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Review

Is MAC the knife that cuts cytochrome *c* from mitochondria during apoptosis?

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Abstract

Apoptosis is a phenomenon fundamental to higher eukaryotes and essential to mechanisms controlling tissue homeostasis. 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 proapoptotic factors. Mitochondrial apoptosis-induced channel (MAC) is the mitochondrial apoptosis-induced channel and is responsible for cytochrome *c* release early in apoptosis. MAC activity is detected by patch clamping mitochondria at the time of cytochrome *c* release. The Bcl-2 family proteins regulate apoptosis by controlling the formation of MAC. Depending on cell type and apoptotic inducer, Bax and/or Bak are structural component(s) of MAC. Overexpression of the antiapoptotic protein Bcl-2 eliminates MAC activity. The focus of this review is a biophysical characterization of MAC activity and its regulation by Bcl-2 family proteins, and ends with some discussion of therapeutic targets.
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Keywords: MAC; mitochondrial apoptosis-induced channel; apoptosis; cytochrome *c*; patch-clamp; Bcl-2; Bax; t-Bid

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

Introduction

Apoptosis is a conserved cell death mechanism essential for normal development and tissue homeostasis in multicellular organisms (reviewed in Danial and Korsmeyer[1], Antonsson[2], Sharpe *et al.*[3], Green and Kroemer[4], Kuwana and Newmeyer[5], Fadeel and Orrenius[6], Lucken-Ardjomande and Martinou[7], Martinez-Caballero *et al.*[8] and Dejean *et al.*[9]). Two major signaling pathways leading to cell death by apoptosis have been identified. They 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.[10] This receptor activation ultimately leads to processes that cleave and activate the initiator procaspases 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 and plasma membrane blebbing.

Mitochondria play a pivotal role in the response of a variety of cell types to a diverse set of apoptotic signals that activate the intrinsic pathway, including DNA damage, growth factor withdrawal, and viral infection. 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.[1–3,6,11] 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 (see other papers in this issue). 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 into the cytosol (recently reviewed in Danial and Korsmeyer[1], Antonsson[2], Sharpe *et al.*[3], Green and Kroemer[4], Kuwana and Newmeyer[5], Lucken-Ardjomande and Martinou[7], Martinez-Caballero *et al.*[8], Dejean *et al.*[9], Juin *et al.*[12]). 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, for example Bid.[13] In some cells, extrinsic caspase 8 cleaves Bid to form t-Bid, which facilitates Bax activation and oligomerization in mitochondria. Thus, t-Bid is generated by the extrinsic pathway and goes on to activate the intrinsic pathway.

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 would rupture the outer membrane, and spill cytochrome *c* and other proapoptotic proteins into the cytosol.[14–16] However, it was recently shown that sustained PTP opening is primarily involved in necrosis and ischemia–reperfusion.[17–19] Cyclophilin-D is a regulator of the PTP. 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.[17] Furthermore, cytochrome *c* release can occur in the absence of mitochondrial depolarization and without loss of outer membrane integrity. These observations indicate that, instead of rupturing, a more selective mechanism of permeabilization is operating, like the formation of a pore in the outer membrane.[11,20–24]

The mitochondrial apoptosis-induced channel (MAC), was detected by directly patch clamping mitochondria isolated from cells in early apoptosis. 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.[20,23,24] Nevertheless, MAC and PTP transient opening may act alone or in combination, depending on cell type and death stimulus, to relocalize Bax to the mitochondria, remodel the cristae, and maximize cytochrome *c* release to amplify the death signal.[22,25]

The focus of this review is a biophysical characterization of MAC, cytochrome *c* permeability, and how Bcl-2 family proteins regulate the permeability of the mitochondrial outer membrane through the formation of MAC. We have also incorporated some personal notes regarding Stan Korsmeyer's contributions to our present understanding of MAC. Early in 1996, a call came into the lab from Stan. The loud commotion of the French Press in the lab made it nearly impossible to hear his exciting ideas about Bcl-2 family proteins and mitochondria. Needless to say, I then entered the field of apoptosis, a field in which Stan was a leading pioneer. This event began our many years of collaborations. Stan generously provided materials in which to expand our studies but also commitment and inspiration. For many years, this visionary scientist facilitated our work and that of so many other labs; he is sorely missed.

Electrophysiological Characterization of MAC

MAC was first detected in patch-clamp experiments on mitochondria isolated from apoptotic FL5.12 cells 12 h after interleukin-3 (IL-3) withdrawal.[20] Stan actually provided this cell line and many of the protocols with which we began this study. Single-channel analysis of MAC was performed in proteoliposomes because the mitochondrial outer membrane is densely packed with voltage-dependent anion-selective channel (VDAC) and translocase of the outer membrane (TOM) channels. Proteoliposomes were formed with mitochondrial outer membranes purified from apoptotic and control cells (Figure 1a). Membrane patches were removed from the proteoliposomes with a micropipette and the current flow through the individual channels was characterized.

MAC is a heterogeneous high-conductance channel. There are multiple subconductance levels and MAC has transitions of up to 2.5 nS (Figure 1b). Although flickering between conductance states is sometimes observed, current traces usually show MAC occupying a stable open conductance state with relatively infrequent transitions.[20,23,24] The activity of MAC is significantly different from the constitutive channels of the mitochondrial outer membrane, TOM and VDAC. The single-channel parameters of peak conductance, transition

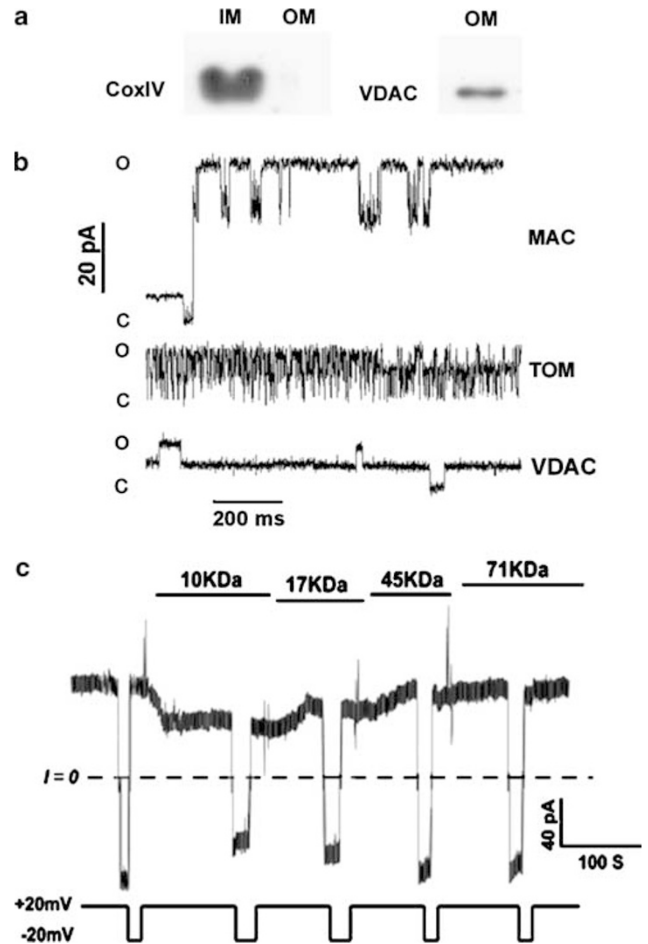

Figure 1 MAC is a high conductance channel of the outer membrane permeable to up to 17 kDa polymers. (**a**) Immunoblots show the presence of the outer membrane protein VDAC but not the inner membrane protein cytochrome oxidase subunit IV (CoxIV) in the outer membranes (OM, 2 μg) purified from mitochondria of apoptotic FL5.12 cells. Inner membranes (IM, 2 μg) are the positive control for CoxIV. (**b** and **c**) MAC activity was reconstituted by incorporating mitochondrial outer membranes of apoptotic FL5.12 cells into proteoliposomes. (**b**) Current traces at 20 mV allow comparisons of the single-channel behavior of MAC, VDAC, and TOM channels. O and C indicate open and closed state current levels, respectively. (**c**) The pore size of MAC was estimated by the polymer exclusion method. Current traces are shown of a 4 nS MAC after sequential perfusion of the bath with media containing 5% w/vol of indicated MW dextrans as the voltage was switched between $\pm$20 mV. 10 and 17 kDa MW dextrans are permeant as they induce a decrease in the current, but 45 and 71 kDa polymers are not. Parts of this figure were reprinted from Pavlov *et al.*,[20] Guo *et al.*,[23] and Martinez-Caballero *et al.*[29]

size, selectivity, and voltage dependence for these channels are in Table 1 and illustrated by the current traces of Figure 1. Note that the conductance of VDAC was 0.68$\pm$0.09 nS in 0.15 M KCl media in these studies,.[20] which approximates the 4 nS typically reported by others in higher ionic strength (1 M KCl) media.[26–28]

The conductance of MAC is both variable and high.[23,24] The mean conductance of MAC of apoptotic HeLa and FL5.12 cells is 3.3 and 4.5 nS, respectively.[20,23,24] MAC is typically a voltage-independent channel,[20,23,24,29] although it has also been reported to occupy less than peak conductances at higher potentials.[30] The channel is slightly cation-selective, which is consistent with MAC's putative role in releasing the

Table 1 Comparison of the properties of mitochondrial outer membrane and BaxΔC20 channels while patch clamping proteoliposomes[a]

	MAC[b] FL5.12	MAC[c] HeLa	hBax[d]	BaxΔC20[e]	TOM[f]	VDAC[f]
Peak conductance (nS)	4.5±2.4	3.3±1.3	3.4±1.0	5.0±3.0	0.7±0.1	0.7±0.1
Ion selectivity	Cation	Cation	Cation	Cation	Cation	Anion
P_K/P_{Cl}	3.0±0.9	ND	4.7±1.3	6.8±1.0	3.6±0.8	0.7±0.1
Voltage dependent	No	No	No	No	Yes	Yes
Pore size (nm)[g]	4.9±1.4	4.2±0.8	4.3±0.7	5.1±1.7	2.0±0.1	2.0±0.2

[a]All these measurements were made 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.[20,23] [c]MAC of staurosporine-treated HeLa cells.[24] [d]hBax channels of yeast expressing *c-myc*-tagged human Bax.[20,53] [e]Recombinant oligomeric BaxΔC20 channels in artificial membranes.[20,24] [f]VDAC and TOM of FL5.12 cells.[20] [g]Calculated from peak conductance using the method of Hille[79] ND, not determined

cationic protein cytochrome *c* from mitochondria early in apoptosis.

The polymer exclusion method was used to measure the pore size of MAC,[23] as has been performed with other channels[31–35] MAC with a conductance of 4 nS is permeable to 10 and 17 kDa, but not 45 and 71 kDa dextran, as there is no current decrease upon introduction of the larger polymers (shown in Figure 1c). The polymer exclusion method indicates that MAC with conductances between 1.5 and 5 nS have pore sizes of 2.9–7.6 nm, which should be large enough to allow the passage of ~3 nm cytochrome *c*.[36]

Is MAC the Cytochrome *c* Release Channel?

Cytochrome *c* modifies the electrophysiological behavior of MAC in a manner consistent with entrance of cytochrome *c* into the pore (Figure 2).[23,24] Physiological concentrations (0.1–1 mM) of cytochrome *c* reduce the current flow through MAC with conductances between ~1.5 and 4 nS.[23,37] However, the effects of cytochrome *c* are complex and are now classified as Type 1 and Type 2.

A Type 1 response is a 4–50% decrease in the conductance that is voltage dependent, reversible, and associated with an increase in current noise (Figure 2a). Similarly, some neurotoxins and other fast blockers are not thought to traverse pores but can modify noise levels and decrease conductance presumably by binding to the open state of the pore.[38,39] However, the presence of cytochrome *c* in the pore would displace small ions, for example potassium, and hence decrease the amount of current through, or conductance of, the channel. Furthermore, the current noise increases as the driving force for cytochrome *c* translocation into the micropipette increases, that is with increased positive voltage (Figure 2b). These effects are comparable to those of translocated molecules on including ATP on VDAC and DNA on α-hemolysin.[26–28,40] Similar effects have also been found with putative translocation of maltodextrins through maltoporins and single ampicillin molecules moving through the general bacterial porin, OmpF (outer membrane protein F).[41,42] Thus, the Type 1 effect provides strong evidence that cytochrome *c* enters the pore of MAC and other data support the notion that MAC is the cytochrome c release channel (see below in this section). It is interesting that hemoglobin has no effect on MAC conductance, indicating this 32 kDa, heme-containing dimer does not permeate through 2–5 nS MAC

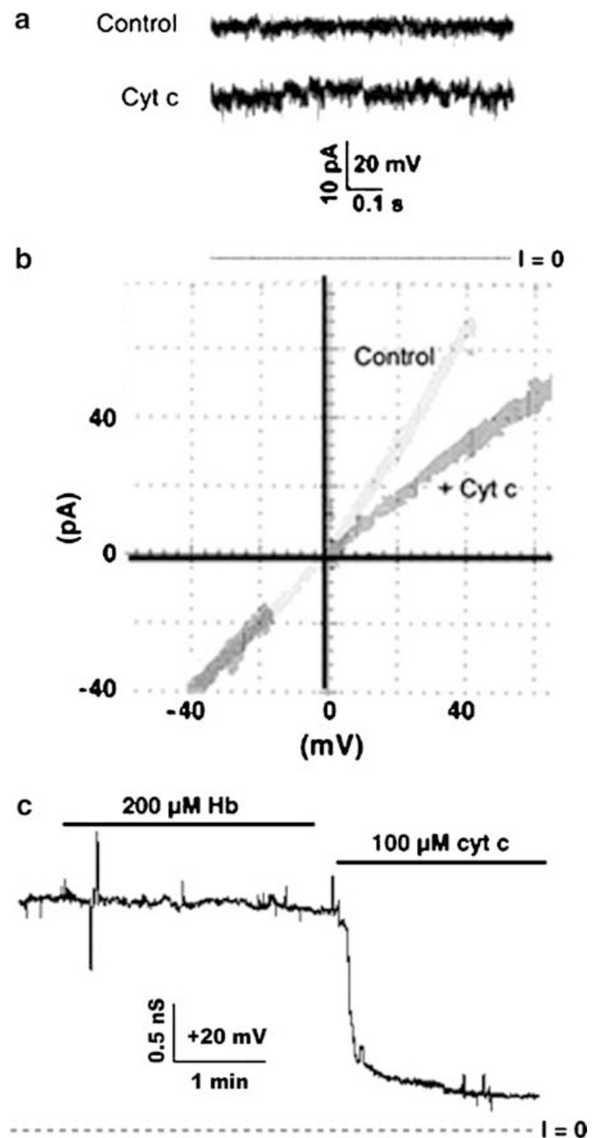

Figure 2 Cytochrome *c* induces Type 1 and Type 2 effects on MAC activity. (**a**) Current traces at +20 mV of MAC in the absence (Control) and presence of 100 μM cytochrome *c* (Cyt *c*) show a decrease in current and increase in noise corresponding to a Type 1 effect. (**b**) Current–voltage relationship for MAC in the presence (+Cyt c) and absence (Control) of cytochrome *c* shows a decrease in current through MAC and increase in noise at bath positive potentials. (**c**) A current trace of MAC illustrates that while hemoglobin (Hgb) has no effect, cytochrome *c* (Cyt *c*) induces a rapid Type 2 reduction in MAC conductance. Spikes in the current trace are due to perfusion of the bath. Reprinted from Guo *et al.*[23]

(Figure 2b). In contrast, the cationic protein ribonuclease A (14 kDa) has the same effects as cytochrome *c* (12.5 kDa).[23]

The Type 2 effects of cytochrome *c* are a 50–90% reduction in conductance that is dose dependent and voltage independent (Figure 2c). This response likely corresponds to a destabilization of the open state of MAC and is not a simple 'plug' because extensive washing typically does not restore conductance of the open state. We speculate that binding sites for cytochrome *c* may exist in the structure of 2–4 nS MAC, which could block the passageway and may be important in synchronization of apoptotic events.

The hypothesis that MAC provides the pathway through the outer membrane for release of cytochrome *c* early in apoptosis is supported by a variety of observations. As described above, the Type 1 effect is consistent with a partitioning of cytochrome c into the pore of $\sim$1.5–4 nS MAC, which is an essential step of translocation. The pore size of MAC estimated by the polymer exclusion method and/or calculated from the peak conductance is sufficient to allow the passage of the 12.5 kDa cytochrome *c*. The onset of MAC activity coincides with cytochrome *c* release in several systems.[20,23,24,29,43] The temporal association of MAC formation and cytochrome *c* release is consistent with biochemical findings. Specifically, proteoliposomes made from apoptotic membranes express MAC activity and fail to retain cytochrome *c* compared with proteoliposomes of control cells.[20] Hence, cytochrome *c* permeability increases early in apoptosis when MAC activity is present. Finally, the effects of Bcl-2 family proteins on MAC activity described below also support the identification of MAC as the cytochrome *c* release channel and provide some answers to questions about the relationship between MAC and Bcl-2 family proteins that were posed to me by Stan more than 10 years ago. In particular, we determined that MAC is, at least in part, composed of Bax and/or Bak and that Bcl-2 blocks MAC formation. These findings unequivocally link cytochrome *c* release with MAC and indicate that MAC is responsible for cytochrome c release in the early stages of intrinsic apoptosis.

Channel-forming Properties of Bcl-2 Family Proteins

X-ray studies of antiapoptotic Bcl-x_L deprived of its hydrophobic C-terminus revealed structural similarities with bacterial toxins capable of pore formation.[44] This observation led to the notion that Bcl-2 family proteins could form channels, which was examined by several groups using planar bilayers and recombinant proteins. The principal results of these investigations are presented in Table 2.[45–49]

Surprisingly, both antiapoptotic and proapoptotic proteins have channel-forming activity in artificial lipid membranes. Typically, the channels formed are slightly cation-selective and voltage independent. Consistent with their homology with bacterial toxins, the activity of all these channels is enhanced at low pH.[50] Unlike Bax and Bak, antiapoptotic Bcl-2 family proteins and t-Bid only form large-conductance channels when they are assayed or pre-inserted at low pH (Table 2).[45,48] Importantly, no channel activity has been detected that can be attributed to Bcl-2 in mitochondria of cells overexpressing this protein.[20,51] These observations raised questions concerning the physiological relevance of channels formed by the antiapoptotic and 'BH3 domain-only' members of the Bcl-2 family.

Recombinant Bax has channel activity that is remarkably similar to that of MAC. Although Bax monomers form channels that up to 1.6 nS, this conductance corresponds to a pore that is too narrow to allow passage of cytochrome *c* (Table 2).[45,46] However, oligomerization of Bax before insertion into planar bilayers allows for the formation of nonselective, voltage-independent channels that show a gradual increase in conductance up to 5.4 nS.[52] The diameter estimated from the peak conductance of 5.4 nS for Bax channels is 5.5 nm, which should easily allow the passage of $\sim$3 nm cytochrome *c*.[36] 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).[24] Like MAC, cytochrome *c* and RNAse A induce Type 1 and Type 2 effects in recombinant BaxΔC20 channels, while hemoglobin has no effect (see current traces and histograms of Figure 3).

The budding yeast *Saccharomyces cerevisiae* is a powerful tool to understand the complexities of the function of Bcl-2 family members because the genome contains no homologs of Bcl-2 family proteins (reviewed in Priault *et al.*[53]). Importantly, heterologous expression of Bax induces cell death and cytochrome *c* release in yeast.[54,55] Independently, Stan and Stephen Manon (U. Bordeaux) provided us with

Table 2 Electrophysiological characteristics of some recombinant pro- and anti-apoptotic Bcl-2 family proteins in a planar bilayers system[a]

	Proapoptotic multidomain		**Proapoptotic BH3-only**	**Antiapoptotic**		
	Bax ΔC19[b]	**Bax ΔC20[c]**	**t-Bid[d] (BidΔN55)**	**Bcl-2 ΔC21[e]**	**Bcl-2 ΔC21[f]**	**Bcl-xLΔ[g]**
Amino acids	1–173	1–172	56–195	1–218	1–218	1–209
His-tag	No	No	No	No	Yes	Yes
Final [ ] (μM)	0.001	0.020	2–4	0.040	2	0.13–3
Peak conductance pH $\sim$7 (nS)	1.50	1.60	0.27	2.10	0.03	0.42
Ion selectivity	Anion	Cation	ND	Cation	Cation	Cation
Voltage dependent	No	Yes	No	No	No	No
Transition size (nS)	0.02–1	0.03–0.25	0.01–0.2	0.08–1.9	0.01–0.03	0.12–0.27
↑activity/insertion at low pH	Yes	Yes	n.d.	Yes	Yes	Yes
Pore size (nm)[h]	3.2	n.d.	1.4	3.9	0.4	1.7

[a]All conductances and transition sizes were normalized to buffer containing 0.15 M KCl, except in [d]where the buffer contains 0.125 M NaCl. Note that these recombinant proteins were used as monomers during these experiments. [b]From Schlesinger *et al.*[45] [c]From Antonsson *et al.*[46] [d]From Schendel *et al.*[48] [e]From Schlesinger *et al.*[45] [f]From Schendel *et al.*[49] [g]From Minn *et al.*[47] [h]Calculated from peak conductance using the method of Hille.[79] ND, not determined

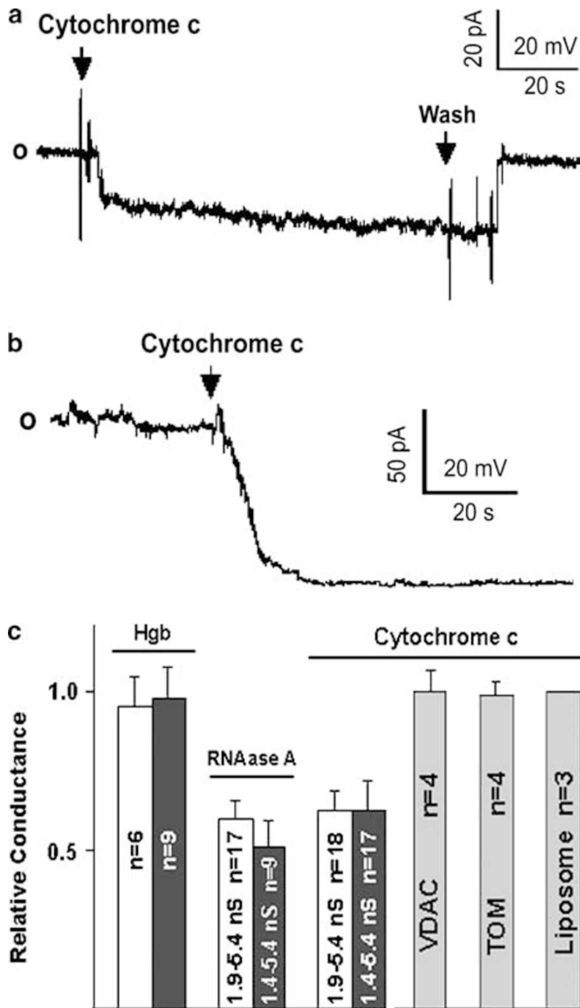

Figure 3 Cytochrome *c* induces Type 1 and Type 2 effects on BaxΔC20 channels. (**a**) A current trace of a BaxΔC20 channel before and after addition of 100 μM cytochrome *c* (Cyt *c*) shows reversal of the effects upon washing (perfusion to remove cytochrome *c*), like the Type 1 effect of cytochrome *c* on MAC in Figure 2. (**b**) A current trace of a BaxΔC20 channel shows a Type 2 effect of perfusing 100 μM cytochrome *c* into the bath. (**c**) Histograms show the conductance of MAC (open) and BaxΔC20 (filled) channels in the presence relative to the absence of 100 μM cytochrome *c*, 100 μM ribonuclease A, or 200 μM hemoglobin as indicated. The effects of 100 μM cytochrome *c* on VDAC, translocase outer membrane channels (TOM), and liposomes conductances are also shown. Mean $\pm$ S.E. is shown and n is the number of independent determinations. Parts of this figure were reprinted from Guo *et al.*[23] and Dejean *et al.*[24]

yeast strains so that we could determine whether a MAC-like activity was associated with Bax expression in mitochondria. Patch-clamp studies of yeast mitochondria expressing human Bax (hBax) in a VDAC-less strain detected a novel channel activity, which again, was similar to MAC activity found in mitochondria of apoptotic cells (Table 1).[8,20,24] This hBax channel activity displays a large peak conductance (3–4 nS), no voltage dependence, and a slight cation selectivity (Table 1). The peak conductance indicates a pore size greater than 4 nm, which should allow passage of cytochrome *c*. These studies beg the question: is Bax a component of MAC?

Regulation of MAC by the Proapoptotic BCL-2 Family Proteins

There is strong evidence that Bax plays a crucial role in MAC channel formation. First, MAC and recombinant oligomeric Bax channels have indistinguishable channel activities that are cytochrome *c* sensitive (Tables 1 and 2; Figures 2 and 3).[24] MAC activity appears when Bax levels increase in mitochondria of apoptotic FL5.12 cells.[20] 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.[24] Although correlative, this evidence supports a mechanistic link between Bax translocation and oligomerization, MAC formation, and cytochrome *c* release that is directly tested by the molecular and immunological studies described below.

Bax is a component of MAC of staurosporine-treated HeLa cells because MAC activity is depleted after immunoprecipitation of oligomeric Bax. In this system, MAC activity is present in total mitochondrial lysates and fractions containing oligomeric, but not monomeric, Bax (Figure 4).[24] This is expected as Bax oligomers form in the outer membrane following Bax translocation to mitochondria at the time that cytochrome *c* is released.[22,24,56,57] Importantly, MAC activity is depleted from solubilized mitochondrial fractions by Bax antibodies raised against an N-terminal epitope of the protein (Figure 4).[24] This epitope is inaccessible in monomeric Bax but becomes exposed following Bax activation. Thus, these antibodies selectively immunoprecipitate oligomeric Bax.[58,59] The concomitant loss of MAC activity and oligomeric Bax by immunoprecipitation indicates that Bax is a component of MAC.

Previous studies using single and double knockout cell lines for Bax and Bak found these two proteins are functionally redundant with respect to their role in apoptosis (see Danial and Korsmeyer[1], Antonsson[2], Sharpe *et al.*[3], Green and Kroemer[4] for a review). Stan generously provided us with these cell lines in order to determine the relationship between MAC and these two proapoptotic proteins using molecular approaches. Cytochrome *c* release occurs in Bax and Bak single knockout cells but not in the double Bax/Bak knockout cells during staurosporine treatment (Figure 4d).[21,24] Similarly, MAC is detected in single knockout, but not the double knockout cell lines during staurosporine treatment (Figure 4c). Although 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 are functionally redundant with respect to MAC.

During apoptosis, the ‘BH3 domain-only protein’ Bid is cleaved to form a C-terminal truncated form referred to as t-Bid. The fragment t-Bid triggers oligomerization of both Bax and Bak in the mitochondrial outer membrane, which causes cytochrome *c* release.[60,61] Furthermore, t-Bid can trigger oligomerization of recombinant monomeric Bax in artificial membranes.[24,52] The oligomerization results in formation of voltage independent and slightly cationic channels with conductances of 1.5–10 nS, which are detected

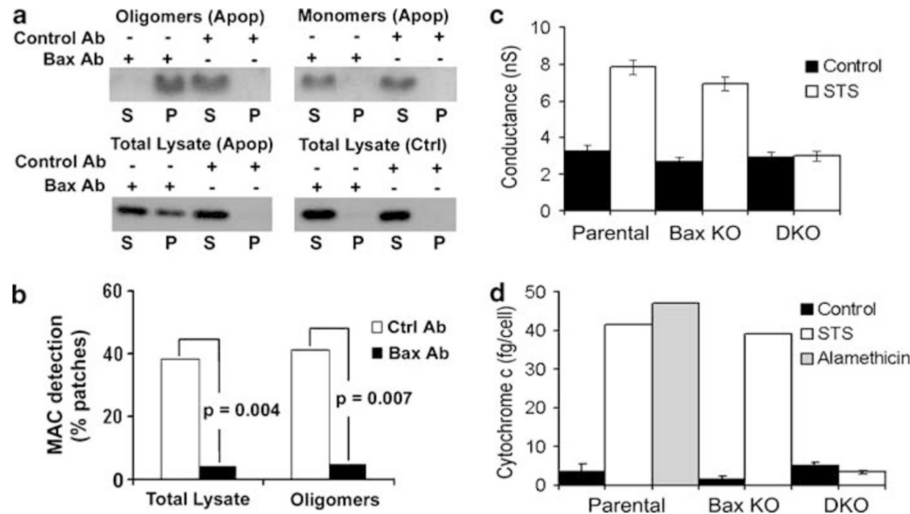

Figure 4 Bax and/or Bak are components of MAC. (**a**) Western blots show the presence of Bax in pellets (P) and supernatants (S) subjected to SDS-PAGE after immunoprecipitation of total mitochondrial lysates and fractions of HeLa cells containing oligomeric and monomeric Bax with anti-Bax antibodies (Bax Ab) or total rabbit IgG (control Ab). (**b**) The supernatants corresponding to immunoprecipitates with anti-Bax antibodies (Bax Ab, closed) or with total rabbit IgG (Control Ab, open) were reconstituted in proteoliposomes and MAC detection frequency was determined by patch clamping. $N = 20–23$ independent patches/condition. *P* values were calculated using the Fisher exact statistical test. (**c**) Mitochondria were isolated from three MEF cell lines, Parental, single Bax KO, and Bax/Bak double KO (DKO) that were (STS) and were not (control) treated with staurosporine. The mean conductance of the outer membrane was measured by patch-clamping isolated mitochondria ($N = 20–23$ patches per condition). (**d**) An ELISA assay was used to assess cytochrome *c* release in the supernatants after permeabilization of the cells with digitonin. Alamethicin (80 μg/ml) was added during digitonin treatment as a positive control for cytochrome *c* release using the method of Polster *et al.*[70] Parts of this figure were modified from Dejean *et al.*[24]

by patch-clamp techniques.[24] Moreover, cytochrome *c* is transported through these t-Bid induced Bax channels, which again makes them very similar to MAC.[24] Future studies will determine whether t-Bid induces MAC activity in isolated mitochondria.

Regulation of MAC by the Antiapoptotic Proteins

Bcl-2 is one of the best studied antiapoptotic proteins in the Bcl-2 family[1–5] and was a central theme of much of the work of the Korsmeyer group. Stan provided us with FL5.12 cell lines that did or did not overexpress antiapoptotic Bcl-2 or mutant Bcl-2 and suggested we determine the relationship between MAC and Bcl-2 family proteins (see below for further details). MAC has never been detected in IL-3-starved FL5.12 cells that overexpress Bcl-2.[20] This result suggests that Bcl-2 can inhibit MAC formation. However, the molecular mechanisms of this inhibition are as yet poorly defined. Recombinant Bcl-2 can form channels in planar bilayers.[45,48] In contrast, no new channel activities are detected when Bcl-2 is overexpressed in FL5.12 or MDA-231 cells, suggesting that this protein does not form channels in native mitochondrial membranes.[20,51] However, channels whose conductance is between 0.75 and 1 nS are detected in isolated mitochondria after addition of caspase-cleaved recombinant Bcl-x_L (ΔN-Bcl-x_L).[62] These channels have conductances and other properties similar to mitochondrial channels detected in squid giant synapses during early stages of hypoxia-mediated apoptosis, when Bcl-x_L is cleaved by caspases.[63] In particular, conductances were detected of up to 3.8 nS in media containing 570 mM KCl in the micropipette.[63] For purposes of comparison, this peak conductance should correspond to about 1 nS in symmetrical 150 mM KCl. Hence, the pore size is expected to be too small to allow for cytochrome *c* transport through the outer membrane and therefore is unlikely to have the same role as MAC during early steps of the intrinsic apoptotic pathway.

Pharmacology of MAC/Bax Channels

MAC is a potential therapeutic target because of its role in the commitment step of apoptosis, that is, cytochrome *c* release. The pharmacological profile of MAC activity is still limited. However, dibucaine, propranolol and trifluoperazine have been identified in patch-clamp experiments as dose-dependent MAC inhibitors. The IC_{50} are 39, 52 and 1 μM for dibucaine, propranolol and trifluoperazine, respectively.[29] In contrast, lidocaine, a structural homolog of dibucaine, has little effect on MAC. In addition, cyclosporine A, a well-known PTP blocker[64–66] has no effect on MAC activity, which reinforces the notion that MAC and the PTP are independent.[29] It has been shown that trifluoperazine and propranolol prevent apoptosis in some cell lines,[67,68] and trifluoperazine and dibucaine also block mitochondrial depolarization induced by glutamate in neurons.[69] Dibucaine, trifluoperazine and propranolol also block cytochrome *c* release from mitochondria induced by recombinant Bax and 'BH3 domain-only' proteins like t-Bid.[70] These studies provide yet another link between Bax and MAC.

Previously, Antonsson and colleagues[71] identified several derivatives of 2-propanol that blocked cytochrome *c* release induced by t-Bid in isolated mitochondria; some have IC_{50} in

the nanomolar range. Recently, two novel agents were found to block the channel activity of recombinant Bax in planar bilayers and inhibit release of cytochrome *c* induced by t-Bid.[72] These drugs may be more specific than dibucaine, propranolol, and trifluoperazine, as they did not block apoptosis induced by staurosporine in cell lines deficient in Bax. Importantly, both agents were effective in blocking apoptosis of neurons in an animal model of global brain ischemia. Inhibition of MAC/Bax channel activity could be an efficient target to prevent apoptosis and therefore reduce tissue damage following ischemic injuries in the brain and presumably other tissues.[72]

Future Perspectives

As shown in the model of Figure 5, MAC is central to the commitment step of apoptosis. The formation of MAC can be triggered by the BH3 domain-only protein t-Bid and this event corresponds to an oligomerization of Bax and/or Bak. As predicted by Stan, MAC formation is prevented by over-expression of antiapoptotic Bcl-2. Once formed, MAC facilitates the release of cytochrome *c*, which initiates activation of executioner caspases and cell death. Hence, MAC is the 'knife' that cuts cytochrome *c* from mitochondria. It has been suggested that the oligomerization of Bax mediated by t-Bid is dependent on one or more mitochondrial proteins.[73] 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. Recent work by Youle, Jensen, Koehler, Scorrano and others has revealed a role for mitochondrial shape in apoptosis.[74–78] In particular, mitochondria typically fragment during apoptosis and inhibition of fission blocks cell death. Are the fission and fusion proteins Drp1, Opa1 or endophilin B candidates for Bax partners? Interestingly, Youle and Karbowski[75] hypothesize that BCL-2 family proteins control apoptosis by regulating the morphogenesis pathways of mitochondria (see also the reviews from RJ Youle and L Scorrano in this issue). The relationship between MAC activity and these pathways may be enlightening with regard to mechanisms of fission and fusion as well as cytochrome *c* release in apoptosis.

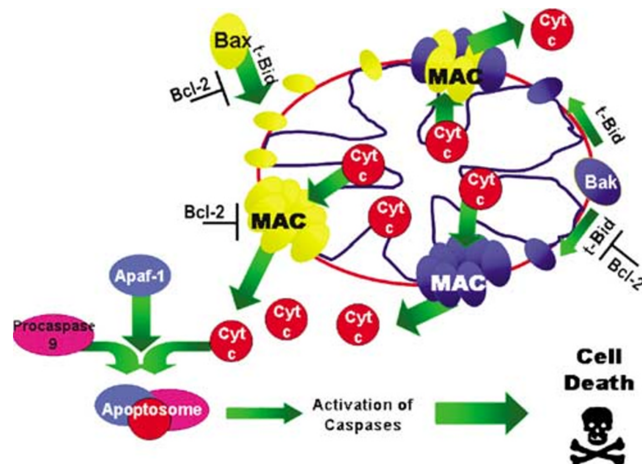

Figure 5 MAC provides a pathway for cytochrome *c* to exit the mitochondria during apoptosis. Model shows that Bcl-2 family members play a critical role regulating MAC formation. Induction of the intrinsic pathway results in Bax translocation to mitochondria and oligomerization. The BH3 domain-only protein t-Bid facilitates activation and oligomerization of Bax and Bak to form MAC. The antiapoptotic protein Bcl-2 suppresses MAC formation. 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. Model was modified from Dejean *et al.*[9]

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