membrane and therefore is unlikely to have the same role as MAC during early steps of the intrinsic apoptotic pathway.

8. Does the formation and activity of MAC require partners other than oligomeric bax?

It has been suggested that the oligomerization of Bax mediated by t-Bid is dependent on one or more mitochondrial proteins [71]. Although oligomeric Bax has been shown to be a component of MAC, no endogenous proteins resident in the outer membrane are clearly implicated in the structure of MAC. Early studies suggested VDAC interacted with recombinant monomeric Bax, and this same recombinant Bax allowed the release of cytochrome *c* from liposomes containing purified VDAC [72]. These studies were followed by the observation that mitochondria from VDAC1-less yeast strains did not release cytochrome *c* after Bax expression [73]. It was also reported by the same group that this interaction between monomeric Bax and VDAC formed a novel large pore that is permeable to cytochrome *c* [74]. However, it was recently shown by others that neither recombinant monomeric nor oligomeric Bax were able to interact with VDAC in electrophysiological experiments [75]. Moreover, several other groups have generated data indicating that VDAC1 was not essential for Bax-mediated cytochrome *c* release in yeast [63,71,76]. Therefore, the involvement of VDAC in early apoptosis remains a subject of debate. Nevertheless, VDAC is not present and therefore is not a component of 260 kDa fractions from apoptotic HeLa cells that express MAC activity and contain oligomeric Bax [20,42].

Importantly, new lines of evidence have emerged supporting roles for mitochondrial proteins in MAC formation. Other isoforms of VDAC may play an integral regulatory role in apoptosis and MAC formation as VDAC2 inhibits Bak activation through their mutual interaction [77]. The first alpha helix of Bax contains a functional mitochondrial targeting sequence which is exposed upon Bax activation and may be responsible for Bax translocation [78]. Finally, Bcl-2 interacts with Tom20, a receptor of the protein import complex of the outer membrane, i.e., the TOM complex [79]. Together, these observations suggest the TOM complex and VDAC2 may play a role in Bax or Bak stabilization in the outer membrane and then, MAC formation. Future studies are needed to identify additional components of MAC and proteomics after immunoprecipitation of oligomeric Bax should provide a logical approach.

Lipids may be functional and/or structural elements of MAC. It has for instance been hypothesized that Bax and even t-Bid may destabilize lipid bilayers and that ceramide

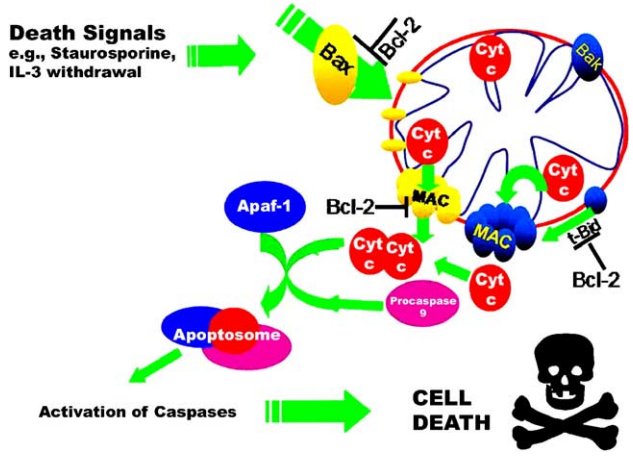

Fig. 4. MAC provides a pathway for cytochrome *c* to exit the mitochondria during apoptosis and MAC formation is a critical target for regulation of the mitochondrial apoptotic response by Bcl-2 family members. Induction of the intrinsic pathway results in Bax translocation to mitochondria and oligomerization. 'BH3 domain only' proteins facilitate Bax activation, as well as Bax and Bak oligomerization to form MAC. MAC could be predominantly composed of either Bax or Bak. There may be other components and Bax and Bak may or may not form hetero-oligomers. MAC formation results in the release of cytochrome *c* and ultimately apoptosome formation, caspase activation and cell death.

can interact with mitochondrial membranes [80–83]. These effects could then orchestrate the formation of lipidic pores large enough to allow the passage of cytochrome *c* [82–85]. Pharmacological studies of MAC have shown that amphiphilic cations such as trifluoperazine, dibucaine, and propranolol block the current flow through MAC with IC_{50}'s of 1–50 μM [65]. These results suggest that MAC activity is highly sensitive to membrane fluidity, because all these agents are known to modify this bilayer property.

9. Conclusion

The central role of mitochondria in apoptosis became apparent as the regulatory role of Bcl-2 family members in releasing mitochondrial apoptotic factors was resolved. In this review, data indicating MAC is a critical target of this family of proteins are presented (Fig. 4). MAC is the putative cytochrome *c* release channel which forms during early apoptosis and the formation of MAC is integral to the intrinsic pathway [19]. Bcl-2 family proteins regulate intrinsic apoptosis and MAC formation. Oligomeric Bax is a component of MAC and Bcl-2 overexpression inhibits MAC formation. Moreover, Bax and Bak are functionally redundant regarding MAC formation and cytochrome *c* release. Finally, recombinant t-Bid activates MAC in mitochondria which contain endogenous levels of Bax and Bak. These lines of evidences support the hypothesis that Bcl-2 family proteins regulate MAC formation and the activation of this channel leads to cytochrome *c* release, apoptosome formation, and finally cell death.

Although apoptosis is an essential physiological process, it is also involved in a wide range of pathological conditions. For example, increased apoptosis has been associated with neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's diseases (for review, see [4]). Also, decreased apoptosis is involved in some cancer and autoimmune disorders. A better understanding of the molecular mechanisms underlying early events of apoptosis should facilitate development of treatments for these diseases that have higher efficacies. In this respect, MAC is a potential therapeutic target whose activity could be modulated in order to increase or decrease apoptosis. Molecular and pharmacological studies are underway to establish clearer links between MAC activity and different pathological conditions in which the apoptotic pathway is known to be amenable to such interventions.

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