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Minireview

Mitochondria: execution central

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Abstract Mitochondria play an essential function in eukaryotic life and death. They also play a central role in apoptosis regulation, reflected by the convergence of Bcl-2 family members on the mitochondrial outer membrane, and the presence of 'death factors' in the intermembrane space. Mitochondrial structure and function must be taken into consideration when evaluating mechanisms for cytochrome *c* release. The core machinery for caspase activation is conserved from *Caenorhabditis elegans* to man, and we consider parallels in the role of mitochondria in this process.

Key words: Mitochondrion; Apoptosis; Caspase; Cytochrome *c*; Bcl-2 homolog

1. Introduction

Mitochondria play a critical role in the control of apoptosis. In response to specific stimuli they release cytochrome *c*, which is a necessary cofactor for caspase activation by Apaf-1. Mitochondria also sequester other pro-apoptotic factors. The unique structure of this organelle is well understood in the context of its function in electron transport and ATP synthesis. However, the contribution of mitochondrial architecture to cytochrome *c* release is less clear. Here, we consider the role of mitochondria in regulation of apoptosis, and discuss the currently proposed models for cytochrome *c* release.

2. Mitochondrial architecture

Mitochondria are organized into several distinct spaces, based on reconstructions of mitochondria imaged by electron tomography [1,2]. The matrix is filled with the enzymes of the tricarboxylic acid cycle. Projecting through the matrix space are hollow flattened or tubular structures, the cristae. The crista membranes are continuous with the rest of the inner mitochondrial membrane, and contain the electron transfer complexes. The intermembrane space can be functionally separated into two compartments, the cristal lumen and the intermembrane space that lies between the inner membrane and the outer membrane. It is estimated that 85–97% of the cytochrome *c* resides in the cristal lumen, with the remainder in the intermembrane space [3,4]. Cytochrome *c* is electrostatically associated with cytochrome *c* reductase and cytochrome oxidase, and is also associated with cardiolipin, which is asymmetrically distributed across the lipid bilayer of the inner membrane. Also present in the intermembrane space/cristal lumen are procaspases 2, 3, and 9, and apoptosis initiating factor (AIF) [5,6]. The points where cristae join the rest of the inner membrane are termed crista junctions [2]. Crista junctions are probably highly dynamic and should not be thought of as rigid structures. Mannella et al. have observed that under stress, the cristae can pinch off, thereby preventing communication with the rest of the intermembrane space [7]. The F_0F_1 ATPase decorates the matrix side of the crista membranes. The inner membrane/crista membrane is highly resistant to proton leakage, allowing the buildup of a significant membrane potential (about −220 mV). The accumulated protons on the outer face of the inner membrane (in the lumen of the cristae) leak back down their electrochemical gradient through the F_0F_1 ATPase to drive ATP synthesis. Cardiolipin is an anionic phospholipid that is only found in the mitochondrial inner membrane and is believed to contribute to the proton-impermeable nature of the inner membrane.

The outer mitochondrial membrane is generally considered to be permeable to small molecules (<1000 Da) because of the presence of a very abundant protein, the voltage-dependent anion channel (VDAC). VDAC comprises as much as 20% of the protein of the outer membrane and is ordinarily open. Bcl-2 is anchored in the outer mitochondrial membrane and projects into the cytosol. Recent data point to an interaction between VDAC and Bcl-2 family members [8,9], although this is controversial [10].

The outer membrane is dimpled with contact points where the outer and inner membranes are closely juxtaposed. Contact points may represent two different biochemical entities: the protein translocation machinery made up of the translocase outer and inner membrane complexes (reviewed in [11]), and the components of the nucleotide transport system, including VDAC, the adenine nucleotide translocase (ANT), and creatine kinase. These latter components are also constituents of the permeability transition pore (PTP), whose opening is often believed to herald mitochondrial destruction and certain cell death. The PTP can be reconstituted with VDAC, ANT, and cyclophilin D (reviewed in [12]). The association of cyclophilin D with the PTP is important, as it underlies the ability of cyclosporin A to prevent pore opening and mitochondrial dysfunction with cyclosporin A [13]. Additional proteins that may co-purify with the PTP include hexokinase, the peripheral benzodiazepine receptor, creatine kinase, and Bax [14].

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Abbreviations: AIF, apoptosis initiating factor; ANT, adenine nucleotide translocator; CPT I, carnitine palmitoyl transferase I; IAP, inhibitor of apoptosis protein; PTP, permeability transition pore; VDAC, voltage-dependent anion channel

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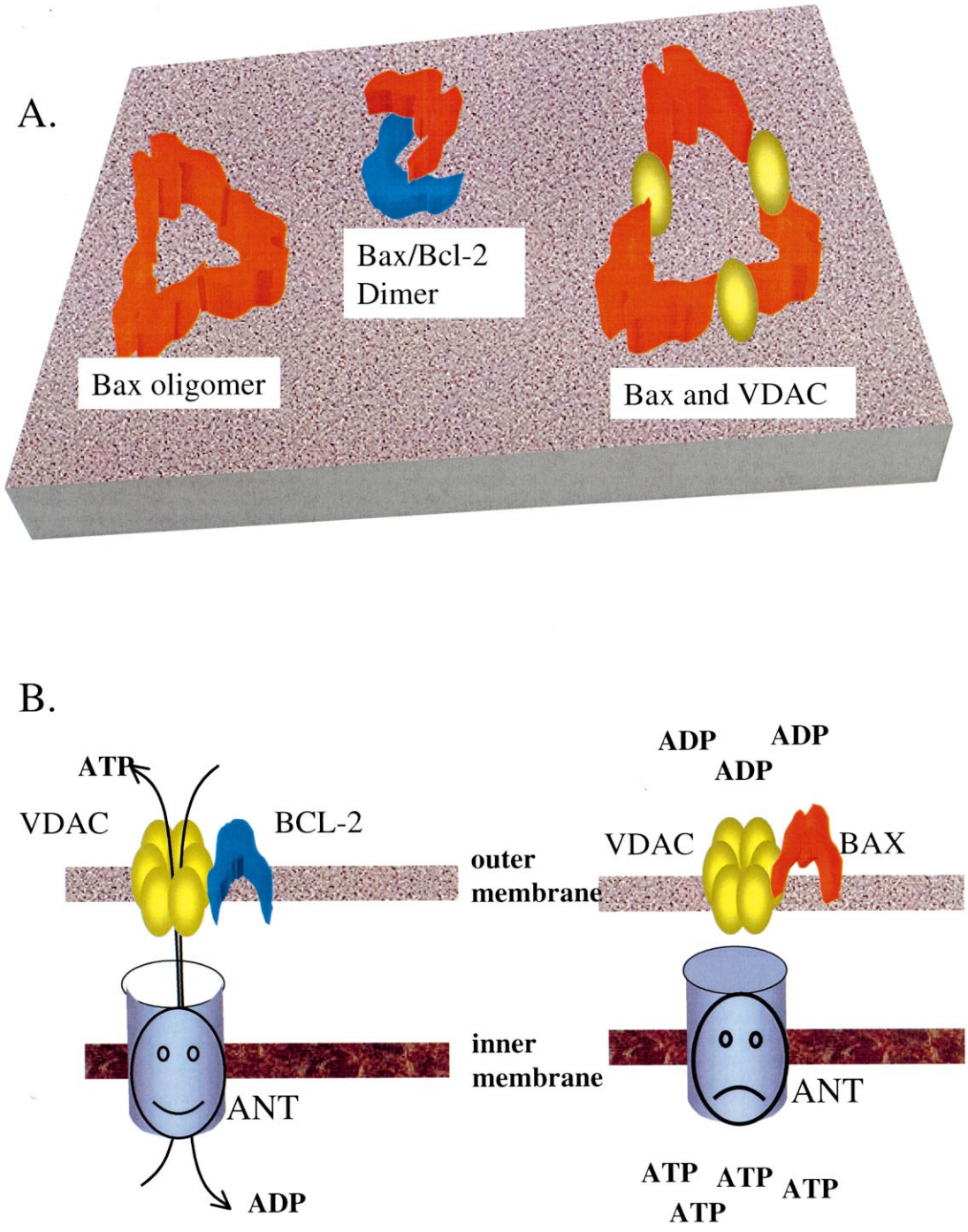

Fig. 1. A: Bax, the pore former. Bax forms oligomers which insert into the mitochondrial outer membrane to generate ion-conducting channels. Dimerization with Bcl-2 or Bcl-x_L prevents channel formation. In Tsujimoto's model, shown on the right, Bax joins with VDAC to form large channels. Bax inserts into the outer membrane and interacts with VDAC to form channels that are large enough to allow the passage of cytochrome *c*. B: Bcl-2, friend of VDAC. Bcl-2 and Bax affect VDAC to regulate ion conductance, including that of adenine nucleotides. Bcl-x_L or Bcl-2 could simply act as an ion channel to dissipate a potential across the outer membrane, permitting VDAC to remain open. Closure of VDAC leads to accumulation of ATP in the intermembrane space, ANT distress, and matrix swelling, followed by rupture of the outer membrane and cytochrome *c* release.

3. Mechanisms of cytochrome *c* release

Because of the pivotal role of cytochrome *c* in caspase activation, much effort has been directed at delineating the mechanism of its release from mitochondria during apoptosis. Bcl-2 and related anti-apoptotic family members prevent its release, while Bax and the BH3-only pro-apoptotic homologs promote its release, perhaps by different mechanisms (recently reviewed [15]). For the most part, pro-apoptotic homologs reside in the cytosol, and in response to a stimulus, translocate to the mitochondrial membrane, where they interact with other proteins to achieve cytochrome *c* release. For instance, Bad is bound to protein 14-3-3 after phosphorylation. Dephosphorylation by protein phosphatase 1A or calcineurin permits

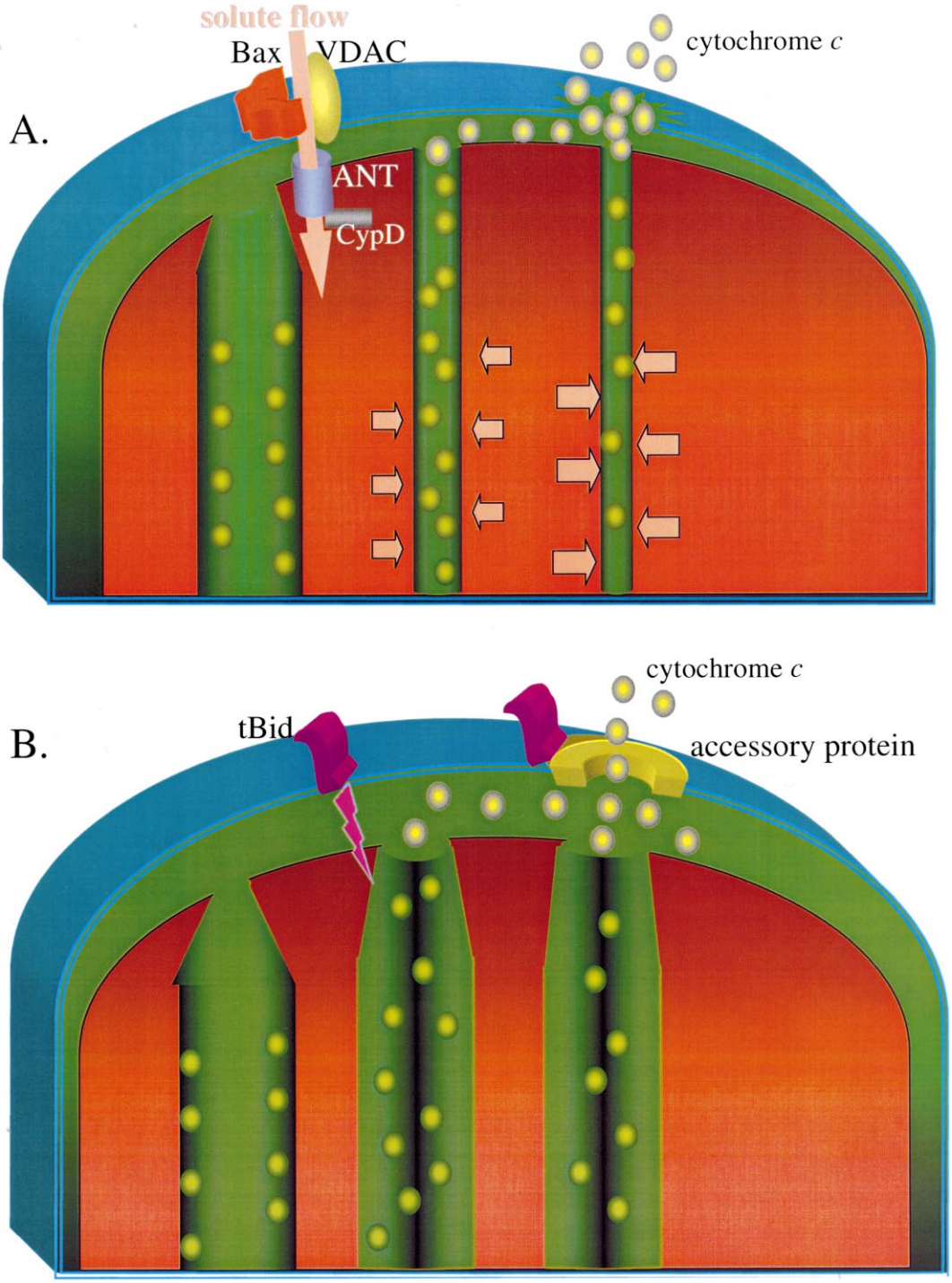

Fig. 2. A: Bax, a component of the PTP. Bax joins forces with other constituents of the PTP (ANT and cyclophilin D (CypD)) to direct its opening, leading to matrix swelling and compression of cristae (shown by arrows), and eventual rupture of the outer membrane and release of cytochrome *c* (shown towards the right side of the diagram). B: Cytochrome *c* release is a two-step process. Truncated Bid (tBid) interacts with the mitochondria to disturb (lightning bolt) the association of cytochrome *c* with the electron transfer complexes of the crista membranes. Once dissociated (shown in middle crista), the cytochrome may interact with other death molecules, and will be free to diffuse into the cytosol once the outer membrane is breached, perhaps by a permeability-enhancing factor or accessory protein (shown towards the right side of the diagram).

its translocation to mitochondria [16,17]. Some studies suggest that Bax translocates from cytosol to mitochondria while others suggest that mitochondrial Bax undergoes a conformational change, which is enhanced by interaction with Bid [18–20]. Bid is proteolysed by caspase 8 and the C-terminal portion of the molecule interacts with the mitochondria, possibly dependent on the presence of Bax [18,21,22]. Several models for the mechanism of cytochrome *c* release have been proposed, based on experimental evidence. They will be discussed below.

3.1. Bax, the pore former

Because of the structural similarity of Bcl-x_L to the pore-forming bacterial diphtheria toxin, it was thought that Bcl-2 family members might function by acting as pores in the mitochondrial outer membrane ([23] and reviewed in [24]). Indeed, when inserted into artificial lipid bilayers (after treatment at pH 4), they do show ion conductance [25]. After regulated cytochrome *c* release was described in 1996 [26], it was hoped that the channels formed by Bax and friends might be large enough to allow passage of cytochrome *c* (Fig. 1). However, evidence in support of the notion that Bax might form large multimers capable of passing a molecule of 12 000 Da has not been forthcoming.

3.2. Bax opens VDAC

Tsujimoto's group provided evidence that Bax and VDAC, when inserted into artificial lipid bilayers, form high-conductance channels whose conductance is blocked by Bcl-x_L [27]. More recent data have allowed them to refine the model to suggest that Bax interacts with VDAC to form a much larger channel capable of accommodating cytochrome *c* (Fig. 1A) [28].

3.3. Bcl-2, friend of VDAC

The ion conductance capacity of Bcl-2 has led Thompson and coworkers [29] to propose that it may form an alternative channel in the mitochondrial outer membrane that will permit the transit of adenine nucleotides. They provide evidence to show that apoptosis is associated with impaired mitochondrial adenine nucleotide exchange, resulting in increased matrix ATP and elevated levels of cytosolic ADP, which is prevented by the expression of Bcl-2 or Bcl-x_L [9,30]. Since nucleotides ordinarily pass unimpeded through VDAC in the outer membrane, they suspect that Bax may cause VDAC to close (Fig. 1B). The perturbation to nucleotide exchange is postulated to lead to permeability transition and cytochrome *c* release.

3.4. Bax, a component of the PTP

Opening of the PTP has been proposed to lead to cytochrome *c* release. The PTP is depicted in Fig. 2A. Opening of the PTP permits influx of solutes <1500 Da, resulting in depolarization of the inner membrane. Swelling of the matrix and redistribution of the redundant cristal membranes may lead to rupture of the outer membrane. Pore opening is regulated by voltage, matrix pH, oxidative stress, electron flow, and by two of the pore constituents, cyclophilin D and ANT ([31,32], reviewed in [12]). Bax is thought to regulate the channel activity of the ANT [14,33]. A number of studies have detected cytochrome *c* release in the absence of mitochondrial depolarization or evidence of swelling [34–36]. PTP opening and swelling does not represent a universal mechanism for cytochrome *c* release but may play a role in some settings.

3.5. Cytochrome c release is preceded by dissociation

We found that cytochrome *c* ceased to participate in electron transfer early in apoptosis, before it could be detected in the cytosol of cells disrupted by the gentle method of nitrogen cavitation [37]. However, disruption of the outer mitochondrial membrane by homogenization could release the cytochrome from mitochondria of apoptotic but not normal cells [38]. Later in the course of apoptosis, the outer membrane was disrupted and cytochrome *c* could be detected in cytosol. These results indicate that cytochrome *c* dissociates from the electron transfer chain first, and in a second step, passes through the outer mitochondrial membrane (Fig. 2B). Truncated Bid was shown to mediate the dissociation of cytochrome *c* [22,39,40]. Recent evidence suggests that an accessory factor may mediate permeability of the outer membrane [41].

4. Mitochondria and caspases

Caspase activation is considered to be the *sine qua non* of apoptosis. Both a receptor-mediated and a mitochondrial pathway to caspase activation have been described and are shown in Fig. 3. In the receptor-mediated pathway, Fas ligand or tumor necrosis factor α binds to its receptor, causing receptor aggregation and recruitment of death adapter molecules on the cytoplasmic side of the membrane. Procaspase 8 and/or 10 is recruited to the complex, where it can undergo proximity-induced processing to the active form of a p20/p10 heterotetramer [42]. The activated caspase 8 (or 10) can then cleave procaspase 3, which serves as an efficient executioner to cleave multiple targets within the cell, including caspase-activated DNase [43]. Caspase 8 also targets the mitochondria through the proteolytic activation of Bid. The C-terminal fragment translocates to the mitochondria, where it mediates cytochrome *c* release. Although anti-apoptotic Bcl-2 family members may prevent the amplification mediated by cytochrome *c* release, they are generally ineffective at preventing the direct activation of caspase 3 by caspase 8 after Fas ligation [44]. This has led to a classification of cell lines as type I or type II based on whether they can generate enough caspase 3 directly or whether they depend upon the Bcl-2-inhibitable step of mitochondrial amplification [45].

A second pathway of caspase activation exists and is activated in response to a variety of cellular stresses, including DNA damage, protein kinase inhibition, and loss of survival signaling. In this pathway, termed the mitochondrial pathway, a pro-apoptotic member of the Bcl-2 family, such as Bax or Bid, associates with the mitochondria and directs the dissociation and eventual release of cytochrome *c* to the cytosol, where it can associate with another key factor, Apaf-1 [46]. Apaf-1 binds cytochrome *c*, dATP or ATP, and forms a large ($\sim$700 kDa) multimeric complex, termed the apoptosome, which also includes molecules of caspase 9 and 3 [47]. Caspase 9 is activated when bound to Apaf-1, where it processes caspase 3 to its active form [48].

While caspases are abundant in the cytoplasm of transformed cell lines, it appears that caspase 9 may be restricted to the mitochondria in neurons and cardiomyocytes [49]. Caspases and another pro-apoptotic molecule, AIF, are released from mitochondria along with cytochrome *c* in response to stimuli that induce the permeability transition [50]. Recently, another mitochondrial factor was identified, named Smac, for second mitochondrial activator of caspases [51] or Diablo (direct IAP binding protein with low p*I*) [52]. This protein, which is released from mitochondria along with cytochrome *c*, binds to inhibitor of apoptosis proteins (IAPs), thereby lowering the threshold for caspase activation. The functional significance of mitochondrial caspases remains unclear, other than to be released from mitochondria during apoptosis. Internal mitochondrial targets of caspases have not been identified. Although the opening of the PTP has been noted to be

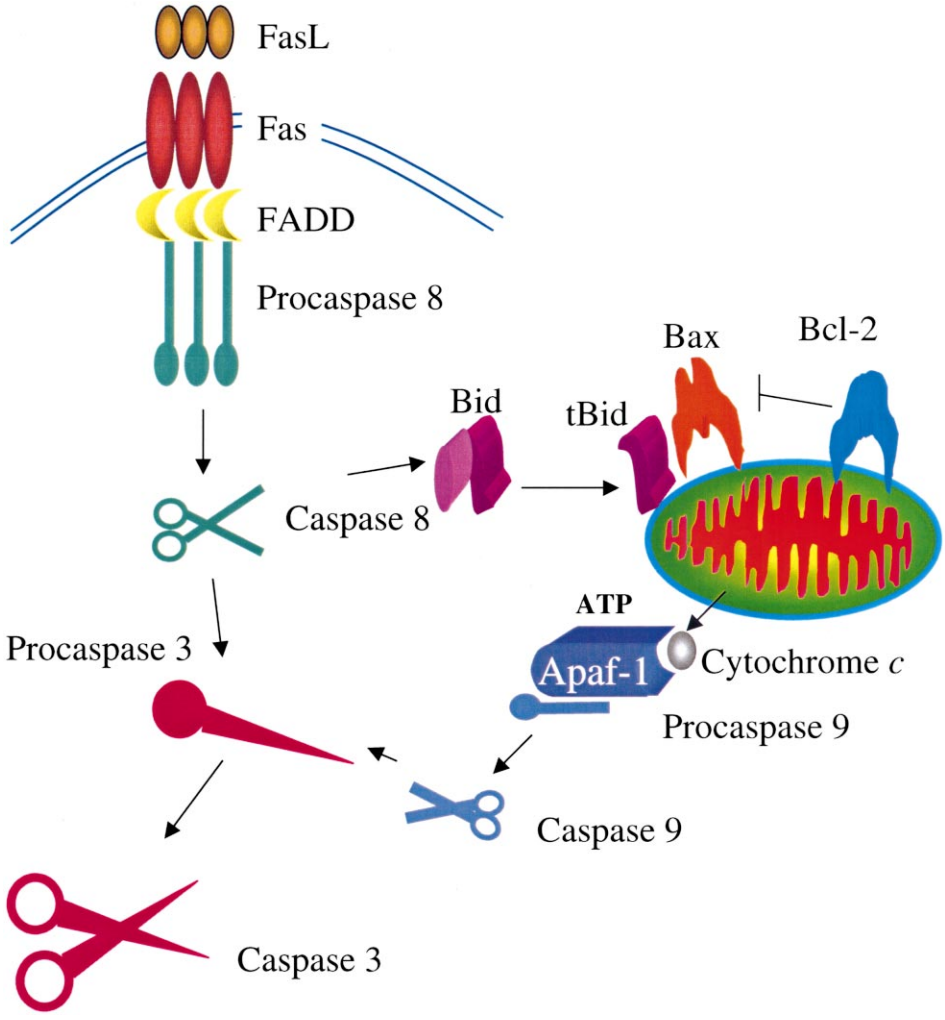

Fig. 3. Two pathways to caspase activation. In the receptor pathway, caspase 8 is recruited to a death-inducing signal complex (DISC) at the plasma membrane, after Fas ligand (FasL) binds and aggregates Fas. FADD, a death adapter molecule, recruits procaspase 8, which undergoes proximity-induced processing. The activated caspase 8 can then proteolytically activate procaspase 3. Various cellular stresses cause a pro-apoptotic Bcl-2 family member (e.g. Bax) to bind to the mitochondrial outer membrane, leading to cytochrome *c* release. Bcl-2 opposes the effect of Bax. Apaf-1 binds cytochrome *c* and dATP or ATP, and recruits caspases to form a large complex (the apoptosome). There is cross-talk between the pathways, as caspase 8 can cleave Bid to generate the active truncated Bid (tBid), which can bind to mitochondria and direct the release of cytochrome *c*.

downstream of caspase activation, proteolysis of pore constituents has not been reported [53].

5. Insights from parallel systems

Substantial parallels exist between the prototypic system of *Caenorhabditis elegans* and more complex mammalian systems. Developmentally programmed cell death in the nematode has been shown to be controlled by a small number of genes, all of which have counterparts that are conserved across evolution [54]. The *ced3* gene encodes a protein that corresponds to caspase 3 [55]. It is activated by association with the product of *ced4*, which shares homology with Apaf-1 [56]. Cell death is prevented by the expression of *ced9*, which is homologous to Bcl-2 [57]. Based on overexpression studies in heterologous systems, it has been concluded that CED-9, which is localized to the mitochondrial membrane, binds CED-4 and prevents its interaction with CED-3 [58]. Expression of EGL-1 (mammalian homolog: Bax) displaces CED-4, permitting apoptosis to proceed [59].

Studies of mammalian homologs have been controversial, but for the most part have failed to demonstrate an interaction between Bcl-2 family members and Apaf-1 [60,61]. Most investigators have concluded that the 700 kDa caspase activation complex, termed the 'apoptosome', forms in the cytosol [47]. This has been based on immunofluorescence and cell fractionation studies that detect most Apaf-1 in the cytosol. While cytochrome *c* is required for mammalian caspase activation, there is no evidence that it plays a role in *C. elegans*. Yet both Bcl-2 and CED-9 are associated with the mitochondria. Do they simply function to sequester pro-apoptotic molecules, or is their role in the cell more complex? One hint for an additional role for Bcl-2 has been provided by the observation that Bcl-2 interacts with carnitine palmitoyl transferase I (CPT I) in a yeast two-hybrid and in co-precipitation experiments [62]. Given that carnitine inhibits caspases [63] and sphingomyelinase [64,65], regulation of the levels of carnitine and palmitoylcarnitine by CPT I and indirectly by Bcl-2 could have a significant influence on the ability of the cell to undergo apoptosis. However, evidence

for regulation of CPT I by Bcl-2 has not been demonstrated.

6. Summary

Several mechanisms for cytochrome *c* release have been proposed. However, even before cytochrome *c* has been released, it has ceased to function in electron transport, leading to a switch to glycolysis. The cytochrome *c* that has dissociated from the electron transport complexes may be available to interact with other pro-apoptotic molecules sequestered in the mitochondria. Conflicting evidence concerning interactions between Bcl-2 and Apaf-1, as well as the mitochondrial localization of caspases, AIF, and perhaps other molecules, suggests the possibility that the apoptosome may nucleate at the mitochondrial outer membrane before diffusing into the cytosol. Mitochondria play a central role in the maintenance of cell survival through energy production and in the initiation of cell death through the release of multiple death molecules.

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