LESSON 5: BIOCHEMISTRY OF APOPTOSIS – BCL-2 FAMILY MEMBERS AND MITOCHONDRIA

(P. Vandenabeele)

- Structure and function of mitochondria
- Link between glycolysis and oxidative phosphorylation
- Bcl-2 subfamily: structure and function
- BH3 only subfamily: structure and function
- Bax subfamily: structure and function
- BH3-only proteins as sentinels for cellular integrity
- How do Bcl-2 family members modulate mitochondrial integrity

Article 7:

Article 8:
Bcl-2 family members as sentinels of cellular integrity and role of intermembrane space proteins in apoptotic cell death. Acta Haematologica 111, 7, 2004
Selective cell suicide is crucial for sculpting the embryo, maintaining tissue homeostasis, shaping the immune repertoire, terminating immune responses and restricting the progress of infections. Moreover, disturbed regulation of this vital physiological process underlies many diseases, including cancer, autoimmunity and degenerative disorders. As cells perform their choreographed ‘dance of death’, they shrink and bleb violently, undergoing chromatin condensation and internucleosomal DNA cleavage, before being tidily packaged into vesicles that are rapidly engulfed by other cells. Although the importance of cell death during development had long been recognized, Kerr, Wyllie and Currie were the first to propose that the stereotypic nature of ‘apoptosis’, as they coined the process, reflected an underlying genetic programme.

The cancer connection

A central player in that genetic programme, and the link between apoptosis and cancer, emerged when BCL2 (B-cell lymphoma 2), the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death, rather than promote proliferation. This unexpected discovery gave birth to the concept, now widely embraced, that impaired apoptosis is a crucial step in tumorigenesis. Indeed, a defective suicide programme endows nascent neoplastic cells with multiple selective advantages. The cells can persist in hostile niches (for example, where cytokines or oxygen are limiting), escape the death that is often imposed as a fail-safe mechanism by other oncogenic changes and evolve into more aggressive derivatives. Finally, defective apoptosis facilitates metastasis, because the cells can ignore restraining signals from neighbours and survive detachment from the extracellular matrix. So, neoplastic progression in no small measure reflects loss of normal apoptotic mechanisms.

Impaired apoptosis is also a significant impediment to cytotoxic therapy. The mutations that favoured tumour development dampen the response to chemotherapy and radiation, and treatment might select more refractory clones. Nevertheless, most tumour cells still remain sensitive to some apoptotic stimuli, and more rational therapy should emerge from clarifying how particular agents elicit apoptosis and which apoptotic pathways remain open in individual tumours.

So, how do Bcl2 and related proteins monitor cellular well-being and decide whether the suicide programme should be activated? Re-evaluation of this hotly debated issue is timely, because recent findings challenge many prevailing notions. How oncogenic changes impinge on the Bcl2 family, how impaired apoptosis affects therapy and how direct targeting of these regulators could lead to more effective treatment of cancer and other diseases in which apoptosis is perturbed are also explored.
Death circuits

The molecular circuitry for apoptosis began to emerge when an exciting convergence of studies in mammals and the nematode Caenorhabditis elegans revealed that the worm protein CED-9 is the functional homologue of Bcl2 (REF. 17), and that CED-9 regulates activation of an aspartate-directed cysteine protease (CED-3)15, now called a caspase. To prevent unscheduled cell suicide, each caspase is synthesized as a pro-enzyme that typically requires processing at caspase cleavage sites to generate the active enzyme19. Once an initiator caspase is activated, it processes others that cleave a host of cellular proteins. So, a chain reaction of caspase activation is the cell's death sentence.

So far, two principal pathways for activating caspases have been discovered (fig. 1 and box 1). The more ancient, which is induced by diverse intracellular stresses, including cytokine deprivation and genotoxic damage, is regulated by Bcl2 and its relatives. Progression through the pathway usually leads to the activation of caspase-9 on a scaffold that is formed by apoptotic protease-activating factor 1 (Apaf1). This activation occurs after Apaf1 has interacted with cytochrome c that is released from damaged mitochondria20. A more-recently evolved pathway is triggered when 'death receptors' on the plasma membrane, engaged by cognate ligands of the tumour-necrosis factor (TNF) family, recruit caspase-8 through the adaptor protein FAS-associated death domain (FADD)21.

The BH3-only proteins are sentinels that detect developmental death cues or intracellular damage. In healthy cells, they are restrained in diverse ways, including sequestration on the cytoskeleton. When unleashed by death signals, they switch off survival function by inserting their BH3 domain into a groove on their pro-survival relatives.

• Either Bax or Bak is required for apoptosis, but how they are activated or countermanded by Bcl2 remains uncertain. During apoptosis, Bax and Bak oligomerize in the mitochondrial outer membrane and probably breach its integrity, freeing pro-apoptotic proteins such as cytochrome c, which allows activation of caspase-9.

• The pro-survival Bcl2-like proteins can prevent cytochrome c release, and hence caspase-9 activation. They probably also regulate the activation of several other caspases, independently of mitochondrial damage.

• Impaired apoptosis is a central step towards neoplasia. Pro-survival Bcl2-like proteins can promote tumorigenesis, and certain pro-apoptotic relatives act as tumour suppressors. Moreover, the expression of family members is affected by other tumorigenic alterations (for example, p53 mutation).

• Conventional cytotoxic therapy indirectly induces apoptosis, but more effective outcomes should be achieved by direct activation of the apoptotic machinery. Promising approaches include impairing expression of pro-survival Bcl2-like proteins or identifying drugs that mimic the action of BH3-only proteins.

Summary

• Apoptosis, the cell-death programme that is mediated by proteins called caspases, is essential for tissue homeostasis, and its perturbed regulation underlies many diseases, including cancer. Commitment to apoptosis in response to diverse physiological cues and cytotoxic agents is governed by proteins of the Bcl2 family.

• Bcl2 and several pro-survival relatives associate with the mitochondrial outer membrane and the endoplasmic reticulum/nuclear membrane and maintain their integrity. Initiation of apoptosis requires not only pro-apoptotic family members such as Bax and Bak that closely resemble Bcl2, but also distant cousins that are related only by the small BH3 protein-interaction domain.

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The Bcl2 clan

In mammals, Bcl2 has at least 20 relatives, all of which share at least one conserved Bcl2 homology (BH) domain (fig. 2). The clan includes four other anti-apoptotic proteins: Bcl-xL, Bcl-w, A1 and Mcl1, and two groups of proteins that promote cell death: the Bax and Bcl2 3-only families. Members of the Bcl2 death family22 have sequences that are similar to those in Bcl2, especially in the BH1, BH2 and BH3 regions, but the other pro-apoptotic proteins have only the short BH3 motif (hence their name) — an interaction domain that is both necessary and sufficient for their killing action. Both types of pro-apoptotic proteins are required to initiate apoptosis: the BH3-only proteins seem to act as damage sensors and direct antagonists of the pro-survival proteins, whereas the Bax-like proteins act further downstream, probably in mitochondrial disruption (see below).

The pro-survival family. Bcl2 and its closest homologues, Bcl-xL and Bcl-w, potently inhibit apoptosis in response to many, but not all, cytotoxic insults (fig. 1). Their hydrophobic carboxy-terminal domain helps
Box 1 | Caspases: the engine of cellular destruction

The dozen or so caspases in a mammal are synthesized as inactive precursors\(^1\)\(^1\)\(^2\)\(^1\)\(^4\). The long prodomain on those that initiate apoptosis promotes self-association and binding to activating adaptor or scaffold proteins. When procaspase-8 molecules are concentrated through their recruitment to ligated death receptors by Fas-associated death domain (FADD), they undergo autocatalysis, releasing the p10 and p20 subunits that form the active (tetrameric) enzyme. Caspase-9 is instead activated — in the presence of ATP and cytochrome c — by an allosteric change on a heptameric scaffold of apoptotic protease-activating factor 1 (Apaf1) proteins termed the apoptosome\(^1\)\(^3\)\(^4\). Effector caspases (3, 6 and 7) have short prodomains and are activated by the initiator caspases; once processed by caspase-8 or -9, caspases -3 and -7, in turn, process caspase-6. Other caspases with a long prodomain (1, 2, 4, 5 and 10 in humans and 1, 2, 11 and 12 in mice) might also serve as initiators (see text for details), as might granzyme B, a serine protease that is released from cytotoxic lymphocytes\(^1\)\(^5\)\(^6\).

SCAFFOLD PROTEINS

Proteins that provide a platform for the assembly of other proteins.

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Figure 2 | Three subfamilies of Bcl2-related proteins. Known \(\alpha\)-helical regions are indicated, as are the four regions \(BH1–4\) that are most highly conserved among family members. Most members have a carboxy-terminal hydrophobic domain that aids association with intracellular membranes, the exceptions being \(A1\) and many of the \(BH3\)-only proteins (\(B\), \(B\), \(B\), and \(P\)). Several other multidomain homologues (for example, \(B\)/\(D\), \(B\)/\(R\), \(B\)/\(G\), \(B\)/\(B\)) have been described, but their function is not yet clear. TM, transmembrane domain.

The BH3-only tribe. BH3-only proteins seem to be sentinels that are charged with triggering apoptosis in response to developmental cues or intracellular damage\(^1\)\(^7\). All programmed death of somatic cells in \(C\). \(E\) requires the single BH3-only protein \(EGL-1\) (REF. 41). The eight or more mammalian BH3 proteins (Fig. 2) — most of which are widely expressed — presumably allow more-refined control over cell death. With the possible exception of \(Bd\) (see below), they are thought to act by binding to and neutralizing their pro-survival relatives. Perhaps the small allosteric change that is induced in the pro-survival proteins by the engagement of a BH3 protein affects their association with another protein (see below). The BH3-only proteins cannot kill in the absence of \(B\) and \(B\), and hence must function upstream in the same pathway.

Individual BH3-only proteins are normally held in check by diverse mechanisms (Fig. 4). \(Bm\) and \(Bm\) are sequestered by binding to dynamin light chains that are associated with the microtubules (\(Bm\)) and actin cytoskeleton (\(Bm\))\(^4\)\(^5\). \(Bd\), after phosphorylation by kinases such as \(A\) and protein kinase A, is bound by \(14–3–3\) scaffold proteins\(^4\)\(^6\), whereas \(Bd\) is relatively inactive until proteolytically cleaved\(^4\)\(^7\). \(N\), \(P\), \(H\) and \(H\) are controlled primarily at the transcriptional level\(^4\)\(^8\)–\(^5\), as is their worm counterpart \(EGL-1\) (REF. 41).

Initial knockout studies indicate that individual BH3-only proteins could have specialized physiological roles. \(Bd\), although dispensable for proper development and tissue homeostasis, facilitates the death of hepatocytes that is provoked by anti-Fas antibody\(^4\)\(^9\). \(Bm\) is a principal regulator of haematopoietic homeostasis\(^5\)\(^0\); in its absence, leukaemia cells numbers rise and plasma-cell accumulation provokes the onset of an autoimmune disease that is equivalent to that elicited by the overexpression of \(Bd\) (REF. 31); this onset probably occurs, in part, because \(Bm\) is essential for the elimination of autoactive lymphocytes\(^5\)\(^1\). \(Bm\) also participates in neuronal death\(^5\)\(^2\).
Tissue homeostasis seems to be set by the balance between the pro-survival and BH3-only proteins. Interestingly, the apoptosis that normally decimates the neonatal kidney and immune system of Bcl2−/− mice was ablated by the concomitant loss of even a single allele of Bim.

Individual BH 3-only proteins might transduce specific death signals. Loss of Bim impairs the cytotoxic response of lymphocytes to cytokine deprivation, calcium flux or paclitaxel (Taxol), but not, notably, the cytotoxic response to γ-irradiation. Similarly, Bmf might be required for anoikis. As Noxa and Puma are both induced by p53, they might mediate the apoptosis that is elicited by genotoxic damage or oncogene activation. Clarifying these pathways should have important implications for tumorigenesis and therapy (see below).

Bid seems to promote death by activating Bax and Bak, and it might also inactivate pro-survival relatives. Exposure of its buried BH3 domain requires cleavage within the amino-terminal region — for example, by caspases or granzyme-B (Fig. 4). The cleaved (p7/p15) complex is then myristoylated on p15 and migrates to mitochondria; it is probably attracted by the cardiolipin-rich ‘contact sites’ between the outer and inner mitochondrial membranes. If Bak (or Bax) is present, Bid then very rapidly within a minute triggers cytochrome release and apoptosis. Bid might act by inducing Bax and Bak to oligomerize and form pores in the membrane, but the oligomers do not contain Bid, which seems to form homotrimers in the membrane. The resemblance between Bid and the pore-forming subunit of some bacterial toxins indicates that it might nucleate channel formation by Bax and Bak.

**The Bax family.** Bax and Bak are widely distributed, whereas the little-studied protein Bok is more prevalent in reproductive tissues. Inactivation of Bax affected apoptosis only slightly and disruption of Bak had no discernible effect, but inactivation of both genes dramatically impaired apoptosis in many tissues. So, the presence of either Bax or Bak seems to be essential for apoptosis in many cell types.

Bax and Bak are thought to function mainly at the mitochondrion, but their potential roles elsewhere (for example, the ER) merit attention. Bax is a cytosolic monomer in healthy cells, but it changes conformation during apoptosis, integrates into the outer mitochondrial membrane and oligomerizes. The three-dimensional structure of monomeric Bax closely resembles that of its pro-survival relatives. Intriguingly, the Bax hydrophobic carboxy-terminal helix occludes its BH 1/2/3 hydrophobic groove. As the carboxyl terminus is essential for targeting to mitochondria, the tail presumably flips out after the cell receives stress signals. Even in healthy cells, Bax is an oligomeric integral mitochondrial membrane protein, but it too changes conformation during apoptosis and might form larger aggregates. How the homo-oligomers form is unclear. Perhaps the Bax-like proteins can assume both a BH 3 donor and a BH 3 acceptor conformation within the membrane environment. Alternatively, some molecules might anchor in the

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**Figure 3** | Three-dimensional structures of Bcl-xL and Bax, showing their similarity. a | Bcl-xL, without its carboxy (C)-terminal tail and the unstructured loop between the BH4 and BH3 regions (see Fig. 2). b | Bcl-xL with the BH3 peptide of Bak (brown) bound to its surface groove. The yellow ribbon indicates Bcl-xL residues that precede the hydrophobic C-terminal tail, which had been deleted to facilitate structural analysis. c | Bax, showing its C-terminal tail (yellow) tucked into the groove, but running in the opposite orientation to a BH3 ligand.

**Figure 4** | Diverse modes of post-translational regulation of BH3-only proteins. In healthy cells, BH3-only proteins are held in check by a variety of strategies. Bim and Bmf are sequestered to the microtubules or actin cytoskeleton, respectively, via interaction with a dynein light chain (DLC). Phosphorylated Bad is bound by 14-3-3 scaffold proteins. Bid is synthesized as a precursor, which requires proteolytic cleavage to be fully active. The ‘beak’ in each represents the BH3 domain.

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Cytochrome c is released from the mitochondrial membrane via the cytochrome c-release channel and enables others to assemble on them as ‘daisy chains’ via intermolecular association of grooves and extruded tails.

Bax and Bak oligomers are widely believed to provoke or contribute to the permeabilization of the outer mitochondrial membrane, allowing efflux of apoptogenic proteins\(^a\) (see below). The mechanism, however, is controversial\(^b\). One model, which is based on the structural resemblance of Bcl2 family members and diptheria toxin\(^c\), is that Bax and Bak form channels. Consistent with this hypothesis is the fact that Bax oligomers can form pores in liposomes\(^d\) that allow passage of cytochrome c\(^e\), and that mitochondria from apoptotic cells contain a novel channel\(^f\). Alternatively, Bax might interact with components of the existing permeability transition pore — for example, the voltage-dependent anion channel (VDAC) — to create a larger channel\(^g\), but several studies have found no evidence for such complexes\(^h\).

Liaising for life or death. In lymphocytes, at least, induction of apoptosis by diverse signals (for example, cytokine deprivation) requires Bim\(^i\), as well as Bax or Bak\(^j\). As Bim does not bind to Bax or Bak\(^k\), it must act by preventing the pro-survival proteins from inhibiting the activation of Bax and Bak. How the Bcl2-like proteins antagonize the Bax-like proteins, however, remains unknown. Direct interaction might not occur physiologically, because it is only observed in certain non-ionic detergents\(^l\). Moreover, although high concentrations of the pro-survival proteins prevent Bax oligomerization and channel-forming activity\(^m\), cross-linking reveals no Bcl2-Bax complexes\(^n\).

**Direct or indirect control of caspase activation?**

The ongoing debate about how the Bcl2 family controls apoptosis hinges on whether its pro-survival members control caspase activation directly\(^o\) or only indirectly, by controlling mitochondrial integrity\(^p\). In other words, does caspase activation occur independently of mitochondrial disruption or only as a consequence of it? For C. elegans, a direct sequestration model is strongly favoured\(^q\) (FIG. 5a): CED-9, the worm Bcl2, binds the adaptor CED-4 and prevents it from activating the CED-3 caspase until the BH3-only protein EGL-1 displaces CED-4. B (Mammals): protection of mitochondrial integrity (see text). Bc2 and its anti-apoptotic homologues guard mitochondrial membrane integrity until neutralized by a BH3-only protein. Bax and Bak then form homo-oligomers within the mitochondrial membrane, resulting in the release of cytochrome c, which activates Apaf1, allowing it to bind to and activate caspase-9. Other pro-apoptotic molecules that exit the mitochondria include Omi and Diablo, which antagonize the pro-survival proteins prevent Bax oligomerization and Bcl2–Bax complexes\(^r\).
The mitochondrial guardian model (FIG. 5b) readily explains how Bcl2 might control activation of Apaf1 and caspase-9 and, through them, caspase-3, but can this axis account for all stress-induced apoptosis? That possibility seemed to be supported by initial gene-inactivation reports: mice lacking either Apaf1 (REFS 94,95) or caspase-9 (REFS 96,97) often died before birth and had enlarged brains, and apoptosis of several cell types was impaired in vitro55–57. Nevertheless, recent evidence rules out an essential role for Apaf1 and caspase-9 in stress-induced death. Unlike Bcl2 overexpression, the absence of Apaf1 or caspase-9 does not increase lymphocyte numbers in vivo, and lymphocytes and embryonic fibroblasts die at normal rates in response to diverse insults against which Bcl2 protects (V. Marsden, J. M. Adams, and A. Strasser, unpublished observations). Even post-mitotic neurons that lack Apaf1 die normally58, and some Apaf1−/− mice become healthy adults59. Furthermore, the deletion of thymocytes with self-reactivity requires Birn60 but not Apaf1 (REF. 100), fibroblasts lacking both Bax and Bak are more resistant to cytotoxic insults, including overexpression of BH-3-only proteins, than those lacking Apaf1 or caspase-9 (REF. 43); and Bcl2 can protect embryonic stem cells that lack Apaf1 (REF. 101). Finally, the absence of cytochrome c only attenuates apoptosis62. So, the cytochrome-c–Apaf1–caspase-9 ‘apoptosome’ is not indispensable for stress-induced apoptosis. Rather, it acts as a caspase amplification system that is more important in certain cell types (for example, neuronal precursors) than others (for example, lymphocytes).

It could still be argued that activation of the relevant initiator caspase(s) requires mitochondrial disruption, because certain synthetic caspase inhibitors (typically, z-VAD-fmk) have blocked cell death but not cytochrome c release66,103. In other studies, however, such inhibitors have also blocked cytochrome c release104,105. Indeed, caspase-dependent apoptosis can occur without cytochrome c release106,107, whereas certain cells can remain viable for days after disruption of the mitochondrial outer membrane108. So, a mitochondrial breach is neither necessary nor sufficient for apoptosis, and it could often be triggered by caspases rather than being required for caspase activation109.

Bcl2 might regulate multiple initiator caspases

Speculatively, Bcl2 might control the activation of several initiator caspases that act upstream or independently of any mitochondrial breach (for example, caspase-2 and -X in FIG. 6). For instance, Bcl2 can control apoptosis from the ER110–112, and caspase-12, which can process other caspases in the absence of Apaf1 or cytochrome c113, is activated by ER-regulated stress114. So, caspase-2 is another plausible initiator, because elimination of its mRNA by RNA interference (RNAi)115 in certain cell lines inhibits apoptosis, release of cytochrome c and Diablo, and recruitment of Bax to mitochondrial109. However, as mice that lack caspases 1, 2, 8, 11 or 12 develop normally, with only slight defects — if any — in stress-induced apoptosis117, we speculate that several of these caspases redundantly trigger the caspase cascade. Their activation might involve oligomerization by cognate proteins that bear both a caspase-recruitment domain (CARD) and a CED-4-like nucleotide binding domain118. If so, the pro-survival clan might sequester such caspase activators (FIG. 6), just as CED-9 directly constrains CED-4 (FIG. 5a).

The Bcl2-like proteins presumably also function at the mitochondrion to prevent Bax/Bak oligomerization. In the absence of convincing evidence for physical interaction of these opposing factions under physiological conditions (see above), indirect models must be considered. First, if Bcl2 helps to gate a mitochondrial pore, as some (controversial) findings indicate18, engagement of Bcl2 by a BH 3-only protein might allow the release of small molecules that provoke a conformational change in Bax/Bak. Second, if Bcl2 and Bax/Bak compete for an unknown target on mitochondria, the ligation of Bcl2 might free it, allowing Bax to bind and nucleate pore formation. Third, if Bcl2 sequesters caspase activators (see above), their release from Bcl2 might allow an activated caspase to mediate Bax translocation, perhaps via cleavage of a Bid-like protein or an outer mitochondrial membrane.
**Box 2 | Bcl2 and the Rb/Arf/p53 network**

Inactivation of the retinoblastoma (Rb) pathway — for example, by loss of cell-cycle inhibitor Ink4a, which can prevent cyclin-D–Cdk4 from phosphorylating Rb — unleashes the transcription factor E2F, which increases expression of Arf, a protein that is encoded by the same locus as Ink4a (REF. 136). Arf, which is also a transcriptional target of Myc, sequesters Mdm2, a negative regulator of p53. Raised p53 levels can either impose growth arrest, typically by inducing the Waf1 cell-cycle inhibitor, or promote apoptosis through targets such as Bax, Puma and Noxa. The apoptotic targets seem to also require the p53 relative p63 or p73 and Noxa. The apoptotic targets seem to also require the p53 relative p63 or p73 (REF. 152). Circles/ovals denote oncogene products; rectangles denote known or likely tumour suppressors. For more detail, see REFS 4–6,136. AT M, ataxia telengiectasia mutated; Chk2, checkpoint 2; NF-κB, nuclear factor-κB.

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**Bcl2 family and tumorigenesis**

The evidence from human tumours that cancer generally requires impaired apoptosis is not yet overwhelming, but the hypothesis is strongly supported by experimental models. In particular, the oncogenic potential of elevated Bcl2 has been clearly shown in several transgenic mouse models. Bcl2 transgenes that mimic the Bcl2L translocation gave rise to B-lymphoid tumours, and their stochastic onset implied a need for acquired mutation(s) 9,12,134. Myc aided the transformation of Bcl2-expressing cells in vitro, and co-expression of Bcl2 and Myc transgenes dramatically accelerated lymphomagenesis 125, revealing a previously unsuspected strong cooperation between mutations that enforce proliferation and those that inhibit apoptosis. This synergy also occurs in breast 123 and pancreatic β-cell tumours 124,125. It presumably reflects the ability of Bcl2 to counter the apoptosis elicited by Myc under suboptimal growth conditions, and the ability of Myc to override the retardation of cell-cycle entry by Bcl2 (REFS 3,5). Partnerships are not limited to Myc; Bcl2 can also synergize with the chimeric promyelocytic leukaemia–retinoic-acid receptor-α (PM L–RARα) to induce acute promyelocytic leukaemia 126. Although it has been inferred that enforced proliferation plus impaired apoptosis might suffice for fully fledged malignancy 125,126, in most cell types, bypassing of senescence minimally requires the elimination of p53 function 4.

All the Bcl2 pro-survival family members are likely to be oncogenes. Bcl-xL, for example, has been implicated in mouse myeloid and T-cell leukaemias 127. Conversely, members of both pro-apoptotic subfamilies are probably tumour suppressors. Bax or Bak is mutated in some human gastric and colorectal cancers 128,129, as well as in leukaemias 123, and loss of Bax increases tumorigenicity 131–133. Absence of both Bax and Bak can enhance transformation, beyond loss of either alone 4. Bim also seems to be a tumour suppressor: absence of even one Bim allele accelerates Myc-induced lymphomagenesis (A. Egle and S. Cory, unpublished observations). The p53 targets, Noxa and Puma, might mediate p53 apoptotic function, and Bmf might inhibit metastasis 4.

Many oncogenic mutations probably impair apoptosis indirectly, by affecting signal-transduction pathways that promote or repress expression of Bcl2 family members (BOX 2). For example, mutations that increase the activity of nuclear factor-κB (NF-κB) transcription factors (for example, Ras) can enhance the expression of pro-survival family members 124,135. Conversely, mutations that inactivate the retinoblastoma (RB) tumour-suppressor pathway or promote Myc activation upregulate apoptosis inducers such as p53, the targets of which include Bax, Puma and Noxa 136 (BOX 2). Given that the RB/Myc/p53 circuitry is shorted in almost all tumours, some dysregulation of the Bcl2 family during oncogenesis might be almost universal.

**Targeting the apoptotic machinery for therapy**

Most cytotoxic agents, irrespective of their primary targets, are now thought to kill cells predominantly by triggering their apoptosis programme 137. Supporting
that notion, overexpression of Bcl2 renders tumour cells refractory to diverse therapeutic drugs and radiation, in vivo as well as in vitro\(^1\), and selection for drug resistance in cancer cells is often accompanied by upregulation of Bcl2 (Ref 139). Moreover, elimination of Bax from human colorectal cancer cells abolished their apoptotic response to non-steroidal anti-inflammatory drugs\(^2\).

So, the prospect of directly switching on the apoptotic machinery is gaining widespread interest\(^2\). One promising approach is to engage death receptors, such as for TNF-related apoptosis-inducing ligand (TRAIL), because tumorigenesis often spares that arm of the apoptotic response and normal cells are surprisingly refractory\(^2\).\(^3\) (Fig. 1). Other approaches target Bcl2 — for example, Phase III clinical trials with antisense Bcl2 deoxyoligonucleotides are underway\(^4\). Although such studies might establish the value of compromising Bcl2 function, antisense oligonucleotides have a chequered history, as RNA\(^5\) using small RNA duplexes, delivered as synthetic oligonucleotides or expressed from vectors, seems more promising.

**BH3 mimetics.** An exciting approach for manipulating Bcl2 function is to mimic the binding of a BH3 peptide to its groove. The dramatic rescue of the degenerative defects in Bcl2\(^{-/-}\) mice by loss of a single Bim allele\(^6\) indicates that degenerative diseases might be retarded by drugs that modulate the action of Bcl2-3 only proteins. Conversely, small molecules that mimic the BH3 domain and neutralize Bcl2-like function might well be effective against cancer or autoimmune diseases. Several reports have already described small organic molecules that bind to Bcl2 in vitro (albeit with low affinity) and compromise cell viability\(^7\)\(^8\)\(^9\)\(^9\), and the potential of such approaches has been discussed\(^7\).\(^10\).

Why might a BH3 mimetic be more effective than conventional anti-cancer drugs? As most genotoxic drugs act primarily through p53 to induce apoptosis (Box 2), p53 mutation gives tumour cells a decided advantage over normal cells. By targeting Bcl2 directly, the BH3 mimetic would bypass that roadblock. Normal cells must tolerate reduced Bcl2 levels, because mice that lack one Bcl2 allele, or an allele of any pro-survival relative, are completely healthy. The tumour cell might be more vulnerable because of oncogenic changes such as Myc activation, which reduces Bcl2 and Bcl-\(\times\) expression\(^11\) and might prime that of certain BH3-3 only proteins such as Bim. Moreover, BH3 mimetics that target specific family members — for example, Bcl2 and not Bcl-\(\times\) — would allow therapy to be tailored to the dominant pro-survival molecule in that tumour, increasing the therapeutic index.

**Puzzles and prospects**

The decisive first step towards apoptosis occurs when sentinel BH3-3 only proteins respond to developmental cues or damage to particular cellular compartments, but how they register those signals needs clarification. Once unleashed (Fig. 4), the BH3-3 only proteins engage the Bcl2-like anti-apoptosis proteins (Fig. 3b), but how that neutralizes their pro-survival function remains uncertain. A central unresolved issue is the nature of the immediate effectors of Bcl2 function. Rather than acting merely as a guardian of the mitochondrion (Fig. 3a), Bcl2 might act primarily by constraining the activation of several initiator caspases (Fig. 6). RNAi offers new opportunities for testing this (unorthodox) model, and identifying the initiators and their activators. Another puzzle is how Bax and Bak are activated — does this involve caspase activation, for example, by cleavage of Bid-like proteins, or do Bax and Bak instead contribute to caspase activation? Although caspase-mediated apoptosis apparently can occur without disruption of the outer mitochondrial membrane, that step often provides the coup de grâce for the cell by allowing several pro-apoptotic molecules, including cytochrome c, to escape to the cytosol and augment the caspase cascade. The disruption is often attributed to pores formed by Bax or Bak oligomers, but convincing in vivo evidence for these pores is still lacking.

Impairment of apoptosis is a central step in tumorigenesis and many BH3-3 only proteins are likely to be tumour suppressors. Determining which BH3-3 only proteins are activated by specific anticancer agents could lead to a more rational basis for cytotoxic therapy. Finally, even though impaired apoptosis might seem to render the tumour cell invulnerable, it could instead prove to be its ‘Achilles’ heel’, because pharmacological manipulation of the Bcl2 family and other apoptosis regulators is likely to open up new therapeutic opportunities.


This demonstration that human Bcl2 could replace the worm survival gene revealed the marked conservation of the apoptotic machinery.


An illuminating moment, when developmental genetics and mammalian biochemistry converged to reveal that cell death is launched by the activation of a class of cysteine proteases, later called caspases.


Together with reference 24, this paper provided the first evidence that Bcl2 has pro-apoptotic relatives.


First evidence that Bcl2 has pro-apoptotic relatives.


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REVIEWS


Bcl-2 was found to be unable to block apoptosis triggered by the newly discovered ‘death receptor’ pathway, establishing that there are at least two distinct pathways to apoptosis in mammalian cells.


Acknowledgements

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Bcl-2 Family Members as Sentinels of Cellular Integrity and Role of Mitochondrial Intermembrane Space Proteins in Apoptotic Cell Death

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Abstract
In addition to their function as major energy-providing organelles of the cell, mitochondria accomplish a crucial role in apoptosis. The pro-apoptotic BH3-only members of the Bcl-2 family continuously sense the cellular integrity and well-being at various subcellular levels. If these sentinels are induced, released or activated, they converge on the release of mitochondrial intermembrane space proteins such as cytochrome c, the oxidoreductase AIF, endonuclease G, Smac/DIABLO and the serine protease Omi/HtrA2. We discuss how Bcl-2 family members integrate diverse survival and death signals and act as central regulators of apoptosis. Furthermore, we describe the current knowledge on the role of mitochondrial proteins in apoptotic cell death, discuss the molecular mechanisms of their release and the apoptotic role of mitochondria from a phylogenetic and immunological point of view.

Key Words
AIF, apoptosis-inducing factor  Apoptosis  Bcl-2  BH3-only  Cytochrome c  Endonuclease G  Mitochondria  Omi/HtrA2  Smac/DIABLO

Evolution and Internal Structure of Mitochondria
Mitochondria are the major energy-providing organelles of the eukaryotic cell. Beside their role in meeting the demand for ATP by oxidative phosphorylation [1], they exert a pivotal role in the process of programmed cell death (PCD) [2] and are important players in the regulation of calcium homeostasis, in lipid metabolism, in amino acid metabolism as a source of acetyl-CoA and the ureum cycle [3]. The endosymbiont theory postulates that mitochondria evolved from the fusion of a primitive anaerobic eukaryote (host) and a respiration-competent proteobacterium (symbiont) to form a primitive eukaryotic cell with the proteobacterium destined to become the mitochondrion [4]. Most animal cells have a small mitochondrial genome of ~16,5 kb which encodes two rRNAs (12S and 16S), 22 tRNAs and 13 mRNAs that code for subunits of all respiratory complexes except complex II [5]. Mitochondria are typically oval in shape and contain a double membrane, reflecting their prokaryotic origin. The double membrane defines two mitochondrial compartments, the intermembrane space (IMS) between the outer mitochondrial membrane (OMM) and...
inner mitochondrial membrane (IMM), and the internal compartment called the matrix. Two different models prevailed to describe the internal mitochondrial structure. Sjöstrand [6] described the folding of the IMM in cristae as ‘septa’, defining discrete compartments that have no openings into the IMS while Palade [7] observed the cristae shaped as ‘baffles’ with intracisternal spaces communicating freely with the IMS. In the early 1970s, however, it has been shown that steroid-secreting cells like those from the adrenal cortex contain mitochondria with lamellar cristae in fetal life which develop into tubular cristae connected to the IMS at the time mitochondria acquire the enzymes for steroidogenesis [8] (fig. 1A). This shows that the structure of cristae can be influenced by the enzymatic composition of the membrane. Thin sections of mitochondria with tubular cristae nearly always contained also vesicles and at that time, they were assigned as sections of independent membranous spheres [3]. Those early observations are in line with recent reports describing mitochondrial morphology. Recent evolutions in electron microscopy tomography suggest that cristae form tubular or lamellar structures, connected to and continuous with the inner boundary membrane by small tubular structures, termed cristae junctions [9] which may have important functional implications in oxidative phosphorylation and apoptosis as diffusion between internal compartments is restricted (fig. 1B). As mitochondrial respiration relies on diffusion of substrates from the cytosol to the electron transport chain in the IMM, cristae junctions could regulate oxidative phosphorylation. A regulated diffusion of cytochrome c between IMS and IMM could also modulate the release of cytochrome c from the IMS during apoptosis.

As mitochondria are self-replicating organelles and are implicated in a wide range of biochemical pathways in the cell such as Krebs cycle, urea cycle and fatty acid oxidation, it is not surprising that they contain about 1,000 different proteins. Except for 13 mtDNA-encoded ones, these are encoded by nuclear genes and synthesized in the cytosol. Therefore, mitochondria dispose of a well-developed protein import system. Mitochondrial protein import and correct targeting to either the matrix, IMS, IMM or OMM is mediated by integral membrane multi-subunit complexes, referred to as translocases of the outer and inner mitochondrial membranes, TOM and TIM complexes, respectively [10].

**Abbreviations**

ACBP = Acyl-CoA-binding protein; AICD = activation-induced cell death; AIF = apoptosis-inducing factor; ala = alanine; AMID = AIM-homologous mitochondrial-associated inducer of cell death; ANT = adenine nucleotide translocator; Apaf-1 = apoptotic protease-activating factor-1; ATP = adenosine triphosphate; Bad = Bcl-2-ANT = adenine nucleotide translocator; Apaf-1 = apoptotic protease-activating factor-1; ATP = adenosine triphosphate; Bad = Bcl-2 = B-cell lymphoma 2; BH = Bcl-2 homology domain; Bax = Bel-2-antagonist of cell death; Bak = Bel-2-antagonist/killer; Bid = BH3 interacting domain death agonist; Bim = Bel-2 interacting mediator of cell death; BIR = baculovirus IAP repeats; C. elegans = Caenorhabditis elegans; CAD = caspase-activated DNA nuclease; CARD = caspase-activating and recruitment domain; CASH = caspase homolog; CASPER = caspase-8-related protein; Ced = Caenorhabditis elegans death gene; clp = cellular inhibitor of apoptosis; CLARP = caspase-like apoptosis-regulatory protein; Complex I = ubiquinone oxidoreductase; Complex II = succinate-ubiquinone reductase; Complex III = cytochrome bc1; Complex IV = cytochrome oxidase; Complex V = F1F0 ATPase or ATP synthase; CPS-6 = Cds-3 protease suppressor; Cyp-D = cyclophilin D; CaA = cyclosporin A; DARK = Drosophila Apaf-1-related killer; dATP = deoxyadenosine 5′-triphosphate; DD = death domain; DED = death effector domain; DFF = DNA fragmentation factor; DIABLO = direct IAP-binding protein with low PI; DISC = death-inducing signaling complex; DKO = double knockout; DNA = deoxyribonucleic acid; EPO = erythropoietin; EPO = erythropoietin; ER = endoplasmic reticulum; ES cells = embryonic stem cells; FABP = fatty acid-binding protein; FADD = Fas-associated death domain; FADH2 = flavin-adenine dinucleotide (reduced); FLAME-1 = FADD-like anti-apoptotic molecule; HKII = hexokinase II; FLICE = inhibitor interleukin-1β converting enzyme (= MACH); FLIP = FLICE-inhibitory protein; glic = glucose; GM-CSF = granulocyte-colony-stimulating factor; Hsp = heat-shock protein; HtrA2 = high temperature requirement protein A2; IAP = inhibitor of apoptosis protein; IBM = IAP-binding motif; ICAD = inhibitor of caspase-activated DNA nuclease; Ile = isoleucine; IMM = inner mitochondrial membrane; IMS = intermembrane space; IRES = internal ribosome entry site; JNK = c-jump N-terminal kinase; KD = kilodaltons; MACH = MORT1-associated CED-3 homolog (= FLICE); mtCK = mitochondrial creatine kinase; MPT = mitochondrial permeability transition; MRIT = MACH-related inducer of toxicity; mtDNA = mitochondrial DNA; Δψm = mitochondrial transmembrane potential; NGF = neuronal growth factor; NBD = nucleotide-binding domain; NADH = nicotinamide-adenine dinucleotide (reduced); OMM = outer mitochondrial membrane; PARP = poly(ADP-ribose) polymerase; PBR = peripheral benzodiazepine receptor; PCD = programmed cell death; PDZ = postsynaptic density protein, disc large tumor suppressor and Zo-1 tight junction protein; Phe = phenylalanine; PMA = phorbol 12-myristate 13-acetate; PTP = permeability transition pore; PtB = polyomavirus trans-acting binding protein; ROS = reactive oxygen species; SAPK = stress-activated protein kinase; Smac = second mitochondria-derived activator of caspase; tBid = truncated Bid; TCR = T-cell receptor; TIM = translocase of inner mitochondrial membrane; TNF = tumor necrosis factor; TNFR = tumor necrosis factor receptor; TOM = translocase of outer mitochondrial membrane; UCP = uncoupling protein; UV = ultraviolet; Val = valine; VDAC = voltage-dependent anion channel.
Mitochondria and Energy Metabolism

During normal mitochondrial respiration, five respiratory enzyme supercomplexes situated in the IMM mediate the electron transfer from NADH or FADH$_2$ to oxygen as final acceptor (fig. 2A). Cytochrome c, residing in the IMS, functions as an electron shuttle between complex III and IV [1, 11]. The energy released by this electron transfer is used to generate a proton-motive force across the IMM by pumping protons from the matrix to the IMS, a process mediated by complex I, III and IV. This proton-motive force is a combination of the proton concentration gradient (pH) and the membrane electric potential ($\Delta W_m$). The spontaneous backflow of protons to the matrix through ATP synthase is coupled to the synthesis of ATP from ADP and Pi. This coupled backflow of protons is absolutely dependent on the integrity of the IMM. However, not all of the energy available in the electrochemical gradient is coupled to ATP synthesis. Electrons continuously leak out of the mitochondrial respiratory chain leading to the production of oxygen free radicals and some protons are able to move back into the matrix bypassing the ATP synthase. The leakage can be mediated by the uncoupling protein 1 (UCP1) homologs, as UCP1 is responsible for a protein-catalyzed proton conductance found in mitochondria from brown adipose tissue. UCP1 catalyzes the net transfer of protons from the IMS to the matrix uncoupling the electron flow from ATP production. The physiological function of UCP1 in the brown adipose tissue is heat production, since the energy of oxidation dissipates as heat [12]. However, using various model systems, the physiological and biochemical functions of the UCP1 homologs remain unclear [13].

Mitochondria and Cell Death

In addition to their well-established role of providing ATP to drive energy-requiring processes within the cell, mitochondria play a key role in controlling the pathways that lead to PCD in mammals. PCD is a genetically encoded and functional form of cell suicide that is central to the development and homeostasis of multicellular organisms. In the context of embryonic development, three main morphologies of PCD have been described [14]. Type I apoptotic cell death is characterized by morphological changes such as cell shrinkage and extensive chromatin condensation. The formation of autophagic vacuoles inside the dying cell is typical for autophagic or type II cell death, whereas rapid loss of plasma membrane integrity and spillage of the intracellular content distinguish type III PCD. Eventually, the type of cell death will inevitably depend on the cellular context and stimulus, as every cell death program is a net result of self-propagating
Fig. 2. Electron transport chain and hypothetical molecular architecture of the mitochondrial PTP. A The flow of electrons through the electron transport chain is coupled to the translocation of protons from the mitochondrial matrix to the IMS. This process generates a proton gradient across the IMM, the proton-motive force. The movement of protons back into the matrix, down their concentration gradient, is coupled to the synthesis of ATP from ADP and Pi. The exchange of ADP for ATP is mediated by the ANT, which is, next to VDAC, a core component of the transmembrane channel formed at the contact sites between IMM and OMM. VDAC makes the OMM permeable to most small molecules (<5 kDa), allowing free exchange of respiratory chain substrates. Electrons continuously leak out of the mitochondrial respiratory chain producing oxygen free radicals and some protons are able to move back into the matrix circumventing the ATP synthase. This move back can possibly be regulated by UCP1 homologs. Complex I: ubiquinone oxidoreductase, Complex II: succinate-ubiquinone reductase, Complex III: cytochrome bc1, Complex IV: cytochrome oxidase, Complex V: F1F0 ATPase or ATP-synthase, Cyt c: cytochrome c, FADH2: flavin-adenine dinucleotide (reduced), NADH: nicotinamide-adenine dinucleotide (reduced), Q: ubiquinone, UCP: uncoupling protein. B Beside the ANT and VDAC, the PTP involves several other associated proteins: hexokinase II (HKII), mitochondrial creatine kinase (mtCK), cyclophilin D (Cyp-D) and peripheral benzodiazepine receptor (PBR). Different agents and metabolites can either facilitate or inhibit pore opening as depicted in the figure. Proteins or peptides containing the BH3 domain can act on either Bax or Bcl-2 in the OMM. Atr = Atractyloside; CsA = cyclosporin A; glc = glucose; MPTP = mitochondrial permeability transition pore; ΔΨm = mitochondrial transmembrane potential; ROS = reactive oxygen species.
Bcl-2 Family Members as Sentinels of Cellular Integrity

Fig. 3. Different death stimuli converge onto mitochondria with concomitant release of multiple mitochondrial intermembrane space proteins, inducing caspase-dependent or caspase-independent cell death pathways. Different apoptotic stimuli disturb the balance between anti- and pro-apoptotic Bcl-2 family members affecting mitochondrial integrity, which results in the release of apoptotic proteins including cytochrome c (cyt c), Smac/DIABLO, Omi/HtrA2, AIF and endonucleaseG (endoG). Cytochrome c-mediated apoptosome formation induces a caspase cascade. Smac/DIABLO and Omi/HtrA2 can neutralize IAP inhibition of caspases and induce apoptosis in a caspase-independent way of which the mechanism has not been resolved yet. AIF and endoG are involved in caspase-independent nuclear DNA degradation.

signals and signals that suppress the other cell death programs [14]. Mitochondria can be differentially involved in each of these types of cell death, but in this review, attention will be focused on their role in apoptotic signaling pathways. Apoptotic cell death is essentially initiated by two distinct pathways: the extrinsic apoptotic pathway starting with the aggregation of death receptors and the intrinsic apoptotic pathway starting with the release of mitochondrial factors (fig. 3). In the former type, homo-trimeric ligands belonging to the tumor necrosis factor (TNF) family such as TNF and FasL bind to their corresponding ‘death receptors’ (TNFR and Fas respectively) on the plasma membrane leading to receptor trimerization and subsequent formation of the death-inducing signaling complex (DISC). DISC formation involves adaptor proteins such as Fas-associated death domain (FADD), directly interacting with the receptor via its death domain (DD) and recruiting procaspase-8 through its death effector domain (DED), resulting in proximity-induced activation [15–17]. The level of caspase-8 activation in the DISC and the susceptibility to cell death inhibition by Bcl-2 overexpression upon death receptor activation may differ between cell types. This difference allowed to distinguish two types of cells: type I cells, e.g., lymphocytes, in which Bcl-2 overexpression cannot overcome death receptor-induced cell death [18, 19], and type II cells, e.g,
The Bcl-2 family

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<th>Anti-apoptotic Bcl-2 family members</th>
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- **Fig. 4.** Classification of the Bcl-2 family members. Bcl-2 family members can be divided into three subgroups: Bcl-2-like survival factors, Bax-like death factors and BH3-only death factors. The BH3 domain in the pro-apoptotic members is a ligand for the hydrophobic pocket formed by the BH1-BH3 domains of the survival factors. Bcl-2 orthologs are identified in *C. elegans*, viruses and mammals. For more details, see text.

- hepatocytes, in which Bcl-2 inhibits death receptor-induced cell death [20–22]. However, the existence of type II cells has been questioned, arguing that the use of agonistic Fas antibodies does not always mimic Fas receptor activation by Fas ligand [23]. For example, transgenic Bcl-2 overexpressing mice are protected against lethal fulminant hepatitis induced by anti-Fas antibody injection but succumb after Fas ligand injection [20, 21]. Nevertheless, the extrinsic apoptotic pathway sooner or later impinges on the mitochondrial pathway, which at least fulfills an amplification role. The molecular links and control mechanisms that either safeguard or arm the intrinsic pathway are outlined below. Besides the type II extrinsic apoptotic pathway, other stimuli such as growth factor withdrawal, UV irradiation and cytotoxic drugs also impinge on the intrinsic apoptotic pathway and require the release of mitochondrial factors. The molecules that integrate the signaling of these different cell death stimuli and converge on the release of mitochondrial factors are the BH3-only members of the Bcl-2 family.
**Mitochondria and Communication with Bcl-2 Family Members**

Bcl-2, first characterized in a B-cell leukemia cell line as a gene linked to an immunoglobulin locus due to t(14;18) chromosomal translocation [24], is the mammalian ortholog of ced-9 in *C. elegans* [25, 26]. In higher eukaryotes, Bcl-2 has at least 20 homologs [27–31], which are classified in three subgroups: Bcl-2-like survival factors, Bax-like death factors and BH3-only death factors (fig. 4). Anti-apoptotic Bcl-2 and its closest homologs Bcl-xL and Bcl-w contain four Bcl-homology (BH) domains and a hydrophobic C-terminal tail which functions as membrane anchor [32]. Residues from BH1, 2 and 3 form a hydrophobic groove, with which BH3-only death factors interact through their BH3 domain [33–36], whereas the N-terminal BH4 domain stabilizes this pocket. Bax-like pro-apoptotic death factors lack this BH4 domain. Most death stimuli finally converge on the activation of Bax and Bak [37, 38] with subsequent disturbance of the integrity of the mitochondrial membrane. In normal healthy cells, Bax is monomeric and resides in the cytosol or is loosely attached to the mitochondrial membrane. Following a death stimulus, cytosolic Bax undergoes a conformational change inducing its oligomerization and translocation to the mitochondria where it becomes an integral membrane protein [39–41]. Death stimuli cause the membrane-associated Bax to integrate in the OMM and alter the exposition of its N-terminal BH3 domain [42, 43]. In the absence of death signals the N-terminal domain prohibits the transmembrane anchor function, explaining why Bax in healthy cells fails to insert in the mitochondrial membrane. Activated Bax also binds and antagonizes anti-apoptotic Bcl-2 proteins [44]. In contrast to Bax, Bak permanently resides in the OMM but also undergoes oligomerization and activation upon apoptosis [38, 45]. Different molecular mechanisms have been proposed how Bax/Bak may cause disturbance of mitochondrial integrity and will be discussed below. Upstream sensors and mediators of apoptotic cell death are the BH3-only proteins (fig. 5). Yang et al. [46] proposed a ‘displacement model’ in which BH3-only proteins competitively bind to anti-apoptotic Bcl-2 or Bcl-xL, releasing and consequently activating the pro-apoptotic Bax/Bak proteins.

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**Fig. 5.** BH3-only proteins as sentinels for cellular integrity. Different mammalian BH3-only death factors block the action of the Bcl-2-like survival factors. BH3-only proteins can be regulated either at transcriptional or post-translational level. Bax-like death factors are directly or indirectly activated after which they oligomerize and insert in the OMM with concomitant disruption of mitochondrial integrity. Dashed arrow represents data based on Bim-BH3-derived peptide [49].
However, as the BH3-2 and Bcl-xL is not complexed with Bax or Bak prior to the addition of detergent [40, 47], it is more conceivable that BH3-only molecules activate Bax/Bak by allosteric conformational changes of the latter, through either indirect, e.g. interaction with Bcl-2, or direct, e.g. tBid/Bax, interaction [48]. This two-class model is supported by a study using peptides representing the BH3 domains of several BH3-only proteins, which proposed that BH3-only proteins can either directly activate Bax/Bak proteins or sensitize the latter by binding to the hydrophobic pocket of anti-apoptotic Bcl-2 family members [49]. Combined deficiency of Bax and Bak in double knockout (DKO) mice revealed important functions in homeostasis and development as the vast majority of bax/bak DKO mice die around the time of birth and only a small percentage survived to adulthood [50, 51]. Bax/bak DKO mice have increased hematopoietic progenitor cells in the bone marrow and white blood cells in the blood. Transgenic expression of Bax in lymphocytes sensitized to glucocorticoid- and DNA damage-induced cell death and induced also a major reduction of their lymphoid progeny at birth and only a small percentage survived to adulthood [50, 51]. Bax/bak DKO mice have increased hematopoietic progenitor cells in the bone marrow and white blood cells in the blood. Transgenic expression of Bax in lymphocytes sensitized to glucocorticoid- and DNA damage-induced cell death and induced also a major reduction of their lymphoid progeny. BimL and BimS, the latter being the most toxic [71]. Investigation of tissue distribution of Bim demonstrated the expression in hematopoietic, epithelial, neuronal and germ cells [72]. While BimL and BimS are co-expressed at similar levels, BimS was never detected by IP/Western blot analysis. One may speculate that BimS is expressed in only a limited number of cells that need to be killed rapidly or that BimS is not expressed under physiological conditions. BimL and BimS are associated with cellular microtubule complexes by tethering to the dynein light chain LC8. Upon cytokine deprivation, calcium flux, microtubule perturbation (taxol) or cellular damage by UV irradiation, the LC8-Bim complex is released and can bind to and antagonize Bcl-2 [70, 73]. Co-immunoprecipitation studies failed to detect interaction of Bim with Bax, Bad, Bik or Bid [71].

**BH3-Only Members, Sentinels of Life and Death**

In extrinsic apoptotic cell death (see above), amplification of the apoptotic signal occurs through proteolytic activation of BID to truncated BID (tBid) [34, 58]. In front of its BH3 domain, BID contains a protease sensitive loop that can be target for several proteases such as caspase-8 [34, 59], granzyme B [60], cathepsin [61] or calpain [62]. N-myristoylation at the N-terminal glycine of tBid [63] and high affinity binding to cardiolipin through helix 4, 5 and 6 of tBid [64, 65] facilitates its translocation and insertion in the OMM at the contact sites, which are rich in cardiolipin [65]. Insertion also depends on the hydrophobic C-terminus of tBid [45]. tBid mutants with amino acid substitutions in the BH3 domain efficiently translocate to mitochondria but display diminished disruptive effect on the OMM and no cytochrome c release [45, 66]. tBid seems to harbor two distinct signals: one for translocation to the mitochondria and another, the BH3 domain, for interaction with proteins and lipids to exert its pro-apoptotic function. Studies using cells derived from bax and bak DKO mice demonstrate that Bid acts in concert with Bax and Bak by triggering their oligomerization, resulting in the release of cytochrome c [37, 48]. Generally speaking, Bid acts as a cytosolic sentinel for the presence of activated proteases that could be due to CTL attack in case of granzyme B, to lysosomal leakage and DISC formation in case of apoptotic cell death [67, 68] or to calpain activation in endoplasmic reticulum (ER)-stress induced apoptosis [62]. However, studies in bid-deficient mice revealed that this sensing role of Bid might be restricted to certain cell types as only hepatocytes are protected from death receptor-induced apoptosis [69]. Compensation for the absence of Bid by functionally redundant BH3-only members may be more likely.

Another BH3-only member, Bim, seems to play a major and non-redundant role in embryogenesis, in the control of hematopoietic cell death and as a barrier against autoimmunity [70]. Bim occurs as three splice variants, BimEL, BimL and BimS, the latter being the most toxic [71]. Investigation of tissue distribution of Bim demonstrated the expression in hematopoietic, epithelial, neuronal and germ cells [72]. While BimL and BimSL were co-expressed at similar levels, BimS was never detected by IP/Western blot analysis. One may speculate that BimS is expressed in only a limited number of cells that need to be killed rapidly or that BimS is not expressed under physiological conditions. BimL and BimEL are associated with cellular microtubule complexes by tethering to the dynein light chain LC8. Upon cytokine deprivation, calcium flux, microtubule perturbation (taxol) or cellular damage by UV irradiation, the LC8-Bim complex is released and can bind to and antagonize Bcl-2 [70, 73]. Co-immunoprecipitation studies failed to detect interaction of Bim with Bax, Bad, Bik or Bid [71]. The BH3 region is essential for the interaction of BimL with pro-survival Bcl-2 family members and for most of its ability to promote apoptosis. During the attempts to generate bim gene-deficient mice, it was observed that Bim plays an important role during embryogenesis. Only one third of the expected homozygous bim KO offspring was born and a significant portion of bim<sup>−/−</sup> heterozygotes died during prenatal development, indicating there is a gene dosage effect. Bim<sup>−/−</sup> mice...
showed a significant elevation in blood leukocytes reflected by a 2- to 4-fold increase in the number of B and T cells, granulocytes and macrophages and by the occurrence of lymphadenopathies at older age. Furthermore, studies in bim−/− mice showed that the elimination of autoreactive thymocytes is affected [74]. The number of red blood cells and megakaryocytes was normal, but platelets were half the normal amount [70]. This may indicate a Bim-dependent apoptosis-like mechanism for platelet shedding from megakaryocytes. Bim appears to be required for certain apoptotic responses in leukocytes (cytokine withdrawal, Ca2+ flux, taxol, glucocorticoid dexamethasone or γ-irradiation) but not others (phorbol 12-myristate 13-acetate (PMA), Fas ligand or the topoisomerase inhibitor etoposide) [70], most probably due to functional compensation by other BH3-only proteins. Apoptosis induced by growth factor withdrawal has been thought to proceed mainly through Bad and for some apoptotic stimuli through Hrk/DP5 (see below), but at least in B and T cells Bim must be the dominant transducer of this cytotoxic signal. However, besides its release from microtubule complexes, the transcriptional upregulation of Bim is another way to respond to cell death stimuli. In T-cell receptor (TCR)-stimulated thymocytes, Bim expression is upregulated and may be part of the activation-induced cell death (AICD), which protects the organism from an overstimulated immune system [75].

Bmf functions in the same context of maintaining cytoskeleton integrity as Bim. Bmf is also tethered to the dynein light chain LC8 but is regulated by interaction with the myosin V actin motor complex [76]. Consequently, Bmf is activated by apoptotic stimuli that affect the actin cytoskeleton such as anoikis (detachment from the extracellular matrix). UV irradiation can also activate Bmf by releasing the protein, bound to dynein, from the myosin V actin motor complex. Bmf interacts with and antagonizes Bcl-2, Bcl-xL and Bcl-w but does not bind to Bax, Bad or Bid as revealed by yeast two hybrid screen. Overexpression of Hrk/DP5 induces rapid cell death, which is repressed by co-expression of Bcl-2 or Bcl-xL and abolished by deleting the BH3 motif. Immunostaining displayed a Hrk/DP5 expression pattern which was granular and extranuclear, consistent with a localization confined to membranes of intracellular organelles. High expression levels were detected in lymphoid tissues, predominantly spleen and bone marrow, whereas very low expression was detected in kidney, liver, lung and brain [83]. Hrk/DP5 is rapidly induced after growth factor withdrawal in hematopoietic progenitor cells [85]. Hrk/DP5 is also an important mediator in neuronal cell death as its expression is upregulated upon neuronal growth factor (NGF) withdrawal or treatment with amyloid β protein [86] in cultured neurons at the time when these cells are committed to die [84]. Studies with chemical inhibitors have demonstrated that transcriptional activation of the hrk/dp5 gene requires stress-activated/c-jun N-terminal (SAPK/JNK) kinase [87, 88].

NOXA and PUMA, another set of BH3-only proteins, are primarily controlled at the transcriptional level by the transcription factor and tumor suppressor p53. p53 expression is induced by DNA-damaging treatments such as topoisomerase inhibitors (etoposide, doxorubicin) or by γ-irradiation. Aberrant growth signals resulting from expression of oncogenes such as c-myc and ras can upregulate the expression of Noxa and Puma in a p53-dependent way [89–91]. Murine noxa cDNA encodes a 103-amino-acid protein that contains two related 9-amino-acid se-
quences, characteristic for the BH3 motif. NOXA mutants carrying a substitution in one of those BH3 domains had lower pro-apoptotic activities than wild-type NOXA while a mutant with substitutions in both BH3 domains was totally inactive demonstrating the requirement of the BH3 domain for apoptotic activity [89]. Immunofluorescence staining and subcellular fractionation of HeLa cells infected with epitope-tagged noxa revealed co-localization of the protein with mitochondria, which is dependent on a functional BH3 domain [89]. Co-immunoprecipitation studies showed binding of NOXA to Bcl-2 and Bcl-xL, both depending on a functional BH3 motif of NOXA, whereas NOXA failed to bind to Bax [89]. Overexpression of noxa in HeLa cells induced a decrease in Δψm, cytochrome c release and Apaf-1 and caspase-9 activation. Bax is also a known p53 target gene, however DNA damage-induced apoptosis seems normal in thymocytes of bax-deficient mice, indicating that p53-induced BH3-only proteins may compensate for loss of Bax. Introduction of the antisense oligonucleotide to noxa in the hematopoietic cell line BAF-3, inhibited radiation-induced expression of noxa and apoptosis, which is known to be dependent on p53 in this hematopoietic cell line [92, 93].

Human noxa encodes 54 amino acids, containing only one BH3 motif probably corresponding to the second motif of mouse NOXA. The promotor region of human noxa also contains a p53-responsive element and human NOXA is able to induce apoptosis in various cells in a BH3 motif-dependent manner [89]. PUMA exists as four splice variants (α, β, γ, δ), three of which contain a BH3 domain (α, β, γ) [90]. However, only endogenous PUMAα and β could be detected by Western blot analysis in response to p53 activation in p53-inducible human H1299 lung cancer cells. Antisense studies showed the effective contribution of PUMA in p53-mediated apoptosis [90]. Immunostaining revealed co-localization of PUMA with a mitochondriar marker, which was not impaired by a mutation of the BH3 domain. Overexpression of PUMAα and β inhibits growth and induces cytochrome c release in a BH3 domain dependent and Bcl-2 inhibitable way. Depending on the cellular context, DNA damage-induced PUMA and NOXA expression may have different outcomes, namely apoptosis or cell cycle arrest. In fibroblasts, the response to DNA damage is cell cycle arrest while in thymocytes apoptosis is the consequence [89, 90, 94–97]. The reason for the differential response is not clear but it is conceivable that the contribution of additional pro-apoptotic molecules or a lower expression of pro-survival Bcl-2 members favor apoptosis in thymocytes. This balance might be different in fibroblasts resulting in growth arrest instead of apoptosis [89, 97].

For most BH3-only proteins, it is still unclear whether the cytotoxic mechanism is solely dependent on the inactivation of the survival function of Bcl-2 or whether another BH3-dependent mechanism contributes to the cytotoxic action, as is the case for tBid. Also, the survival function of Bcl-2 is not yet well understood in molecular terms. In this review, Bcl-2 and other family members are mainly described as proteins associated with and functioning in the context of mitochondria. However, it should be kept in mind that the majority of Bcl-2 is found at other subcellular locations such as nuclear envelope and ER [98, 99]. Therefore, it is conceivable that the anti-apoptotic effects of Bcl-2 and the pro-apoptotic effects after interaction with BH3-only proteins may be not solely exerted at the level of mitochondria. Moreover, also the pro-apoptotic Bax and Bak, which are the eventual direct or indirect targets of the BH3-only proteins, have been reported to reside in other organelles than mitochondria [100, 101].

**Release of Apoptogenic Factors from the Intermembrane Space of Mitochondria during Cell Death**

Beside cytochrome c, a well-known apoptogenic factor [102], other factors that trigger caspase-dependent and caspase-independent death processes are also released from mitochondria after specific death stimuli (fig. 3). We performed an in vitro study in which proteins released from recombinant tBid-treated mouse liver mitochondria were identified by mass spectrometry [103]. Besides cytochrome c, adenosine kinase 2, endonuclease G, Smac/DIABLO and HtrA2/Omi were also found in the supernatants of tBid-treated mitochondria, which were also reported by other groups as mitochondrial factors playing a role in the apoptotic process [104]. We also identified some additional proteins such as polyphosphatidyl serine-binding protein (PTB), acyl-CoA-binding protein (ACBP) and proteins associated with fatty acid metabolism or protein transport such as fatty acid-binding protein (FABP) and a translocase of the inner mitochondrial membrane (TIM). Although many reports support the mitochondriocentric view on cell death, several items remain unclarified. What is the precise role of mitochondria and mitochondrial factors in apoptosis in *C. elegans* and *Drosophila*? What is the role of cytochrome c and the consecutive
intrinsic apoptotic pathway in immune development, regulation and homeostasis? Are caspases associated with or residing within the mitochondria in mammalian cells? In the following part of this review we will describe the potential role of some mitochondrial factors in mammals as compared to C. elegans and Drosophila, and shortly discuss the items and controversies mentioned above.

**Cytochrome c, Activator of the Apoptosome Complex**

Cytochrome c, the first identified apoptogenic IMS protein being released from the mitochondria during apoptosis, has been shown to be required for proteolytic activation of procaspase-3 using a cell-free system [58]. Microinjection of cytochrome c also leads to apoptosis [105]. In mammals, cytochrome c triggers the assembly of the apoptosome, a high molecular weight caspase-activating complex composed of ‘apoptosis protease-activating factor 1’ (Apaf-1), dATP and cytochrome c [28, 102, 106, 107]. The three-dimensional structure of the apoptosome has recently been determined [28, 102, 106, 107]. Apaf-1 is a cytosolic protein that comprises an N-terminal caspase-recruitment domain (CARD), a nucleotide-binding domain (NBD) and a C-terminal domain containing multiple WD-40 repeats [108]. Under normal conditions, Apaf-1 adopts a ‘closed’ monomeric conformation, in which the C-terminal WD-40 repeat domain is folded back on the CARD domain. Under apoptotic conditions, binding of cytochrome c to the WD-40 repeat domain [109] facilitates the binding of dATP to the nucleotide-binding domain [110]. These events convert Apaf-1 to an ‘open’ heptameric platform, exposing the CARD domain. The heptameric structure with hub and spokes forms the metaphoric wheel of death [107]. The CARD domains are exposed in the center of the wheel and act as a docking region for the recruitment of procaspase-9 [102, 106, 107]. Once bound to the platform, caspase-9 is activated, even without requirement of processing [111], and the executioner procaspase-3 is recruited and proteolytically activated [112]. The importance of cytochrome c, Apaf-1 and caspase-9 for the execution of intrinsic apoptosis in mammals has been confirmed by targeted disruption of the corresponding genes. *Cytochrome c* null mice exhibit impaired developmentally mediated apoptosis, resulting in prenatal death [113, 114]. Despite their severe phenotype and developmental delay, *cytochrome c* knockout embryos seem capable of developing differentiated tissues derived from the three germ layers, indicating that cytochrome c, unlike AIF (see below), is not essential for cavitation during early embryogenesis [113, 115]. Mouse embryonic stem cells and fibroblasts derived from these knockout mice demonstrate defects in response to a variety of apoptotic stimuli and no procaspase-3 activation occurred in any of the knockout cells. In view of the essential functions of cytochrome c in oxidative phosphorylation and in the intrinsic apoptotic pathway, it is impossible to pinpoint the molecular mechanism of the embryonic phenotype. Targeted replacement of the *cytochrome c* gene by a mutant that lacks apoptogenic properties but still retains the electron transfer activity [116] would allow evaluating the contribution of cytochrome c in apoptosis.

**Smac/DIABLO and Omi/HtrA2, Propagators of Caspase-Dependent and Caspase-Independent Cell Death**

Human Smac (Second mitochondria-derived activator of caspase) and its murine ortholog DIABLO (Direct IAP-Binding protein with Low PI) are nuclear-encoded mitochondrial proteins of 29 kD containing a mitochondrial targeting sequence, which are processed to a 23-kD mature protein that translocates to the cytosol after an apoptotic stimulus [103, 117, 118]. Smac/DIABLO acts as a dimer and can interact with inhibitor of apoptosis proteins (IAPs), like XIAP, cIAP1 and cIAP2, which in turn inhibit caspases through their characteristic baculovirus IAP repeat (BIR) domains. The inhibitory effect of cIAPs on caspases through mere interaction is 100- to 1,000-fold weaker than that of XIAP [119]. It has been demonstrated that the C-terminal RING zinc-finger domains of XIAP, cIAP1 and -2 possess E3 ubiquitin ligase activity [120, 121], which can mediate autodegradation and degradation of interacting proteins such as TRAF2 [122] and caspase-3 and -7 [120, 123] preventing their anti- and pro-apoptotic functions respectively. Recently, cIAP1 and cIAP2, but not XIAP, were shown to degrade Smac in a proteasome-dependent way [124]. cIAP-mediated Smac ubiquitination and degradation requires specific interaction between cIAPs and Smac through the BIR domain and N-terminal Ala-Val-Phe-Ile IAP-binding motif (IBM) motif, respectively. A similar N-terminal IBM is also present in Omi/HtrA2 and caspase-9 [125] as well as in the *Drosophila* proteins Hid, Grim, Reaper and Sickle [126–128], although for the rest these proteins lack any sequence homology. The IBM motif of Hid, Grim and Reaper prevents DIAP-1 and -2 from inactivating cas-

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pases, while DIAP-1 directs Hid and Grim to proteasome-
dependent degradation through its E3 ubiquitine ligase
activity [124]. At odds with this elegant Smac/DIABLO-
IAP antagonism is the pro-apoptotic nature of Smac β, a
splice variant of Smac that lacks the mitochondrial target-
ing sequence and sensitizes apoptosis induced by death
ligands and chemical stimuli independent of its IBM
motif [129]. This pro-apoptotic function of Smac β is
exerted by the C-terminal domain. The physiological mi-
tochondrial function of Smac/DIABLO is unknown and
diablo knockout mice are apparently normal [130]. This
observation suggests the existence of redundant factors
compensating for the loss of Smac/DIABLO, possibly
Omi/HtrA2 (see below), or that Smac/DIABLO has no
essential and general role in apoptosis during develop-
ment. However, studies using cell lines suggest that Smac/
DIABLO is important in the negative regulation of B-cell
differentiation. IFN-γ, a factor that sensitizes apoptosis
induces de novo synthesis of Smac/DIABLO and its
release in the cytosol [131]. This sensitization of apoptosis
by Smac/DIABLO has been exploited in experimental
therapeutic models. Transfection of full-length smac
cDNA or administration of peptides based on Smac’s
IBM motif sensitizes various tumor cells in vitro and
malignant glioma cells in vivo for TRAIL- and cytotoxic
drugs-induced apoptosis [132].

Omi/HtrA2, a mammalian serine protease residing in
the mitochondria, was identified as a 49-kD protein [133,
134], which shows homology with the bacterial endopro-
tease DegP/HtrA (high-temperature requirement), sup-
porting the mitochondrial endosymbiosis theory [4].
Omi/HtrA2 seems to be involved in conditions of cellular
stress as it is upregulated after heat shock, in ischemia-
reperfusion and during ER stress [133, 134]. The 49-kD
precursor Omi/HtrA2 contains an N-terminal mitochon-
drial signal sequence and is processed to the 37-kD
mature form which is released into the cytosol after an
apoptotic trigger, where it contributes both to caspase-
dependent and caspase-independent cell death [103, 135–
140]. Like Smac/DIABLO, mature Omi/HtrA2 contains
an N-terminal IBM motif allowing it to compete for the
interaction between IAPs and caspases. In contrast to
Smac/DIABLO that preferentially binds to the BIR3 do-
main of XIAP, Omi/HtrA2 has a higher affinity for the
BIR2 domain of XIAP [136, 137, 140]. Whether Omi/
HtrA2 is also target for IAP-mediated ubiquitination and
proteasome-dependent degradation is currently un-
known. The catalytic serine protease moiety of Omi/
HtrA2 is followed by a C-terminal PDZ (postsynaptic
density protein, disc large tumor suppressor and Zo-1
tight junction protein) domain [134], a domain generally
involved in protein-protein interactions that serves as an
intra-molecular sensitive regulator of the protease activity
[141]. The catalytic activity is indispensable for the cas-
pase-independent cytotoxic activity of Omi/HtrA2, i.e.
separate from its IAP antagonism [136, 137, 141]. Omi/
HtrA2 has also been shown to compete with caspases for
binding to XIAP. Although Omi/HtrA2 and Smac/DIA-
BLO have similar features, both proteins have a distinct
tissue distribution pattern. Smac/DIABLO is most abun-
dant in heart, liver, kidney and testis with little or no
expression detected in skeletal muscle, lung, thymus and
brain [117, 118], whereas Omi/HtrA2 is expressed ubi-
quitously [133, 134]. This may suggest a redundant func-
tion of Smac/DIABLO and Omi/HtrA2 in certain cell
types. Until now, no specific physiological substrates or
targets for the catalytic activity and the PDZ domain of
Omi/HtrA2 have been described. Therefore, the precise
function of Omi/HtrA2 in cell death remains elusive.

Apoptosis-Inducing Factor (AIF) and
Endonuclease G, Mitochondrial Mediators of
Nuclear Chromatin Condensation and DNA
Fragmentation

The mammalian mitochondrial protein AIF was iden-
tified as a 57-kD flavoprotein sharing homology with bac-
terial, plant and fungal oxidoreductases [142]. AIF bear-
ing both mitochondrial and nuclear signal sequences is
normally confined to the mitochondrial IMS or, as argued
by Arnoult et al. [143], peripherally associated with the
IMM, but translocates to the nucleus in response to apop-
togenic stimuli. In the nucleus, AIF is implicated in large-
scale (~ 50 kb) DNA fragmentation and peripheral chro-
matin condensation, but not in oligonucleosomal DNA
laddering [142, 144]. Beside nuclear effects, overexpres-
sion of AIF induces other typical characteristics of apo-
potic cell death, such as exposure of phosphatidylserine
on the plasma membrane and the dissipation of vγm
[142]. How AIF exerts its cytotoxic activity remains elu-
sic. AIF does not contain intrinsic nuclease activity and
its oxidoreductase activity is not required for its apop-
togenic function, but rather may even prevent cell death by
oxygen radical scavenging [144, 145]. AIF appears to be
involved in PCD very early in mammalian development
as aif-null mice die at a very early stage during embroyogen-
esis [115]. It is unclear to what extent the phenotype of
aif-deficient mice can be attributed to cell death associat-
ed or other functions of AIF. Embryonic stem (ES) cells
from aif-deficient animals were shown to be sensitive to most conventional apoptotic triggers, such as etoposide, staurosporin and UV irradiation. They are nevertheless resistant to growth factor deprivation-induced cell death [115]. AIF-dependent cell death can be genetically uncoupled from Apaf-1 and caspase-9 expression as was shown in Apaf-1−/− and caspase-9−/− ES cells, indicating that AIF acts independent of the apoptosome [115]. The existence of AIF homologs in plants and fungi suggests that the role of AIF in cell death may represent a caspase-independent ancestral pathway [146]. AIF-homologous mitochondrion-associated inducer of cell death (AMID) is an AIF-homologous flavoprotein [147] that is presumably associated with the OMM. Overexpression of AMID in 293T cells induced apoptotic cell death, which could not be inhibited by Bel-2, CrmA or z-VAD-fmk. Although the molecular action mechanism of AMID-induced cell death is unclear, caspases are also dispensable.

endonuclease G, a 30-kD protein containing a mitochondrial signal sequence removed after import into IMS, is encoded by a nuclear gene [148, 149]. Originally, endonuclease G was described as a protein involved in mitochondrial DNA replication, however as a recent report demonstrates the exclusive presence of endonuclease G in the IMS, its role in replication becomes less likely [150]. Endonuclease G is released from mitochondria during apoptosis [148, 149] and subsequently translocates to the nucleus, where it is involved in nuclear DNA breakdown independent of caspase activity or the caspase-activated deoxyribonuclease CAD/DFF40. In healthy cells, CAD/DFF40 is tethered in the cytosol by its inhibitor ICAD/DFF40. Apoptosis stimuli lead to a caspase-3-mediated cleavage of ICAD, translocation of CAD to the nucleus and degradation of nuclear DNA [151, 152]. However, icad/dff45-deficient mice or transgenic mice lacking the ICAD-caspase-3 cleavage site are phenotypically normal [153]. The same holds true for caspase-9–/– mice [154]. Nevertheless, caspase-independent DNA fragmentation in vivo when phagocytosed by phagocytes [153, 154]. However, these mutant thymocytes undergo DNA fragmentation in vivo when phagocytosed by macrophages, most likely by the lysosomal DNase II from the phagocyte [154]. The same holds true for cd–/– thymocytes [155]. Embryonic fibroblasts from icad/dff45-deficient mice still show residual DNA fragmentation after induction of apoptosis by TNF or UV radiation [148, 153, 154]. This indicates that CAD is not the only DNase implicated in apoptosis. CAD has also been shown dispensable for the early stage chromatin condensation or high molecular weight DNA fragmentation using a cad-deficient cell line [156]. It has been questioned if endonuclease G could account for this CAD-independent DNA fragmentation. Wang et al. [157] described endonuclease G-mediated DNA laddering of isolated nuclei in contrast to our observations of higher order DNA degradation [149]. In addition, in in vitro assays a higher concentration of endonuclease G is required to induce DNA degradation as compared to CAD/DFF40 [148, 152]. This suggests that endonuclease G alone may not be sufficient but may act in concert with other nucleases or cofactors such as exonucleases and DNaseI to degrade the DNA [157]. What is the relation between AIF and endonuclease G-induced DNA degradation? Both proteins may act together to ensure nuclear degradation when caspase-mediated DNA breakdown is compromised and they may be responsible for the caspase-independent DNA degradation observed in plants, fungi and protozoa [146, 158]. In this respect, recent genetic studies in C. elegans have provided evidence for a cooperative role of the endonuclease G ortholog cps-6 and the AIF ortholog waf-1 in the breakdown of DNA during developmental cell death [149, 159, 160]. As the CAD ortholog is lacking in C. elegans, this caspase-independent Cps-6/WAF-1-mediated DNA degradation may represent an ancestral pathway.

Other Mitochondrial Factors

The role of other mitochondrial factors [103, 125] released from tBid-treated mitochondria is still elusive. Fatty acid-binding protein, which binds free acids and thus may be implicated in intracellular lipid transport, could be linked to the phospholipid transfer role of tBid [161]. Polypyrimidine tract-binding (PTB) protein is an RNA-binding protein necessary for efficient translation of some mRNAs, which contain internal ribosomal entry sites (IRESs). Mitchell et al. [162] demonstrated the involvement of PTB in the activation of the Apaf-1 IRES. Mitochondrial localization of PTB can be questioned as the protein lacks a mitochondrial localization signal. Acyl-CoA-binding protein (ACBP) is a 20-kD monomer capable of activating m-calpain by decreasing the Ca2+ concentration required for the activation of m-calpain [163]. As mentioned earlier, calpain can proteolytically activate Bid [62] and ACBP may thus affect mitochondrial integrity in the absence of caspase activity. In certain cell types the presence of procaspases (-2, -3, -8, -9) in the IMS has been reported [164–168], however we could not identify any procaspase or active caspase in the purified mitochondria from different cell types using Western
blotting neither in apoptotic nor in non-dying conditions [169]. Moreover, except for procaspase-2, none of the caspases seems to contain a mitochondrial localization signal [169] questioning the possibility that procaspases get into mitochondria.

**How Are Mitochondrial Intermembrane Space Proteins Released?**

The different models that have been proposed to explain how cytochrome c is released from the IMS during apoptosis can be divided into two main paradigms. The first one encompasses two models sharing the prediction of OMM rupture as a result of swelling of the mitochondrial matrix. The second paradigm suggests the formation of a pore in the OMM, large enough to allow passage of cytochrome c and other IMS proteins. One model of the first paradigm invokes loss of the IMM permeability barrier as the cause of matrix swelling. Mitochondria are known to be endowed with a latent mechanism called the mitochondrial permeability transition (MPT), involving the opening of a non-specific pore, known as the mitochondrial PTP, formed at the contact sites between IMM and OMM [170]. Opening of the PTP allows free flow between the cytosol and the mitochondrial matrix of any small molecule (<1,500 daltons), including protons. An important consequence of an activated MPT is uncoupling of oxidative phosphorylation (see above) whereas the entry of water and solutes will induce swelling of the mitochondrial matrix. The core components of the PTP are the voltage-dependent anion channel (VDAC) in the OMM, which makes the OMM permeable to most small molecules (<5 kD) and the adenine nucleotide translocator (ANT) found in the IMM [170] (fig. 2B). Bcl-2 family members have been shown to regulate the opening of the PTP by interacting with the main components of the pore [171, 172]. The second model of the first paradigm proposes closure instead of opening of the VDAC. This closure leads to hyperpolarization of the IMM and subsequent damage of the OMM due to defective exchange of ATP between mitochondria and cytosol [173]. Bcl-xL, e.g., is able to prevent this VDAC closure and OMM damage [174].

For some stimuli, it has however been shown that opening of the PTP occurs after cytochrome c release and caspase activation [102, 175] and some reports mentioned that cells committed to die still exhibit a mitochondrial transmembrane potential ($\Delta \psi_m$), questioning the paradigm discussed above. A second paradigm proposed therefore the formation of a specific channel in the OMM rather than total OMM rupture. This hypothesis was confirmed by the observation that during apoptosis induced by a variety of stimuli, all cytochrome c is released from mitochondria with a normal $\Delta \psi_m$ within 5 min. The caspase-dependent drop in $\Delta \psi_m$ follows cytochrome c release, supporting the formation of an OMM channel prior to disturbance of the IMM [176]. Loss of cytochrome c without a drop in $\Delta \psi_m$ could be explained by the postulation of Mootha et al. [177] that cytochrome c can freely re-enter the IMS and participate in electron transport to maintain the $\Delta \psi_m$. Another possibility is that not all cytochrome c is released at a time. The initially released pool of cytochrome c might be the loosely bound or soluble fraction from the IMS whereas the second pool might comprise the fraction more tightly associated with the IMM and participating in electron transport [178, 179].

The formation of these specific channels is predominantly mediated by Bax-like death factors. Bax and Bak can insert into mitochondrial membranes and form oligomers in vitro on liposomes and isolated mitochondria [180, 181] and recent electron microscopy studies showed that Bax and Bak come together in large aggregates on the surface of apoptotic mitochondria [182].

Other channel-forming models propose the cooperation between Bax and VDAC or ANT to form lipid or protein-lipid complexes [171, 172, 181, 183]. Bid may also display channel-forming activity by itself [184, 185] as the lipid transfer activity of Bid as proposed by Esposti [161, 186] may contribute to a change in lipid composition of the OMM. However, studies on cells derived from bax and bak DKO mice suggest that Bid acts in concert with Bax and Bak by triggering their oligomerization, resulting in the release of cytochrome c [37, 48]. Cardiolipin has been shown to be necessary for tBid/Bax-mediated permeabilization [64, 187]. A study on isolated mitochondria provided evidence that the amount of cytochrome c released from mitochondria is determined by the amount of cardiolipin peroxidation and the degree to which the electrostatic interaction between cytochrome c and cardiolipin is disrupted [178, 179].

The mechanisms underlying mitochondrial cytochrome c release might also be applicable to the release of other IMS proteins. On the other hand, Martin and colleagues [188] demonstrated that release of cytochrome c from the IMS is caspase-independent whereas release of Smac/DIABLO is caspase-dependent and occurs downstream of cytochrome c release, in response to different cell death stimuli. This suggests that the cytochrome c-releasing pore may differ from the pore releasing Smac/
DIABLO which could be due to the size of the released proteins, respectively ~15 and ~50 kD as Smac/DIABLO forms dimers [189].

A Mitochondrio-Centric View on the Apoptotic Universe, a Too Dogmatic Paradigm?

Mitochondria are generally described as key players in the apoptotic process. Recent studies, using knockout cells and mice, rule out an essential role for Apaf-1 and caspase-9 in stress-induced death. Some Apaf-1–/– mice become healthy adults [190], bax–/–bak–/– fibroblasts are more resistant to cytotoxic insults than those lacking Apaf-1 or caspase-9 [191] and the absence of cytochrome c only attenuates stress-induced apoptosis in cell lines established from early cytochrome c–/– embryos [113]. In addition, Marsden et al. [192] showed that apoptosome formation is dispensable for hematopoietic homeostasis, using an in vivo reconstitution model consisting of fetal liver stem cells from Apaf-1 or caspase-9 knockout mice. The absence of Apaf-1 or caspase-9 does not increase lymphocyte numbers in vivo, and lymphocytes and embryonic fibroblasts die at normal rates in response to diverse death stimuli. However, as Bcl-2 overexpression in the haematopoietic system results in increased numbers of lymphocytes and resistance to many apoptotic stimuli [192, 193], Bcl-2 may act as a regulator of caspase activation independent of the apoptosome. The cytochrome c-dependent formation of the apoptosome may not be an essential trigger but rather an amplifier of the caspase cascade [31, 192]. In Saccharomyces cerevisiae, cytochrome c is only implicated in oxidative phosphorylation and incapable of initiating apoptosis in a cell-free system [194]. Furthermore, although Drosophila has an Apaf-1 ortholog with WD-40 repeats (DARK), caspase activation in the fly does not seem to require cytochrome c [195]. As the apoptosome does not seem not to be essential in the intrinsic death pathway, one can speculate there will be (an)other initiator caspase(s) acting upstream of mitochondria instead of the downstream caspase-9 [196]. Lassus et al. [197] proposed caspase-2 as a potential candidate. Although caspase-2 knockouts have no remarkable phenotype, caspase-2 seemed to be necessary for DNA damage-induced apoptosis in E1A-transformed fibroblasts as ablation of caspase-2 prevents cytochrome c and Smac/DIABLO release in these cells as well as translocation of Bax to mitochondria. Taken together, these findings demonstrate the importance of other mitochondrial apoptogenic proteins than cytochrome c. Some of them like Smac/DIABLO and Omi/HtrA2 may, next to a caspase-dependent pathway, also initiate a caspase-independent one, while others like AIF and endonuclease G initiate caspase-independent cell death pathways. However, the profound phenotype of Bax/Bak DKO may also suggest that other target organelles and released factors thereof may be implicated.

In most reports, pro- and anti-apoptotic functions of Bcl-2 members are described in function of their mitochondrial localization. However, it is also important to pay attention to other subcellular distributions of Bcl-2 family members. As mentioned above, the majority of Bcl-2 is found at other subcellular locations such as nuclear envelope and ER [98, 99]. Bel-XL and Bcl-2 have been found in complex with procaspase-8 and Bap31 in the vicinity of the ER [198], and Bax and Bak are also associated with the Golgi apparatus, ER and nuclear envelope [100, 101, 199]. In that respect, it was shown that Bax and Bak operate at the ER as well as mitochondria as an essential gateway for selected apoptotic signals [101]. These data suggest that other organelles than mitochondria are implicated in apoptotic pathways independent or in concert with mitochondrial factors.

Conclusions

Next to their major role in energy metabolism, mitochondria exert a role in apoptosis by the release of IMS proteins, although this mitochondrial contribution to apoptosis may not be as exclusively as often suggested. BH3-only death proteins, crucial sensors of cellular integrity, converge most signals on the mitochondria with subsequent activation of Bax/Bak death factors that impair mitochondrial integrity. BH3-only proteins can bind to anti-apoptotic members of the Bcl-2 family but how they neutralize their anti-apoptotic function is still uncertain. Again, not all functions of the anti-apoptotic Bcl-2 family members can be attributed to mitochondria, suggesting interplay between different organelles during the cell death process. Upon disruption of the OMM, a set of apoptogenic proteins is released, which can either in a caspase-dependent or caspase-independent way lead to apoptosis. Cytochrome c–mediated apoptosome formation seems to be dispensable for most stress-induced signals and haematopoietic homeostasis. Released AIF and endonuclease G propagate DNA fragmentation independent of caspases. Smac/DIABLO and Omi/HtrA2 share an IBM motif leading to competition with IAP-mediated inhibition of caspases but also induce caspase-indepen-
dent cell death. The mechanisms contributing to the release of IMS proteins are still unresolved. However, despite these impressive data on the molecular mechanisms of mitochondrial factors in caspase-dependent and caspase-independent processes, in general, a picture is emerging that other organelles besides mitochondria, including ER, lysosomes and the Golgi apparatus are also major points of integration of pro-apoptotic signaling or damage sensing [100]. The apoptotic regulatory systems operating at the different membrane sites may either respond differentially to individual stimuli or may cooperate to elicit apoptotic signals.

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References


Habe: B91: ZAHAE396XA.4FF: ZUF9 E1: 11X2

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Habe: B91: ZAHAE396XA.4FF: ZUF9 E1: 11X2


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Bcl-2 Family Members as Sentinels of Cellular Integrity


