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Review

Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death

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Abstract

Programmed cell death (PCD) is essential for normal development and maintenance of tissue homeostasis in multicellular organisms. While it is now evident that PCD can take many different forms, apoptosis is probably the most well-defined cell death programme. The characteristic morphological and biochemical features associated with this highly regulated form of cell death have until recently been exclusively attributed to the caspase family of cysteine proteases. As a result, many investigators affiliate apoptosis with its pivotal execution system, i.e. caspase activation. However, it is becoming increasingly clear that PCD or apoptosis can also proceed in a caspase-independent manner and maintain key characteristics of apoptosis. Mitochondrial integrity is central to both caspase-dependent and-independent cell death. The release of pro-apoptotic factors from the mitochondrial intermembrane space is a key event in a cell's commitment to die and is under the tight regulation of the Bcl-2 family. However, the underlying mechanisms governing the efflux of these pro-death molecules are largely unknown. This review will focus on the regulation of mitochondrial integrity by Bcl-2 family members with particular attention to the controlled release of factors involved in caspase-independent cell death.
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1. Caspase dependence of apoptosis

This family of intracellular proteases was first implicated in apoptosis by genetic analysis in the nematode, *Caenorhabditis elegans*, with the identification of *ced3* and *ced4*, cell death genes essential for developmental apoptosis [1]. *Ced3* encodes a caspase that is activated by the upstream 'trigger'*ced4* resulting in the death of 131 cells of the 1090 generated during normal development [2,3]. The critical role of both *ced3* and *ced4* in apoptosis is well established. *C. elegans* mutants lacking either *ced3* or *ced4* or carrying loss of function mutations have a complete absence of developmental cell death [2]. To date, seven caspases have been identified in the fruit fly, *Drosophila melanogaster*, drICE, DCP-1, DCP-2/DREDD, Dronc, Decay, Strica/Dream and Damm/Daydream [4]. At least DCP-1 is essential for early *Drosophila* development as deletion of DCP-1 causes larval lethality and promotes melanotic tumour development [5]. Caspases therefore appear to play a crucial role in developmental apoptosis in both *Drosophila* and *C. elegans*.

A more complex network of apoptotic machinery has evolved in higher organisms. Since the recognition that *ced3* has sequence identity with the mammalian cysteine protease interleukin-1-converting enzyme (ICE), a family of at least 13 related cysteine proteases has been identified in mammals, existing in the cell as inactive precursors that undergo proteolytic processing and activation. Once activated, initiator caspases can proteolyse additional effector caspases, generating a proteolytic cascade that cleaves key structural components as well as proteins critical for cell survival in a highly sequence-specific fashion, ultimately resulting in the systemic and controlled destruction of the cell. At least two major pathways of caspase activation have been characterised. The extrinsic, receptor-mediated pathway entails ligand binding to cell surface receptors, receptor oligomerisation and recruitment and activation of initiator caspases such as caspase-8, and the mitochondrial pathway involving caspase activation from within the cell.

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This intrinsic pathway is under the control of the Bcl-2 family and initiated by release of cytochrome *c* and Smac/Diablo from the mitochondrial intermembrane space. Cytochrome *c* associates with Apaf-1 to promote the activation of caspase-9 which can then activate effector caspases, while Smac/Diablo functions by binding to the inhibitors of apoptosis proteins (IAPs) thereby disrupting their ability to inhibit caspases 3, 7, and 9 [6,7]. The caspase cascade may also be initiated at the endoplasmic reticulum (ER) through activation of caspase-12 following ER stress. Very little is currently known regarding the consequences of caspase-12 activation, although it has recently been reported that at least in vitro recombinant caspase-12 cleaves and activates procaspase-9. ER stress therefore may induce a caspase cascade involving activation of caspase-9 independently of cytochrome *c* release [8]. Interestingly, this pathway is also regulated by Bcl-2 proteins [9].

Table 1
Examples of caspase-independent cell death

Cell death stimulus	Cell type	Reference
Tumour necrosis factor alpha (TNF-alpha)	Human neutrophils	[133]
Vitamin D compounds	Breast cancer cells (MCF′ 7’s)	[134]
Depletion of heat shock protein 70	Breast cancer cells	[135]
UVB	Jurkat T cells	[99]
HIV-1 infection	Primary CD4+ T cells	[136]
Tumour suppressor gene ARHI	Ovarian and Breast cancer cells	[137]
Bax	PC12 cells (neuronal cell line)	[138]
Ras	Human glioma and gastric cancer cells	[139]
Staurosporine	L1210/0 cells	[140]
Dexamethasone	Dendritic cells	[141]
Differentiation-inducing factor, (DIF)	Insulin-secreting INS-1 cells	[142]
Inhibition of nuclear factor kappaB (NFkappaB)	Human T lymphocytes	[143]
AIF	Apaf-1$^{-/-}$ or caspase-3$^{-/-}$ cells	[81,92]
Cathepsin inhibition	Human leukaemia and lymphoma cells	[144]
Cross-linking of several surface receptors including CD2, CD4, CD45, CXCR4 and CD99	CD4+ T cells, Human peripheral T lymphocytes, CD4 + CD8+ thymocytes and Ewing’s sarcoma cells	[145–148]
Oxidative stress	Retinal cells	[149]
Hydroxyl radical	Human tumour cells	[150]
Nitric oxide	PC12 and HeLa cells	[151]
Thymocyte development	Thymocytes	[152]
Light	Mouse photoreceptor cells in vivo	[11]
Mutation in Rod cGMP phosphodiesterase	Mouse photoreceptor cells in vivo	[10]

There is no doubt that caspases play a central role in many apoptotic systems and that many of the typical morphological features associated with apoptosis such as internucleosomal DNA fragmentation, as well as membrane blebbing and apoptotic body formation can be orchestrated by caspase activation. It would be somewhat naive, however, to believe that caspases are indispensable for programmed cell death (PCD). Emerging evidence from an increasing number of experimental systems supports the belief that apoptosis can occur independently of caspase activation (Table 1). Cell death in these systems often occurs with only partial condensation of chromatin and without internucleosomal DNA fragmentation, but maintains many other features of apoptosis, such as cytoplasmic and nuclear shrinkage, mitochondrial depolarisation, membrane blebbing, and externalisation of phosphatidylserine. Furthermore, this laboratory has recently described caspase-independent apoptosis in three different in vivo models of retinal degeneration, all of which display internucleosomal DNA fragmentation [10,11]. Evidence from the above studies supports the concept that cell death can proceed in a caspase-independent manner while maintaining key characteristics of apoptosis.

Further support for the existence of caspase-independent apoptosis comes from studies on yeast. Yeasts lack homologues of *ced3*, *ced4*, *ced9* and caspase coding sequences in their genome, yet still undergo PCD under conditions of stress [12]. Moreover, a *Saccharomyces cerevisiae* cdc 48 cell cycle mutant has been described that results in cell death featuring membrane blebbing, chromatin condensation and fragmentation, and phosphatidylserine exposure [13]. This yeast mutant provides strong evidence that some cytoplasmic and nuclear hallmarks of apoptosis can occur independently of caspases.

2. Bcl-2 proteins and caspase-independent apoptosis

So what is responsible for apoptotic-like features in cells where caspase activation is not detected or where caspase inhibitors have been employed? Several candidates, which are capable of exerting at least some of these features, reside within the mitochondrion. These include apoptosis inducing factor (AIF), endonuclease G (endoG) and Omi/HtrA2 (discussed in detail later). While it remains elusive as to how exactly these proteins are released from mitochondria, their discharge requires permeabilisation of the OMM, an event that is under the tight control of the Bcl-2 family. This family consists of both pro- and anti-apoptotic members that elicit opposing effects on mitochondria. Pro-apoptotic members can induce mitochondrial membrane permeability, which results in the release of these factors, while anti-apoptotic members such as Bcl-2 preserve mitochondrial integrity and therefore potentially block the release of these soluble intermembrane proteins.

The family possess up to four conserved Bcl-2 homology domains (BH) corresponding to α helical segments and can be divided into three categories:

(1) The multi-domain pro-apoptotic members such as Bax, Bok and Bak comprised of BH1, BH2 and BH3 domains.
(2) The 'BH3-only' pro-apoptotic members such as Bid, Bik, Bad, Bim, Noxa, PUMA and the recently identified Spike. These share sequence homology only in the BH3 domain.
(3) Anti-apoptotic members such as Bcl-2, Bcl-x_L and Bcl-w, all of which exert anti-cell death activity and share sequence conservation within all four BH domains. Many of these members contain a hydrophobic carboxy terminal domain, which targets them to intracellular membranes such as the OMM, ER and nuclear envelope [14]. Further recent evidence suggests that FKBP38, a member of the immunophillin family, may be crucial in targeting Bcl-2 and Bcl-x_L to mitochondria [15].

The exact mechanism(s) by which Bcl-2 proteins protect against or induce permeabilisation of the OMM is controversial and still under intense investigation. At least two models have been proposed, both of which involve members of the Bcl-2 family (Fig. 1). Bcl-2 proteins themselves may form channels in the OMM or they could interact with and regulate pre-existing channels such as the Permeability Transition Pore (PTP). These two mechanisms are not necessarily mutually exclusive and may occur in a sequential fashion depending on cell type, stimulus and level of stress. There is substantial evidence for and against both models and this is discussed in several excellent reviews

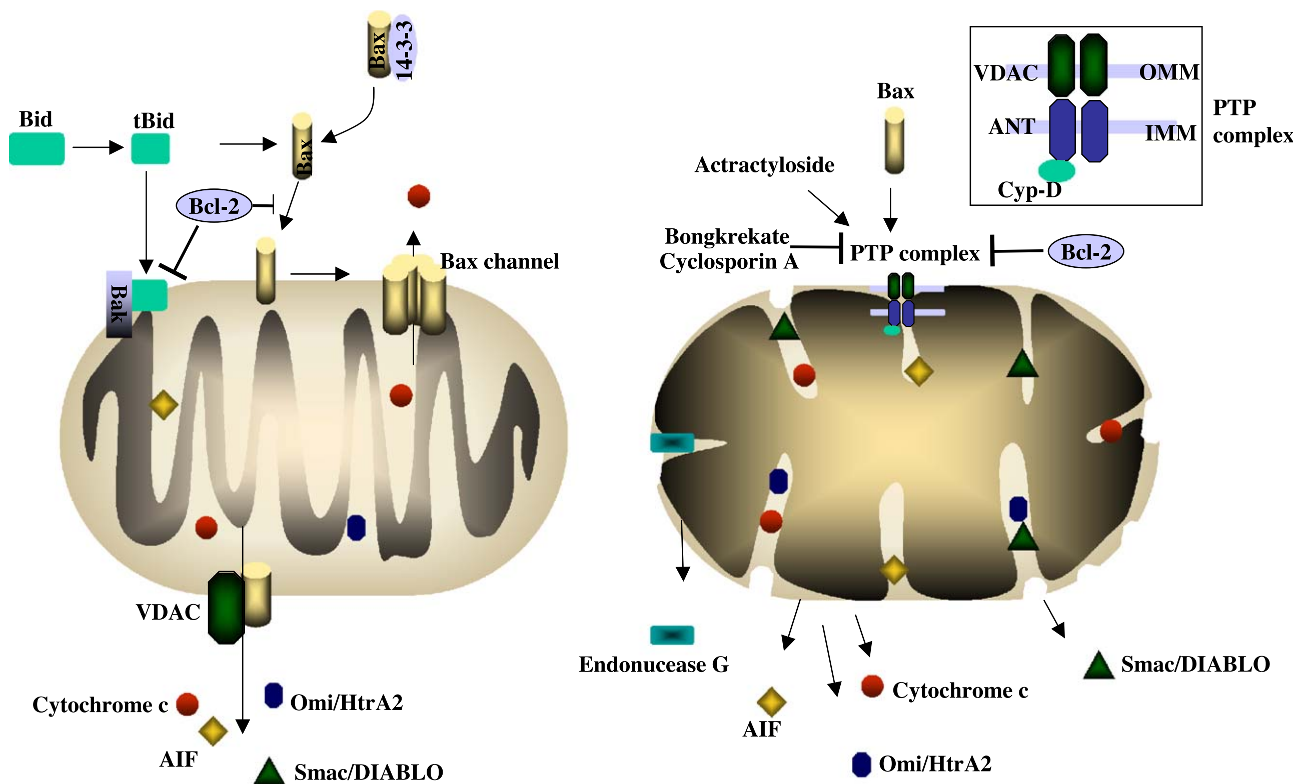

Fig. 1. Two models proposed for mitochondrial membrane permeabilisation. (A) Channel formation by Bcl-2 family members. Bax is normally found in the cytosol of healthy cells. The release of Bax from its negative regulator 14-3-3ε can occur by both caspase-dependent and caspase-independent mechanisms allowing Bax translocation from cytosol to mitochondria. Interaction of Bax with tBid stimulates a conformational change in Bax, insertion to the OMM and Bax oligomerisation. The resultant pore formed requires four Bax molecules and is capable of transporting cytochrome *c*. Whether or not larger proteins can pass through this pore or if Bax can undergo further oligomerisation to form larger channels remains to be elucidated. Following processing, tBid can also translocate to mitochondria where it inserts into the OMM and induces a conformational change in Bak and Bak homo-oligomerisation. The anti-apoptotic protein Bcl-2 prevents tBid insertion, Bax translocation and Bax/Bak oligomerisation in mitochondrial membranes. Alternatively, Bax could interact with other proteins in mitochondrial locations such as the VDAC to form larger pores through which apoptogenic proteins involved in caspase-independent cell death such as AIF (57 kDa), endoG (30 kDa) and Omi/HtrA2 (50 kDa) are released. (B) Opening of the PTP. The PTP is a complex primarily made up of the VDAC in the outer membrane, ANT in the inner membrane and cyclophilin D in the matrix. It is postulated that opening of the PTP results in loss of mitochondrial membrane potential, an influx of fluid into the matrix which results in swelling and rupturing of the OMM and the release of pro-apoptotic proteins from the intermembrane space. In this way, all soluble intermembrane proteins including caspase-independent factors such as Omi, endoG and AIF are released following disruption of mitochondrial integrity. Various inhibitors of the PTP that favour the closure of this channel including cyclosporin A (acts on cyclophilin D) and Bongkrekic acid inhibit mitochondrial membrane permeabilisation and in many instances cell death. Actractyloside, which is a ligand of the ANT, induces PTP opening and the release of pro-death factors. It is proposed that pro-apoptotic Bcl-2 family members like Bax and Bak cause opening of the PTP while anti-apoptotic members such as Bcl-2 and Bcl-x_L favour closure of this channel.

[14,16–19]. Both proposed mechanisms are to some extent compatible with the release of soluble intermembrane proteins that play a role in caspase-independent cell death and are therefore discussed below.

3. Channel formation by Bcl-2 family members

The idea that Bcl-2 family members themselves form channels in the OMM was originally based on the structural similarity between Bcl-x_L and the pore-forming domains of several bacterial toxins, a key feature of which is the ability to form ion channels in biological membranes [20]. Several members of the Bcl-2 family such as Bax [21,22], Bcl-2 [23], Bcl-x_L [24], and cleaved Bid [25] have since been reported to form channels in artificial membranes.

Bax is probably the best studied and characterised pro-apoptotic Bcl-2 protein that is capable of forming channels under physiological conditions. Importantly, Bax is activated in several instances of caspase-independent cell death, supporting a role for Bax channels in the release of caspase-independent death factors. Bax is normally found in the cytosol of healthy cells and is reported to translocate to the OMM in response to apoptotic stimuli and release intermembrane space proteins [26,27]. Bax translocation involves a conformational change that exposes the amino and carboxyl termini leading to mitochondrial translocation [28,29]. Cleavage of Bax is proposed to enhance its cell death function [30]. In this way, cleavage of Bax at its N-terminus by the calcium activated protease calpain is reported to generate a potent pro-apoptotic 18-kDa fragment capable of mediating cytochrome *c* release and apoptosis induced by etoposide and staurosporine [31]. Oligomerisation of Bax also appears to be required for channel formation [32]. This is supported by several studies demonstrating that Bax forms homo-oligomeric complexes in mitochondrial membranes [33,34]. A recent study has also demonstrated that Bax oligomerisation following mitochondrial insertion may trigger a conformational change in resident Bak resulting in Bak homo-oligomerisation, an event previously reported to be mediated by Bid [35]. Overexpression of the anti-apoptotic protein Bcl-2 not only prevents the oligomerisation of Bax but also has been reported to inhibit both the Bax conformational change and mitochondrial translocation [28,33].

While Bax translocation and integration into the mitochondrial membrane in several apoptotic systems is well established, the regulation of this translocation is poorly understood and exactly how Bax remains inactive in healthy cells is still ambiguous. Tsujimoto's group [36] have recently improved our understanding of this mechanism in their study describing the negative regulation of Bax by a member of the 14-3-3 family, a group of proteins which are implicated in apoptosis through their interaction with pro-apoptotic molecules such as Bad. In the study carried out by Tsujimoto's group, 14-3-3ε was found to interact with Bax in the cytoplasm of living cells and Bax underwent dissociation from this protein by caspase-dependent and independent mechanisms. These results suggest that 14-3-3ε may play a crucial role in the negative regulation of Bax activity in living cells and more significantly demonstrate that Bax translocation to mitochondria can occur independently of caspase activation.

Oligomerisation and/or activation of Bak and Bax can also be induced by Bid, a pro-apoptotic 'BH3-only' Bcl-2 family member. This mechanism is particularly important during death receptor signalling as determined by the inability of Bid deficient mice to undergo Fas-induced hepatocellular apoptosis [37]. While some studies implicate full-length Bid in the mitochondrial apoptotic pathway, apoptotic signals that result in the processing of Bid to truncated forms (tBid) result in a greatly improved inducer of this pathway [38,39]. Caspase-8 was the first protease shown to be responsible for Bid processing [40]. However, it is now clear that cleavage of Bid can be achieved by several other proteases in a caspase-independent manner, including the serine protease Granzyme B (GrB) [41–43], lysosomal proteases [44,45] and calpain [46,47]. Once processed, tBid translocates to mitochondria and induces mitochondrial membrane permeabilisation and the release of apoptotic molecules. The mechanism by which tBid causes the permeabilisation of the OMM is poorly defined. One proposed mechanism is through interaction with Bax or Bak [42,48–50]. tBid has increased affinity for Bax-like factors and could act by stimulating the mitochondrial insertion and oligomerisation of Bax/Bak. Alternative mechanisms include channel-forming activity [25,51] or interaction with the PTP complex. [52]. Multiple mechanisms are also thought to be responsible for the suppression of Bid-induced apoptosis by Bcl-2. While Bcl-2 does not inhibit Bid processing or tBid translocation, this anti-apoptotic protein does prevent tBid insertion, Bax translocation and Bax/Bak oligomerisation in mitochondrial membranes [53].

Regardless of the mechanism, it is widely accepted that both Bax and tBid can induce mitochondrial membrane permeabilisation. Clearly, the disruption of mitochondrial integrity by these proteins can occur in a caspase-independent manner as determined by the inability of caspase inhibitors to prevent this phenomenon [54]. This is not surprising considering calpains, lysosomal proteases and GrB are all capable of processing Bid to produce tBid and calpains can cleave Bax to generate another powerful pro-death molecule, as previously mentioned. Additionally, calpain activation has also been shown to result in Bcl-x_L cleavage [55]. Cleavage of Bcl-x_L during the execution phase of cell death has previously been shown to convert Bcl-x_L from a protective to a lethal protein [56,57]. The pro-apoptotic activity of Bcl-x_L elicited by proteolytic cleavage could be due, at least in part, to enhanced pore forming activity. This is supported by the demonstration that C-terminal cleavage fragments of Bcl-x_L, but not their full-length anti-apoptotic counterpart, permeabilise both

mitochondria and pure lipid bilayer membranes to cytochrome *c* [58]. There is substantial evidence, therefore, in favour of the production of potent death inducers and resultant mitochondrial membrane permeabilisation in a caspase-independent manner. If the Bcl-2 family regulate outer membrane permeabilisation via their channel-forming activities, the question remains: are these channels nonselective, thereby allowing the passage of all soluble intermembrane proteins including caspase-independent prodeath factors? If so, the pore formed must be large enough to allow passage of relatively high molecular weight proteins (e.g. AIF, 57 kDa). In relation to Bax-forming pores, it has been demonstrated that purified Bax rapidly forms membrane pores that release fluorescein-isothiocyanate-conjugated cytochrome *c* (FITC-cytochrome *c*). The Bax pore capable of transporting cytochrome *c* required four Bax molecules and has an estimated pore size of 22 Å, theoretically not large enough to transport larger proteins [59]. Would a sufficient density of Bax molecules in the mitochondrial membrane form a pore large enough to release other apoptosis promoting proteins? This would require further oligomerisation, and such a channel has yet to be identified in vivo. Additionally, it is possible that Bax could interact with other proteins in mitochondrial locations such as the voltage-dependent anion channel (VDAC) to form larger pores. This theory continues to be explored and is discussed later.

Evidence in favour of the formation of nonselective channels comes from studies carried out on in a *Xenopus* cell-free system [60]. Using this system, recombinant tBid and Bax were found to induce the release of multiple intermembrane proteins including, in addition to cytochrome *c*, adenylate kinase (25 kDa), and sulfite oxidase (104 kDa). Importantly, the ultrastructural morphology of mitochondria remained unchanged, indicating that the release of these soluble factors does not require mitochondrial swelling, a feature more characteristic of necrosis than PCD. Further support comes from Vandenabeele's group, who recently undertook a matrix-assisted laser desorption ionisation post-source decay (MALDI-PSD) analysis of proteins released form isolated liver mitochondria treated with recombinant truncated Bid [61]. Several mitochondrial proteins were identified to be released including endoG, Omi/HtrA2, Smac/Diablo and adenylate kinase-2. In contrast, the matrix protein adenylate kinase 3 was absent, indicating that unlike soluble intermembrane proteins, matrix proteins do not exit mitochondria, at least under these conditions. In further support of the nonspecific release of intermembrane proteins, Sutton et al. [62] have recently demonstrated the release from mitochondria of the pro-apoptotic mediators cytochrome *c*, Smac/Diablo, and HtrA2/Omi. This study was carried out in GrB treated cells and potentially involves tBid. These studies therefore support the conclusion that all intermembrane proteins that have been measured are released in a tBid- or Bax-dependent way.

4. Bcl-2 regulation of the PTP

The PTP is a complex primarily made up of the VDAC in the outer membrane, adenine nucleotide translocator (ANT) in the inner membrane and cyclophilin D in the matrix. The underlying mechanism behind the opening of this pore, its involvement in apoptosis and the role of Bcl-2 proteins in its regulation is under continuing debate. It is proposed that pro-apoptotic Bcl-2 family members like Bax and Bak cause opening of the PTP while anti-apoptotic members such as Bcl-2 and Bcl-x_L favour closure of this channel.

In support of the regulation of the PTP by Bcl-2 proteins, Bax, Bak and Bcl-x_L have been reported to interact directly with the VDAC and modulate its activity [63,64], while Bax and Bcl-2 have been reported to interact with ANT [65,66]. Recently, a direct interaction that is enhanced during apoptosis has been reported between the BH3-only protein Bim and VDAC [67]. In this study, Bim induced loss of membrane potential and cytochrome *c* release and microinjection of the anti-VDAC antibody significantly reduced Bim-induced apoptosis, indicating that like Bax and Bak, Bim can also directly activate the VDAC. In contrast, other BH3-only proteins such as Bid (and its truncated form tBid) and Bik do not bind to VDAC [68]. In further support of the regulation of the PTP by Bcl-2 proteins, various inhibitors of the PTP that favour the closure of this channel such as cyclosporin A, Bongkrekic acid and Koenig's polyanion abrogate cytochrome *c* release and loss of membrane potential, effects induced by addition of recombinant Bax to mitochondria [65,69,70]. Cyclosporin A has also been shown to inhibit apoptosis induced by overexpression of Bax in Jurkat T cells [71]. Additional evidence for this model comes from Tsujimoto's group who have demonstrated Bax/Bak-induced loss in membrane potential and resultant cytochrome *c* release in wild-type but not VDAC1-deficient yeast mitochondria [63]. This group was also the first to provide direct evidence that the VDAC plays an essential role in apoptotic mitochondrial changes and apoptosis in mammalian cells [72]. In this study, anti-VDAC antibodies were employed to block VDAC activity. These antibodies were found not only to block Bax-induced cytochrome *c* release but, more significantly, inhibited apoptosis induced by etoposide, paclitaxel, and staurosporine. Of note, while these studies suggest that VDAC is necessary for Bax-induced apoptosis in these systems, they do not elucidate whether VDAC acts as a channel or indeed functions as a receptor protein in the mitochondrial membrane.

It is likely that the PTP plays a role in some apoptotic systems and clearly there is considerable evidence in support of a role for Bcl-2 family members in the interaction with and regulation of components of this complex. How is the PTP involved in regulating mitochondrial membrane permeability? One possible mechanism is the physical rupture of the OMM. It is postulated that opening of the PTP results in loss of mitochondrial membrane potential, an influx of fluid into the matrix which results in swelling and

rupturing of the OMM and the release of pro-apoptotic proteins from the intermembrane space. This mechanism is compatible with the hypothesis that all soluble intermembrane proteins including caspase-independent factors are released following disruption of mitochondrial integrity. Support for the nonselective nature of mitochondrial membrane permeabilisation is provided by the demonstration that supernatant from mitochondria treated with the PTP opening agent actractyloside (a ligand of the ANT) contains multiple soluble intermembrane proteins, including, in addition to known apoptogenic factors, antioxidant enzymes, proteins involved in protein import and catabolic enzymes [73]. This study therefore confirms the nonselective nature of mitochondrial membrane permeabilisation due to PTP opening. A recent report by Chang and Johnson [74] provides additional evidence in favour of the involvement of the PTP in caspase-independent cell death. This study demonstrated that cyclosporin A (acts on cyclophilin D) inhibited caspase-independent death of NGF-deprived sympathetic neurons, suggesting that PTP opening is a critical event in caspase-independent neuronal cell death, at least in this system.

Another explanation for mitochondrial membrane permeabilisation is the release of pro-death molecules through channels formed by interactions between individual components of the PTP and Bcl-2 family members such as the VDAC-Bax channel [75]. This model proposes that after activation, Bax directly interacts with and forms a large pore with the VDAC, through which apoptogenic proteins such as cytochrome *c* are released [16]. In this way, Bax can trigger caspase-dependent apoptosis. Whether or not larger proteins involved in caspase-independent cell death such as AIF (57 kDa) and endoG (30 kDa) are released through the Bax-VDAC channel remains to be elucidated. The majority of studies on this channel has focussed exclusively on cytochrome *c* release and therefore does not address the issue of the translocation of other factors under these conditions. One particular study has reported that Bax/VDAC liposomes allow the passage of cytochrome *c* but are impermeable to a 50-kDa protein [63]. How then is Smac/Diablo released? This caspase co-activator acts as a multimeric protein with an apparent molecular weight of 100 kDa [76]. It could be argued that a prerequisite for caspase activation and caspase-dependent apoptosis is the removal of IAP inhibition [77] and it is likely therefore that Smac/Diablo is released along with cytochrome *c* during apoptosis to counter the inhibitory activity of IAPs and promote cytochrome *c*-dependent caspase activation [76,78]. One would expect, therefore, that the channel formed by the proposed VDAC-Bax interaction is at least large enough to allow translocation of this protein. Theoretically then, any soluble intermembrane protein with a molecular weight of up to 100 kDa could be released through this channel. This of course assumes that cytochrome *c*, Smac/Diablo and other intermembrane proteins are released via the same pathway. In support of this hypothesis, overexpression of Bcl-2 not only inhibits the efflux of cytochrome *c* but also regulates the release of Smac/Diablo, AIF, endoG and Omi/HtrA2, suggesting that these molecules may exit via the same route [79–83]. Release kinetics studies of these proteins may provide us with additional evidence as to whether or not these intermembrane molecules are released via the same pathway. There are conflicting reports, however, regarding the release kinetics of these proteins. For example, the release of AIF precedes that of cytochrome *c* in several models of apoptosis induction [69,81,84]. However, another report places AIF release downstream of cytochrome *c* release [85]. In a similar way, the kinetics of Smac/Diablo release from mitochondria in response to UV treatment is the same as that of cytochrome *c* release [76,78], again suggesting that the same mechanism responsible for cytochrome *c* release is responsible for the translocation of Smac/Diablo. In contrast, other reports suggest that Smac/Diablo and cytochrome *c* may exit the mitochondria by different pathways [86,87]. Omi/HtrA2 has been reported to be released together with cytochrome *c* and Smac/Diablo during apoptosis [88] and, similarly, both endoG and cytochrome *c* are observed simultaneously in the supernatants of tBid treated mitochondria [89]. The conflicting results regarding the release kinetics of AIF and Smac/Diablo may be explained by the techniques employed, the affinity of the antibodies used and the stimulus employed to induce mitochondrial membrane permeabilisation. Finally, although similar release kinetics might suggest analogous exit routes, different kinetics do not necessarily suggest distinct mechanisms of release but may imply the sequential action of a shared pathway.

5. Mitochondrial intermembrane proteins involved in caspase-independent cell death

There is no doubt that pro-apoptotic Bcl-2 proteins such as Bax and Bid play a crucial role in PCD via the induction of OMM permeabilisation and the resultant efflux of apoptogenic proteins. However, not only is the molecular mechanism involved in this regulation under dispute but also the exact proteins released. Is this family responsible for the release of cytochrome *c* alone or do other pro-apoptotic molecules translocate via the same or alternative pathways?

Irrespective of the mechanism responsible for mitochondrial membrane permeabilisation, the release of proteins from the mitochondrial intermembrane space appears to be nonselective. The nonselective nature of mitochondrial membrane permeabilisation is confirmed by the two recent publications discussed above employing either the PTPC-opening agent actractyloside or recombinant tBid and isolated mitochondria [61,73]. In both studies, several mitochondrial proteins belonging to a broad molecular mass range were identified to be released and included several proteins capable of mediating caspase-independent cell death including relatively small proteins such as endoG

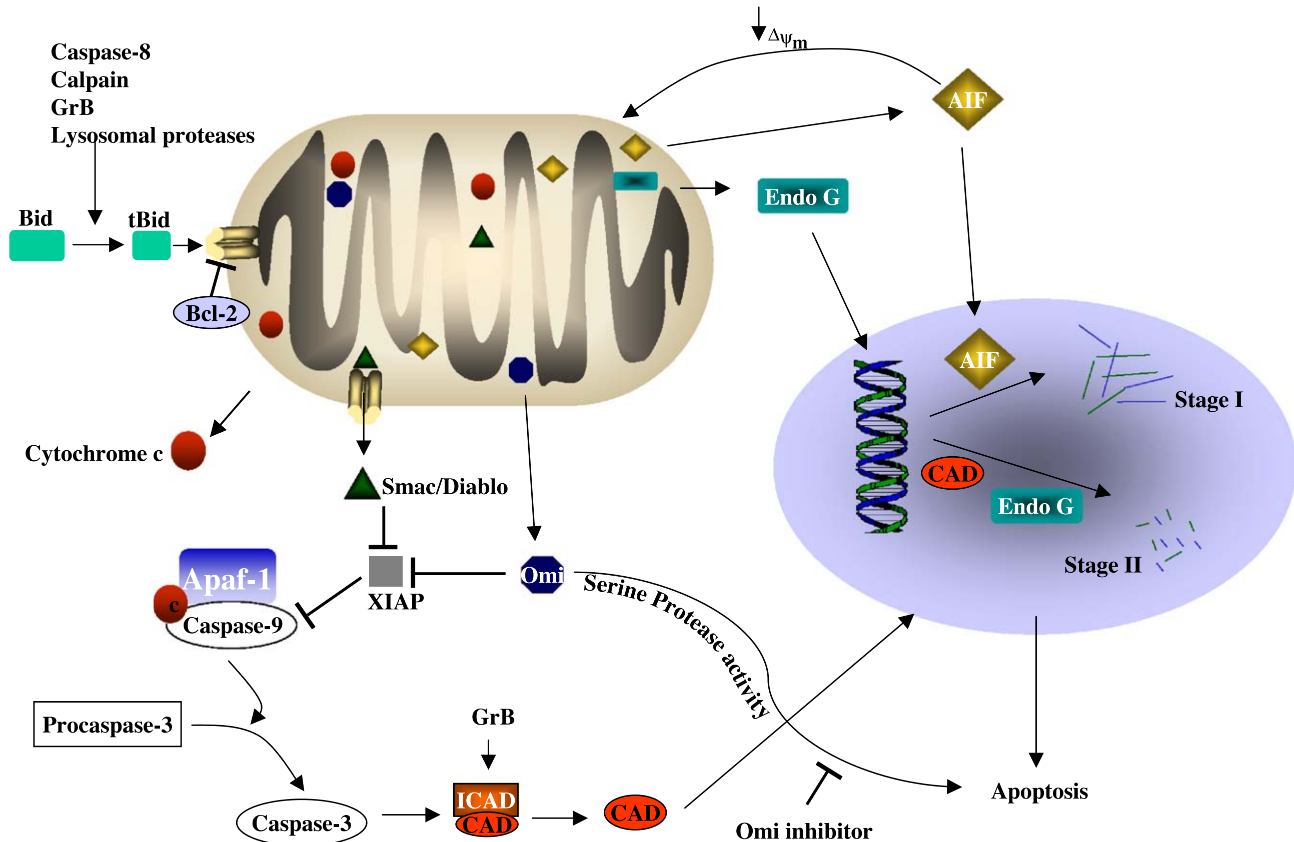

Fig. 2. Release of apoptogenic factors from mitochondria and their involvement in caspase-dependent and-independent cell death. Cleavage of Bid can be achieved by several proteases including in addition to caspase-8, the serine protease GrB, lysosomal proteases and calpain. Once processed, tBid translocates to mitochondria and induces mitochondrial membrane permeabilisation and the release of apoptotic molecules. These molecules may be released through Bax channels (formed as a result of the interaction between tBid and Bax), channels incorporating Bax and various components of the PTP complex or as a result of PTP opening, mitochondrial swelling and rupturing of the OMM (See Fig. 1). Regardless of the mechanism, several proteins are released from the mitochondrial intermembrane space following disruption of mitochondrial integrity, and these proteins play crucial roles in both caspase-dependent and -independent PCD. Cytochrome *c*, once released, associates with Apaf-1 to promote the activation of caspase-9, which can then activate caspase-3. Cleavage of ICAD by caspase-3 triggers activation of the CAD endonuclease, which mediates oligonucleosomal DNA fragmentation (Stage II). GrB can also cleave ICAD in a caspase-independent manner resulting in the activation of CAD. The caspase co-activator Smac/Diablo is released along with cytochrome *c* during apoptosis to counter the inhibitory activity of IAPs and promote cytochrome *c*-dependent caspase activation. Smac/Diablo functions by binding IAPs such as XIAP, thereby disrupting their ability to inhibit caspases 3, 7, and 9. On release to the cytosol, Omi has also been reported to bind XIAP, disrupting the interaction of XIAP with caspase-9 resulting in caspase-9 activation. However, Omi can also induce caspase-independent cell death in mammalian cells independently of its ability to interact with IAPs via its serine protease activity. While the underlying mechanism by which Omi induces caspase-independent cell death is poorly understood, a specific inhibitor of the proteolytic activity of Omi dramatically reduces caspase-independent cell death. AIF, once released to the cytosol, translocates to the nucleus in response to death stimuli and induces peripheral chromatin condensation and large-scale DNA fragmentation (Stage I). AIF in association with a heat-labile cytosolic factor may also permeabilise mitochondrial membranes [81]. Finally, in response to apoptotic stimuli, endoG is also released from the mitochondrion into the cytosol where it translocates to the nucleus and generates oligonucleosomal DNA fragmentation (Stage II). Unlike caspase inhibitors, Bcl-2 effectively protects cells from both caspase-dependent and -independent apoptosis. This is more than likely through its ability to prevent mitochondrial membrane permeabilisation and the resultant release of potent pro-apoptotic molecules.

(30 kDa) as well as larger proteins such as AIF (57 kDa) and the serine protease Omi/HtrA2 (50 kDa) (Fig. 2).

6. AIF

AIF is a 57-kDa mitochondrial flavoprotein, which in healthy cells resides in the mitochondrial intermembrane space. A recent study demonstrating a vital role for AIF in neuron survival in the ageing mouse brain has shed some light on the function of AIF in normal cells [90]. This study was carried out on Harlequin (Hq) mutant mice, which have a proviral insertion in the AIF gene that results in an 80% reduction in mRNA transcript and protein expression. This loss of AIF leads to increased hydrogen peroxide sensitivity that can be rescued by AIF transduction. Ackerman and colleagues therefore propose that AIF acts as a free radical scavenger, specifically acting on hydrogen peroxide. In addition to this crucial antioxidant function in neurons, AIF also displays apoptogenic activity and translocates to the nucleus in response to death stimuli [84]. Once in the nucleus, AIF induces peripheral chromatin condensation and

large-scale DNA fragmentation [81,91]. These effects have been reported to occur following addition of AIF to isolated nuclei and upon microinjection of recombinant AIF protein into cells. Additionally, AIF can affect mitochondria causing a drop in the mitochondrial membrane potential and cytochrome *c* release [69,81]. None of these AIF effects can be prevented by addition of zVAD.fmk indicating that they are caspase-independent [92]. Mitochondrial release of AIF has been reported in several instances of caspase-independent cell death induced by a variety of agents including, H_2O_2 [93], pneumococcus [94], cladribine [95], granulysin [96], peroxynitrite [97], P53 [98], UVB [99], sulfasalazine [100] and PARP-dependent cytotoxity induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, H_2O_2, and *N*-methyl-D-aspartate [101]. The cell death in these systems is characterised by various displays of apoptotic features including cell shrinkage, cell surface exposure of phosphatidylserine, loss of mitochondrial membrane potential and large-scale DNA fragmentation, which cannot be prevented by caspase inhibitors. AIF, therefore, appears to act as a lethal, caspase-independent effector of apoptosis. Further evidence in support of the caspase-independent action of AIF comes from studies carried out on the single cell organism *Dictyostelium discoideum* [102]. Cell death in this organism is associated with the release of the *Dictyostelium* homologue of AIF (DdAIF) from the mitochondria and exhibits many features of mammalian PCD including externalisation of PS, engulfment of dying cells by neighbouring cells and fragmentation of nuclear chromatin. Notably, this organism lacks clear homologues of mammalian caspases.

Recently, definitive genetic evidence provided by Joza et al. [103] establishes an essential role for AIF during early mammalian development. Inactivation of AIF rendered embryonic stem cells resistant to cell death induced by serum withdrawal. Furthermore, AIF was found to be essential for the first wave of PCD required for embryonic morphogenesis and cavitation. Importantly, PCD required for cavitation displayed typical apoptotic morphology, including peripheral chromatin condensation, plasma membrane blebbing, and formation of apoptotic bodies. An essential function for AIF in *C. elegans* has also been demonstrated [104]. Wang and colleagues employed RNA interference to silence the *C. elegans* homologue of AIF (*wah-1*) and found that in *wah-1* RNAi-treated worms the removal of apoptotic bodies was delayed, thus implicating WAH-1 involvement in *C. elegans* PCD.

Significantly, AIF relocalisation from mitochondria to the nucleus appears to be clinically relevant and has been demonstrated to occur in vivo following traumatic brain injury [97] and during photoreceptor apoptosis induced by retinal detachment [105]. In both instances, DNA fragmentation was detected and at least in the retinal detachment model, administration of zVAD.fmk failed to prevent either photoreceptor apoptosis or retinal functional damage. Interestingly, this laboratory has also described a caspase-independent pathway of photoreceptor apoptosis in vivo, which cannot be prevented by zVAD.fmk and retains key characteristics of apoptosis [11]. Rescue of photoreceptor degeneration by overexpression of Bcl-2 has been reported in this model [106]. Targeting mitochondrial dysfunction, therefore, in diseases such as Retinitis Pigmentosa, where photoreceptor apoptosis can occur independently of caspase activation, may be of more therapeutic benefit than caspase inhibitors. AIF itself may also represent an important therapeutic target for protection against cell death. This is supported by the crucial contribution of AIF to at least some PCD pathways and is underscored by the effect of AIF neutralising antibodies [81]. These antibodies have been demonstrated to inhibit the effects of microinjected AIF [69,81] and, importantly, AIF antisera significantly decrease caspase-independent cell death induced by DNA damage [98]. Intriguingly, HSP70 may also be a useful anti-apoptotic agent in conditions where caspase activation does not occur, due to its ability to interact with AIF, and protects cells against death induced by AIF overexpression, staurosporine and menadione [107]. The same may also be true for the treatment of cancer. Classic therapeutic strategies often target caspase-dependent apoptosis; however, many cancer cells are resistant to apoptotic stimuli that induce caspase-dependent cell death. This is true in relation to lung cancer cells that are resistant to a broad spectrum of apoptotic stimuli, including receptor stimulation, cytotoxic drugs and gamma-radiation. Triggering an AIF-mediated caspase-independent pathway as recently described by Joseph et al. [108] may circumvent the resistance of these cells to treatment.

Exactly how AIF exerts its functions remains elusive. It has recently been suggested that AIF-induced nuclear apoptosis requires a direct interaction with DNA [109]. This study revealed the crystal structure of human AIF, revealing a positive electrostatic potential and interaction of AIF with DNA in a sequence-independent manner. More importantly, AIF mutants lacking DNA-binding ability failed to induce cell death while retaining nuclear translocation. The recent cloning of the *C. elegans* homologue of AIF (*wah-1*) and investigation into the mechanisms by which it mediates apoptosis in the nematode have led to the proposal that AIF may cooperate with endoG to promote DNA degradation [104]. In this study, WAH-1 and CPS-6 (the worm orthologue of mammalian endoG) were found to interact in vitro and cooperate together to efficiently degrade DNA in a concentration-dependent manner. Importantly, coexpression of *csp6* and *wah-1* in touch receptor neurons had a synergistic effect on cell killing, further supporting the conclusion that these proteins cooperate to promote DNA degradation and cell death. It has yet to be determined whether mammalian AIF can cooperate with endoG or indeed other proteins to promote apoptosis in mammalian cells.

The mechanism(s) responsible for AIF release from the mitochondrial intermembrane space are still unclear and whether or not it is released via the same pathway as cytochrome *c* remains to be elucidated. However, there is

little doubt that Bcl-2 family members regulate AIF efflux and subsequent caspase-independent cell death. Overexpression of Bcl-2 in a variety of systems inhibits the mitochondrial release of AIF and cell death. These include apoptosis induced by granulysin [96], TNFα [110], cladribine [95], TRAIL [111], overexpression of AIF constructs [91], staurosporine [81,84] and camptothecin in Apaf$^{-/-}$ neurons [98]. Bax has also been reported to play a role in the regulation of AIF release. Translocation of Bax to mitochondria and subsequent AIF release has been described in several systems including hypothyroidism during rat cerebellar development [112] and apoptosis of Env-induced syncytia [69]. Bax expression leads to an increase in AIF efflux from mitochondria [85]. Furthermore, Bax has been shown to mediate mitochondrial depolarisation and AIF release in neurons [98]. In this study, camptothecin was found to induce AIF release in Bax$^{+/+}$ neurons but not in neurons deficient of Bax (Bax$^{-/-}$). tBid is also associated with AIF release and cleavage of Bid has been demonstrated to occur in conjunction with AIF translocation from mitochondria to the nucleus [110,111]. Furthermore, treatment of isolated liver mitochondria with truncated Bid results in AIF release [113]. Finally, the genetic data obtained from *C. elegans* add further support to the regulation of AIF release by Bcl-2 family members. In a similar way to AIF, WAH-1 localises to the mitochondria, and is released to promote DNA fragmentation. Significantly, global expression of EGL-1 (the nematode homologue of the mammalian pro-apoptotic 'BH-3 only' subfamily) causes the release of WAH-1 from mitochondria [104]. Therefore, the pro-apoptotic function of AIF and the regulation of its release by Bcl-2 family members may represent an evolutionary conserved pathway to DNA degradation and cell death.

7. EndoG

While AIF translocation to the nucleus is associated with large-scale fragmentation of DNA (~ 50 kb), apoptotic DNA cleavage can occur in two stages and this initial large-scale cleavage may be followed by a second stage of oligonucleosomal DNA fragmentation (DNA laddering). The best characterised endonuclease to date capable of inducing both chromatin condensation and DNA fragmentation is caspase activated DNase (CAD) [114]. CAD requires cleavage of its inhibitor, ICAD, by caspase-3 for nuclease activity [115]. However, in cells lacking caspase-3, ICAD can still be cleaved in response to apoptotic stimuli, suggesting that caspases other than caspase-3 or indeed other proteases may also cleave this inhibitor and induce DNA fragmentation [116]. In this way, GrB has been shown to induce DNA fragmentation in the presence of broad range caspase inhibitors [117]. Direct cleavage of ICAD by GrB in a caspase-independent manner has also been demonstrated both in vitro and in vivo and mutational analysis has revealed a common cleavage site of caspase-3 and GrB for ICAD processing [118]. These studies provide a caspase-independent pathway for GrB in mediating apoptotic cell death.

It is now evident, however, that other endonucleases may be essential for DNA fragmentation, particularly during caspase-independent apoptosis. The involvement of endonucleases other than CAD in apoptosis is supported by a recent report demonstrating oligonucleosomal DNA fragmentation in ICAD knockout mice after traumatic brain injury [119]. Additionally, transgenic mice expressing a caspase-resistant mutant ICAD develop normally and exhibit residual DNA fragmentation [120]. These studies indicate that CAD is not the only DNase implicated in apoptosis. One alternative endonuclease is endoG, a mitochondrial enzyme originally proposed to participate in the replication of mitochondrial DNA [121]. Unlike CAD, endoG does not require cleavage for activation but is activated by a change in its subcellular localisation. Originally believed to be a matrix protein due to its proposed involvement in mitochondrial DNA replication, repair and degradation, recent data describe the exclusive location of endoG within the mitochondrial intermembrane space [122]. This location is necessary in order for it to participate in DNA degradation as mitochondria in cells undergoing apoptosis retain most matrix proteins. In response to apoptotic stimuli, endoG is released from the mitochondrion into the cytosol where it translocates to the nucleus and generates oligonucleosomal DNA fragmentation [89]. EndoG-induced DNA fragmentation occurs independently of caspase activation [82,89]. The release of endoG from mitochondria was originally described by Li et al. [89] following treatment of isolated mitochondria with tBid. Importantly, in this study, mitochondrial efflux of endoG was not observed in Bcl-2 transgenic mice indicating that the Bcl-2 family may be involved in the regulation of endoG release. Shortly after this discovery, the in vivo occurrence of endoG release from mitochondria was reported during Fas-induced apoptosis in the liver [82].

The nematode counterpart of endoG, CPS-6, also cleaves DNA into nucleosomal sized fragments and was the first mitochondrial protein identified to be involved in PCD in *C. elegans* [123]. This study also provides the first genetic evidence that DNA degradation is an important component of cell death execution. Loss of CPS-6 activity actually delays the progression of apoptosis, indicating that CPS-6 may contribute to cell killing. The finding that mammalian endoG can replace the function of CSP-6 in the nematode, together with their identical subcellular localisation and DNA degradation properties, may be indicative of an evolutionary conserved DNA degradation pathway.

8. Omi/HtrA2

Omi/HtrA2 is a novel mammalian serine protease that has extensive homology to the bacterial HtrA (high tem-

perature requirement A) heat shock protease [124]. In bacteria, HtrA has a dual role, acting as chaperone at normal temperatures and an active protease at elevated temperatures [125]. Mammalian Omi, similar to endoG and AIF, exhibits pro-apoptotic activity and resides in mitochondria of healthy cells and is released to the cytosol on apoptotic stimulus [88,126]. The translocation of Omi from mitochondria to cytosol has also been confirmed in vivo [80]. In this study, administration of anti-Fas antibodies to mice induced the release of Omi from liver mitochondria. The subcellular localisation of this intermembrane protein appears to be under the regulation of Bcl-2 proteins. tBid induces release of mitochondrial processed Omi while Bcl-2 completely prevents this event [80,88,126]. Recently, Bax has also been demonstrated to play a pivotal role in the control of Omi release from mitochondria [127]. In this study, treatment of human colon cancer cells with thapsigargin resulted in a conformational change in Bax, mitochondrial translocation and the release of Omi together with cytochrome *c* and Smac/Diablo into the cytosol. Importantly, the absence of Bax blocked the thapsigargin-induced efflux of these proteins, indicating that Bax is essential for mitochondrial permeabilisation and Omi release in this system.

Moderate expression levels of Omi can sensitise cells to UV and staurosporine induced apoptosis while higher levels can induce apoptosis even in the absence of apoptotic insult [88,126,128]. In addition, removal of endogenous Omi by RNA interference results in a significant reduction in cell death [126]. The pro-apoptotic activity of Omi is attributed to both its serine protease activity and its ability to antagonise IAPs. There is some evidence that Omi functions as an IAP inhibitor thereby inducing caspase-dependent apoptosis in a manner similar to Smac/Diablo. Omi protein is synthesized as a precursor that is processed in the mitochondria to produce the mature protein. Processing exposes an internal tetrapeptide at the amino terminus. Smac/Diablo and the *Drosophila* IAP antagonists Reaper, Grim and Hid share a similar sequence motif, the Reaper Grim Hid (RGH) motif, that is sufficient to bind IAPs. On release to the cytosol, Omi has been reported to bind XIAP through this RGH domain, disrupting the interaction of XIAP with caspase-9, resulting in caspase-9 activation [88,126]. Omi has also been reported to antagonise XIAP inhibition of caspase-3 in vitro [83]. The N-terminal IAP-binding motif is essential for its ability to bind IAPs [83,88].

Omi can also induce caspase-independent cell death in mammalian cells independently of its ability to interact with IAPs via its serine protease activity [88,128]. This is supported by a recent study demonstrating that UV treatment results in the release of Omi into the cytosol, an event that cannot be prevented by zVAD.fmk. These results suggest that translocation of Omi from mitochondria to cytosol does not depend on caspase activity [88]. However, the underlying mechanism by which Omi induces caspase-independent cell death is poorly understood and remains to be elucidated. The demonstration that Omi can process itself to generate an RGH domain at the N-terminus leads to some speculation that it may be capable of cleaving other proteins to reveal similar motifs [126]. It is also possible that Omi could act on the nucleus as the processed form of this protein has been detected there [129]. Future identification of the physiological substrates for Omi will significantly contribute to our understanding of the mechanism by which Omi induces cell death. Very recently, a specific inhibitor of the proteolytic activity of Omi was identified and found to dramatically reduce caspase-independent cell death induced by overexpression of Omi [130]. This inhibitor or similar Omi specific inhibitors will also help us to elucidate the role of Omi in caspase-independent cell death.

9. Conclusion

Caspases appear to provide a critical function in developmental cell death observed in *C. elegans* and *Drosophila*. However, in contrast to these classical examples of caspase-dependent cell death, the studies discussed here reporting caspase-independent apoptosis indicate that at least in these models, many of the apoptotic features observed are not regulated by caspase activation. Such features include cytoplasmic and nuclear shrinkage, mitochondrial depolarisation, peripheral chromatin condensation and, in some instances, oligonucleosomal DNA fragmentation. Furthermore, there are now several examples of cells surviving the activation of caspases, suggesting that caspases alone are not sufficient for the induction of apoptosis [131,132].

Although there is increasing evidence to support a role for proteases other than caspases such as calpains, cathepsins, and GrB as molecular regulators of caspase-independent cell death, mitochondria also play a central role in this process by releasing several death-promoting factors. While cytochrome *c* is probably the best characterised protein to translocate from mitochondria to the cytosol, it is now evident that several other apoptotic proteins including AIF, Omi and endoG reside within the mitochondrion and are released following the death stimulus. It is clear that the nuclear events as well as some of the cytosolic features observed during apoptosis can be mediated in a caspase-independent manner by some of the proteins mentioned above. Mitochondrial membrane perforation, therefore, not only serves to induce caspase-dependent apoptosis but also a caspase-independent cell death pathway. Regarding the underlying mechanism responsible for the release of proteins that mediate apoptotic features independently of caspase activation, at present there is limited data contrary to the view that these proteins exit mitochondria via the same pathway as cytochrome *c*. The resultant cell death pathway is likely to be dependent on the presence of post-mitochondrial caspase-dependent factors such as caspases themselves or Apaf-1. Under conditions where these factors

are absent or inhibited, caspase-independent cell death will ensue, mediated at least by some of the proteins discussed previously.

Regardless of the pathway(s) and mechanism(s) involved, there is little doubt that the release of these pro-death factors requires mitochondrial membrane permeabilisation, an event that is undisputedly regulated by members of the Bcl-2 family. As the control of mitochondrial integrity by this family is common to both caspase-dependent and-independent apoptosis, therapeutic strategies which target Bcl-2 proteins may be extremely beneficial for cancer and degenerative diseases where both pathways can contribute to cell death.

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