The biochemistry of apoptosis

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Apoptosis — the regulated destruction of a cell — is a complicated process. The decision to die cannot be taken lightly, and the activity of many genes influence a cell's likelihood of activating its self-destruction programme. Once the decision is taken, proper execution of the apoptotic programme requires the coordinated activation and execution of multiple subprogrammes. Here I review the basic components of the death machinery, describe how they interact to regulate apoptosis in a coordinated manner, and discuss the main pathways that are used to activate cell death.

Multicellular animals often need to get rid of cells that are in excess, in the way, or potentially dangerous. To this end, they use an active dedicated molecular programme. As important as cell division and cell migration, regulated (or programmed) cell death allows the organism to tightly control cell numbers and tissue size, and to protect itself from rogue cells that threaten homeostasis.

Discovered and rediscovered several times by various developmental biologists and cytologists, programmed cell death acquired a number of names over the past two centuries[1]. The term finally adopted is apoptosis, coined by Currie and colleagues in 1972 to describe a common type of programmed cell death that the authors repeatedly observed in various tissues and cell types[2]. The authors noticed that these dying cells shared many morphological features, which were distinct from the features observed in cells undergoing pathological, necrotic cell death, and they suggested that these shared morphological features might be the result of an underlying common, conserved, endogenous cell death programme[3].

Caspases: the central executioners

Most of the morphological changes that were observed by Kerr *et al.* are caused by a set of cysteine proteases that are activated specifically in apoptotic cells. These death proteases are homologous to each other, and are part of a large protein family known as the caspases[4]. Caspases are highly conserved through evolution, and can be found from humans all the way down to insects, nematodes and hydra[5–7]. Over a dozen caspases have been identified in humans; about two-thirds of these have been suggested to function in apoptosis[7,8].

All known caspases possess an active-site cysteine, and cleave substrates at Asp-Xxx bonds (that is, after aspartic acid residues); a caspase's distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site[9]. Caspases have been subdivided into subfamilies based on their substrate preference, extent of sequence identity and structural similarities.

Because they bring about most of the visible changes that characterize apoptotic cell death, caspases can be thought of as the central executioners of the apoptotic pathway. Indeed, eliminating caspase activity, either through mutation or the use small pharmacological inhibitors, will slow down or even prevent apoptosis[7]. Thus, blocking caspases can rescue condemned cells from their apoptotic fate — a fact that has not escaped the notice of the pharmaceutical industry (see review in this issue by Nicholson, pages 810–816).

It slices, it dices, and that's not all!

What exactly do the caspases do that is so important for apoptosis? Activation of caspases does not result in the wholesale degradation of cellular proteins. Rather, caspases selectively cleave a restricted set of target proteins, usually at one, or at most a few positions in the primary sequence (always after an aspartate residue). In most cases, caspase-mediated 'protein surgery' results in inactivation of the target protein (Box 1). But caspases can also activate proteins, either directly, by cleaving off a negative regulatory domain, or indirectly, by inactivating a regulatory subunit (Box 1).

Several important caspase substrates have been identified in recent years. One of the more exciting discoveries has been the elucidation of the mechanism of activation of the nuclease responsible for the famous nucleosomal ladder. First described by Wyllie[10], this nuclease cuts the genomic DNA between nucleosomes, to generate DNA fragments with lengths corresponding to multiple integers of approximately 180 base pairs. The presence of this DNA ladder has been used (and abused) extensively as a marker for apoptotic cell death.

In an elegant series of experiments, the groups of Wang and Nagata showed that the DNA ladder nuclease (now known as caspase-activated DNase, or CAD) pre-exists in living cells as an inactive complex with an inhibitory subunit, dubbed ICAD (ref. 11). Activation of CAD occurs by means of caspase-3-mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit[12–14].

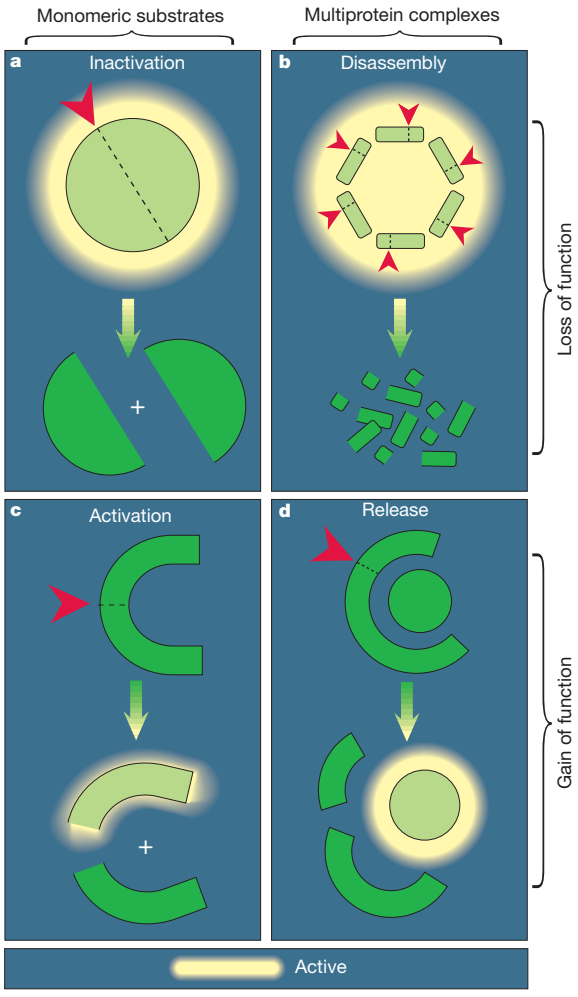
Caspase-mediated cleavage of specific substrates also explains several of the other characteristic features of apoptosis. For example, cleavage of the nuclear lamins is required for nuclear shrinking and budding[15,16]. Loss of overall cell shape is probably caused by the cleavage of cytoskeletal proteins such as fodrin and gelsolin[17]. Finally, caspase-mediated cleavage of PAK2, a member of the p21-activated kinase family, seems to mediate the active blebbing observed in apoptotic cells. Interestingly, in this last case, caspase cleavage occurs between the negative regulatory subunit and the catalytic subunit, and results in a constitutive activation of PAK2 (ref. 18).

Close to 100 additional caspase substrates have been reported over the years, and there will certainly be many more[7,19]. Why are there so many substrates? Perhaps apoptosis is just much more complicated that we currently believe. Indeed, several of the key apoptotic subprogrammes, such as cell shrinking and the emission of pro-engulfment signals (see review in this issue by Savill and Fadok, pages 784–788), are still poorly understood. Alternatively, it is possible that many of the described caspase substrates are not relevant substrates, but simply 'innocent bystanders' that get caught in the action.

Box 1

Camera, lights, action. Cut! Outcome of caspase activity

Proteolytic cleavage by caspases can lead to diverse results, depending on the nature of the substrate and the exact position of the cleavage site in the primary sequence. The simplest, and probably most frequent outcome is loss of biological activity (panels **a**, **b** in the figure below). Caspase substrates range from single polypeptide chain enzymes (for example, polyADP-ribose polymerase) to complex macromolecular structures (for example, the lamin network). Limited proteolysis by caspases can also result in a gain of biological activity (**c**, **d**). In some cases (for example, Bcl-2 or Bcl-x_L), the cleaved products antagonize the full-length protein (dominant-negative forms). In other cases, removal of inhibitory domains or subunits results in increased biological activity (for example, PAK2, Bid and CAD/ICAD).



According to this line of reasoning, there might be little selection against the presence of fortuitous caspase cleavage sites on irrelevant proteins, as the cell is about to stop functioning anyway. Further experimentation might allow this issue to be resolved.

How to activate a caspase

Given the great importance of caspases in the apoptotic process, it is reasonable to propose that a proper understanding of apoptosis will require us to understand how caspases are activated.

As is true of most proteases, caspases are synthesized as enzymatically inert zymogens. These zymogens are composed of three domains: an N-terminal prodomain, and the p20 and p10 domains, which are found in the mature enzyme. In all cases examined so far, the mature enzyme is a heterotetramer containing two p20/p10 heterodimers and two active sites[7]. Although much has been made about the fact that active caspases are dimers containing two active sites, there is no obvious structural reason why this should be so, and it seems quite possible that caspases could exist as active monomers under the right conditions.

Three general mechanisms of caspase activation have been described so far. Each of them is described briefly below (see also Box 2).

Processing by an upstream caspase

Most caspases are activated by proteolytic cleavage of the zymogen between the p20 and p10 domains, and usually also between the prodomain and the p20 domain. Interestingly, all these cleavage sites occur at Asp-X sites — candidate caspase substrate sites — suggesting the possibility of autocatalytic activation[9]. Indeed, the simplest way to activate a procaspase is to expose it to another, previously activated caspase molecule (Box 2). This 'caspase cascade' strategy of caspase activation is used extensively by cells for the activation of the three short prodomain caspases, caspase-3, -6 and -7. These three downstream effector caspases are considered the workhorses of the caspase family, and are usually more abundant and active than their long prodomain cousins.

The caspase cascade is a useful method to amplify and integrate pro-apoptotic signals, but it cannot explain how the first, most upstream caspase gets activated. At least two other approaches are used to get the ball rolling.

Induced proximity

Caspase-8 is the key initiator caspase in the death-receptor pathway (see review in this issue by Krammer, pages 789–795). Upon ligand binding, death receptors such as CD95 (Apo-1/Fas) aggregate and form membrane-bound signalling complexes (Box 3). These complexes then recruit, through adapter proteins, several molecules of procaspase-8, resulting in a high local concentration of zymogen. The induced proximity model posits that under these crowded conditions, the low intrinsic protease activity of procaspase-8 (ref. 20) is sufficient to allow the various proenzyme molecules to mutually cleave and activate each other (Box 2). A similar mechanism of action has been proposed to mediate the activation of several other caspases, including caspase-2 and the nematode caspase CED-3 (ref. 21). Although forced crowding of zymogens clearly is sufficient in many cases to activate caspases[22], it is a rather crude a way to control the fate of a cell. Whereas the basic concept is probably correct, additional levels of regulation surely must exist *in vivo* to modulate the process.

Association with a regulatory subunit

The most complex activation mechanism described so far is the one used by caspase-9. Unlike other caspases, proteolytic processing of procaspase-9 has only a minor effect of the enzyme's catalytic activity[23,24]. Rather, the key requirement for caspase-9 activation is its association with a dedicated protein cofactor, Apaf-1 (Box 2).

Apaf-1 was identified through a biochemical approach as one of two proteins that are required for caspase-9 activation (the other being cytochrome *c*; see below)[25,26]. Initially believed to be required only transiently, for caspase-9 activation, the Apaf-1/caspase-9 complex is now thought to actually represent the true active form of caspase-9 (ref. 23). Thus, we must view Apaf-1 not simply as a caspase-9 activator, but rather as an essential regulatory subunit of a caspase-9 holoenzyme. This holoenzyme — often referred to as the apoptosome — is a very large complex that might well contain several additional proteins[27–29].

In summary, effector caspases are usually activated proteolytically by an upstream caspase, whereas initiator caspases are activated through regulated protein–protein interactions. The actual molecular mechanisms mediating initiator caspase activation are still unclear and, most likely, much more complex that currently understood.

Regulated protein–protein interactions are in fact one of the underlying themes in apoptosis, and whole caspase activation pathways can be drawn without ever invoking a single enzyme (Box 3). I describe below some of the more commonly encountered interaction modules.

The handshakes that seal the fate

Each of the long-prodomain caspases contains in its prodomain a protein–protein interaction module, which allows it to bind to and associate with its upstream regulators. Caspase-8 and -10 contain a death-effector domain (DED), whereas caspase-2 and -9 contain a caspase activation and recruitment domain (CARD). These two domains share little sequence identity, but fold into very similar three-dimensional structures, consisting of six antiparallel α-helices arranged in a Greek key configuration[30]. The same fold is also found in the death domain, a third protein interaction module present in several upstream regulators of apoptosis, such as CD95 and the adapter molecular FADD (ref. 31). It seems likely that the death domain, DED and CARD are derived from a common ancestral domain[30].

The structure of the death domain, DED and CARD perfectly suits their function. The antiparallel helices bundle into a tight core, leaving exposed large surfaces onto which evolution has carved extended protein–protein interaction domains. The particular face of the module that is used for interaction varies greatly from one protein to the next[31–33]. Work so far suggests that the death adaptor modules usually mediate intrafamily interactions (that is, death domain/death domain, DED/DED and CARD/CARD). However, structural analyses show that there is enough surface area left on death domains, DEDs and CARDs to also interact with other proteins. Indeed, death adaptor modules might well act as integration platforms, binding to several different proteins, which could modulate their dimerization and hence caspase activation.

Keep your friends close, but keep your enemies closer

Regulated protein–protein interactions are also key to the understanding of a second set of apoptotic regulators, the Bcl-2 family. This family has been divided into three groups, based on structural similarities and functional criteria (Box 4). Members of group I possess anti-apoptotic activity, whereas members of groups II and III promote cell death.

How do Bcl-2 family members control cell death? Bcl-2 family members seem to spend most of their time simply trying to block each other's next move. Many family members can homodimerize, but more importantly, pro- and anti-apoptotic members can form heterodimers[34–36]. Because each Bcl-2 family member can interact with several other different members, large numbers of heterodimer combinations within a cell are possible. To a first approximation, heterodimerization can simply be thought as resulting in mutual neutralization of the bound pro- and anti-apoptotic proteins. Thus, the problem collapses into comparing overall levels of pro- and anti-apoptotic family members: cells with more pro-death proteins are sensitive to death; cells with an excess of protective family members are usually resistant.

But Bcl-2 proteins clearly will need to do more than just talk to each other if they are to influence cell death. What is the ultimate output from all these interactions? In the nematode *Caenorhabditis elegans*, the anti-apoptotic Bcl-2 homologue CED-9 protects cells from death by directly binding to and sequestering the Apaf-1 homologue CED-4 (ref. 37). Although this is an appealing scenario, a similar interaction has been very difficult, if not impossible, to detect in mammals, at least not under the conditions tested so far[38–40]. Rather, the key function of Bcl-2 family members seems to be to regulate the release of pro-apoptotic factors, in particular cytochrome *c*, from the mitochondrial intermembrane compartment into the cytosol[35,36].

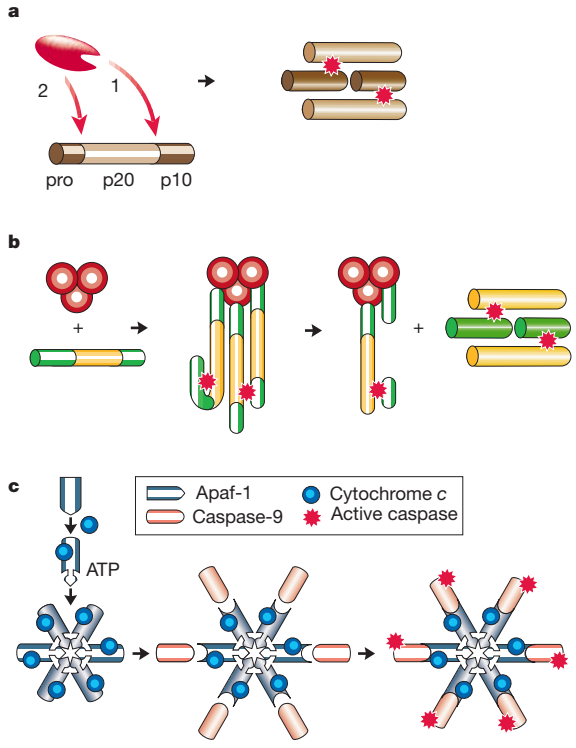
Mitochondria — the forum of death

The mitochondrion is not only the cell's powerhouse, it is also its arsenal. Mitochondria sequester a potent cocktail of pro-apoptotic proteins. Most prominent among these is cytochrome *c*, the humble electron carrier. Work over the past few years has revealed that cytochrome *c* is far from innocuous — in addition to its involvement in mitochondrial oxidative phosphorylation, the protein is one of the components (in addition to the adaptor protein Apaf-1) required for activation of caspase-9 in the cytosol[25].

Box 2

More than one way to skin a cat: mechanisms of caspase activation

Mechanisms of caspase activation include proteolytic cleavage by an upstream caspase (panel **a** in the figure below), induced proximity (**b**) and holoenzyme formation (**c**). Proteolytic cleavage by an upstream caspase is straightforward and effective, and is used mostly for activation of downstream, effector caspases. It is probably also used for induction of apoptosis by non-caspase proteases, such as granzyme B (see review in this issue by Krammer, pages 789–795). In the second mechanism, recruitment or aggregation of multiple procaspase-8 molecules into close proximity somehow results in cross-activation. The actual process is most probably more sophisticated and more tightly regulated than shown in panel **b**. In holoenzyme formation, cytochrome *c* and ATP-dependent oligomerization of Apaf-1 allows recruitment of procaspase-9 into the apoptosome complex. Activation of caspase-9 is mediated by means of conformational change, not proteolysis. Stoichiometry of the apoptosome is not known; it is shown in panel **c** as a hexamer solely for aesthetic reasons.



Exactly how cytochrome *c* manages to cross the mitochondrial outer membrane is not yet known, but it is clear that the Bcl-2 family is intimately involved in the regulation of this process. For example, addition of pro-apoptotic Bcl-2 family members to isolated mitochondria is sufficient to induce cytochrome *c* release, whereas overexpression of Bcl-2 family members will prevent it[35,36,41].

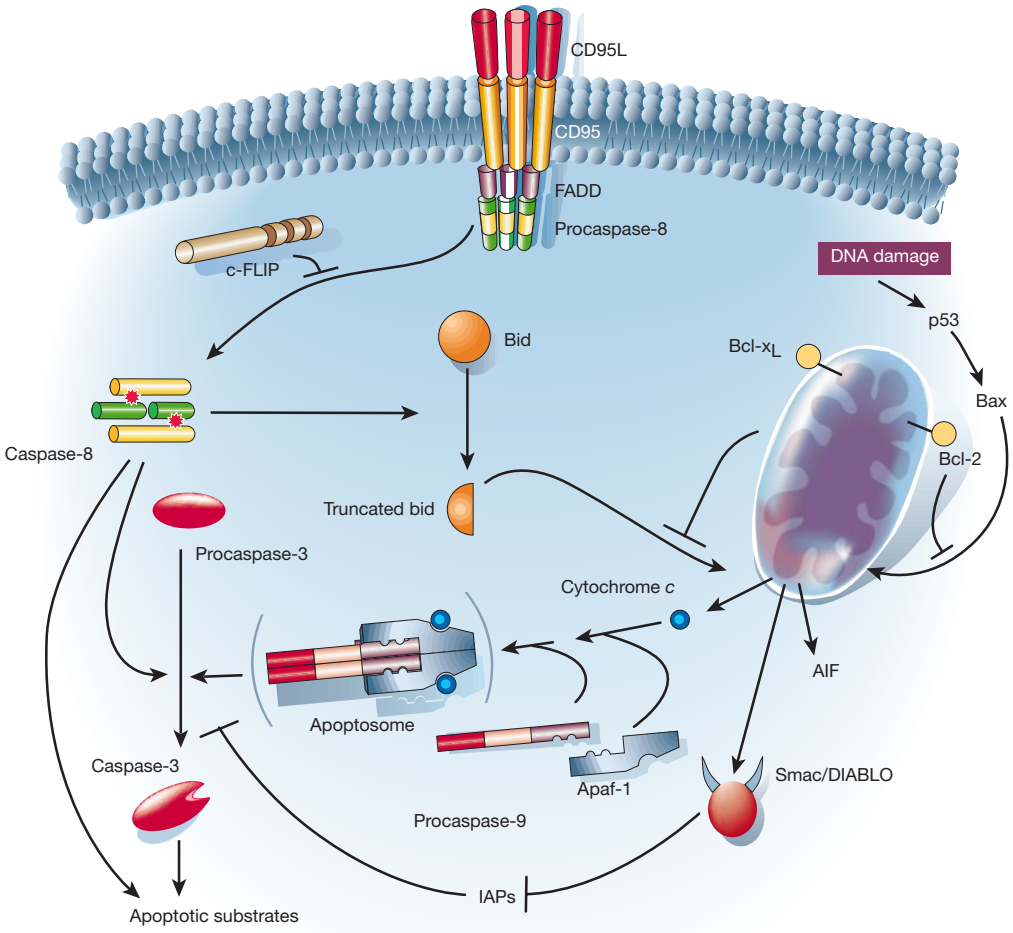
How do Bcl-2 family members regulate cytochrome *c* exit? Several competing hypotheses have been advanced (Box 5); none of them has been proven definitively[34–36,41]. The three basic models are as follows.

Bcl-2 members form channels that facilitate protein transport

Based on the structural similarity of Bcl-x_L to the pore-forming subunit of diphtheria toxin[42], it has been suggested that Bcl-2 proteins might act by inserting, following a conformational change, into the outer mitochondrial membrane, where they could form channels or even large holes. Bcl-2 family members indeed can insert into

Box 3

The roads to ruin: two major apoptotic pathways in mammalian cells



The death-receptor pathway (left pathway in the figure opposite) is triggered by members of the death-receptor superfamily (such as CD95 and tumour necrosis factor receptor I). Binding of CD95 ligand to CD95 induces receptor clustering and formation of a death-inducing signalling complex. This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity (see Box 2). Caspase-8 activation can be blocked by recruitment of the degenerate caspase homologue c-FLIP (ref. 61).

The mitochondrial pathway (right) is used extensively in response to extracellular cues and internal insults such as DNA damage (see review in this issue by Rich *et al.*, pages 777–783). These diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family. Unlike Bcl-2, which seems to spend most if not all of its life attached to intracellular membranes, many group II and group III members, including Bax, Bad, Bim and Bid, can shuttle between the cytosol and organelles[62–65]. The cytosolic forms represent pools of inactive, but battle-ready proteins. Pro-apoptotic signals redirect these proteins to the mitochondria, where the fight for the cell's fate will take place. Activation of pro-apoptotic members can occur through proteolysis, dephosphorylation and probably several other mechanisms[35,36].

Pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate cytochrome *c* exit by a mechanism that is still debated (see text). If the pro-apoptotic camp wins, an array of molecules is released from the mitochondrial compartment. Principal among these is cytochrome *c*, which associates with Apaf-1 and then procaspase-9 (and possibly other proteins) to form the apoptosome. Heat-shock proteins act at multiple steps in the pathway to modulate apoptosis (not shown; see refs. 66, 67).

The death-receptor and mitochondrial pathways converge at the level of caspase-3 activation. Caspase-3 activation and activity is antagonized by the IAP proteins, which themselves are antagonized by the Smac/DIABLO protein released from mitochondria. Downstream of caspase-3, the apoptotic programme branches into a multitude of subprogrammes, the sum of which results in the ordered dismantling and removal of the cell.

Cross-talk and integration between the death-receptor and mitochondrial pathways is provided by Bid, a pro-apoptotic Bcl-2 family member. Caspase-8-mediated cleavage of Bid greatly increases its pro-death activity, and results in its translocation to mitochondria, where it promotes cytochrome *c* exit. Note that under most conditions, this cross-talk is minimal, and the two pathways operate largely independently of each other[62,68].

Clearly, additional death-inducing pathways must exist, as developmental apoptosis is by and large normal in mice defective in the caspase-8 and caspase-9 pathways[7,52].

synthetic lipid bilayers, oligomerize, and form channels with discrete conductances[34]. But it unclear whether such channels would ever be big enough for proteins to pass through.

Bcl-2 members interact with other proteins to form channels

Bcl-2 family members interact with many proteins[34]. One possibility is that pro-apoptotic family members recruit other mitochondrial outer membrane proteins into forming a large pore channel. A particularly attractive candidate for such a protein is the voltage-dependent anion channel (VDAC), as several Bcl-2 family members can bind to it and regulate its channel activity[43]. As the characterized pore size of the VDAC channel is too small to allow proteins to pass through, this model must assume that VDAC undergoes a significant conformational change upon binding to Bcl-2 family members.

Bcl-2 members induce rupture of the outer mitochondrial membrane

It is possible that the Bcl-2 family members control homeostasis of the mitochondria. In this model, apoptotic signals alter mitochondrial physiology (for example, ion exchange or oxidative phosphorylation) such that the organelle swells, resulting in the physical rupture

of the outer membrane and release of intermembrane proteins into the cytosol. The need to form a channel large enough for cytochrome *c* to pass through is thereby neatly bypassed as proteins can be assumed to simply diffuse through the tears in the lipid bilayer.

Mitochondrial homeostasis could be influenced directly by the Bcl-2 family members (for example, through the proposed intrinsic ion-channel activity mentioned above) or indirectly, through modulation of other mitochondrial proteins. The VDAC protein again is a prominent candidate for such regulation, as it is a subunit of the mitochondrial permeability transition pore (PTP), a large channel whose opening results in rapid loss of membrane potential and organellar swelling. Opening of the PTP quickly leads to cytochrome *c* release and apoptotic cell death, and pharmacological inhibitors of the PTP can act as potent inhibitors of cytochrome *c* release, and hence prevent apoptosis[44]. However, cytochrome *c* exit can also occur in the absence of membrane potential loss[41,44], suggesting that the PTP cannot be the sole target of the Bcl-2 family proteins.

Cytochrome *c* exit is an almost universal feature of apoptotic cell death. However, in some cases, it is a very late event. For example, apoptosis induced by death receptors often bypasses the mitochondrial pathway[45]. As might be expected, from the models discussed above, such deaths are relatively insensitive to protection by Bcl-2 (ref. 45), and cytochrome *c* release into the cytosol is likely to be the result of caspase activation, rather than its cause.

Cytochrome *c* is but one of a host of mitochondrial pro-death denizens. Also present in mitochondria and released upon induction of apoptosis are AIF (a flavoprotein with potent but relatively mysterious apoptotic activity[46]), Smac/DIABLO[47,48], and several procaspases, including procaspase-2, -3 and -9 (ref. 44). Release of multiple death-promoting molecules might be necessary to insure swift and certain death — part of the plan to insure that activation of the apoptotic cascade is a one-way proposition.

Apoptotic antidotes and anti-antidotes

Is release of pro-death factors from mitochondria really the point of no return? Several lines of evidence suggest that cells can occasionally still be rescued at this stage — at least for a while. First, pharmacological inhibitors of caspases will often (but not always) rescue cells from apoptosis[49,50]. Second, caspase-3- and caspase-9-knockout mice show reduced neuronal apoptosis during development and a significant defect in apoptosis following insult[7,51,52]. Third, mammals (as well as the fruitfly *Drosophila* and some viruses) carry a family of genes that encode potent caspase inhibitors, known as the inhibitors-of-apoptosis (IAP) proteins[5,53]. There would be little reason for such proteins to exist if they could not influence the apoptotic process.

On the basis of the above, it might seem that cells suffer from a terminal case of indecisiveness when it comes to apoptotic cell death, letting apoptotic signalling go down endless trails but never fully committing (Box 3). But this impression would be wrong. Indeed, quite to the contrary, the apoptotic pathway contains a number of amplification steps and positive feedback loops that insure that a cell will either fully commit to death or completely abstain from it. For example, the fact that procaspases are caspase substrates insures rapid and complete conversion of a pool of proenzymes even if only a few molecules were initially activated[8]. Similarly, there is likely to be positive feedback between caspase activation and cytochrome *c* exit from mitochondria[54,55].

But positive feedback loops do require the presence of buffers and/or dampeners, or even the smallest perturbation would eventually lead to full activation and apoptotic death of the cell. The IAP proteins might well act as such dampeners. It is possible, for example, that IAPs are not meant to protect cells from frontal suicide assaults, but rather to squelch spurious spontaneous caspase activation.

This idea is further supported by the recent identification of a mammalian IAP inhibitor, known as Smac[47] (for second mitochondria-derived activator of caspases) or DIABLO[48] (for direct IAP-binding protein with low pI). As is the case for Reaper, Hid and Grim in *Drosophila* (ref. 56, and see review in this issue by Meier *et al.*, pages 796–801), Smac/DIABLO binds to IAP family members and neutralizes their anti-apoptotic activity. Most interestingly, Smac/DIABLO is normally a mitochondrial protein, but it is released into the cytosol in cells induced to die, presumably following the same exit route as cytochrome *c*.

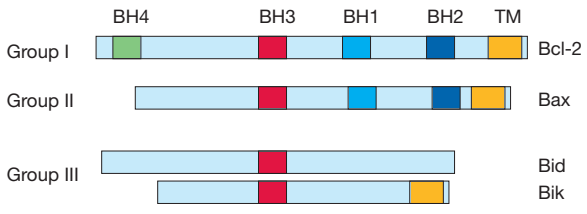
Thus, if a cell is committed to apoptotic death such that it releases its mitochondrial contents, then Smac/DIABLO will sequester the IAP proteins and insure that they do not attempt to stop the programme in its tracks. By analogy, anti-apoptotic Bcl-2 family members can be thought of as buffers that minimize accidental release of mitochondrial contents. Several other buffer zones probably exist, waiting to be discovered.

Would apoptosis, by any other name, be as sweet a death?

Is caspase activation the defining feature of apoptotic cell death? As I mentioned at the beginning of this review, most if not all of the morphological features used to initially describe apoptotic cell

Box 4

Gatekeepers and gatecrashers: Bcl-2 family members



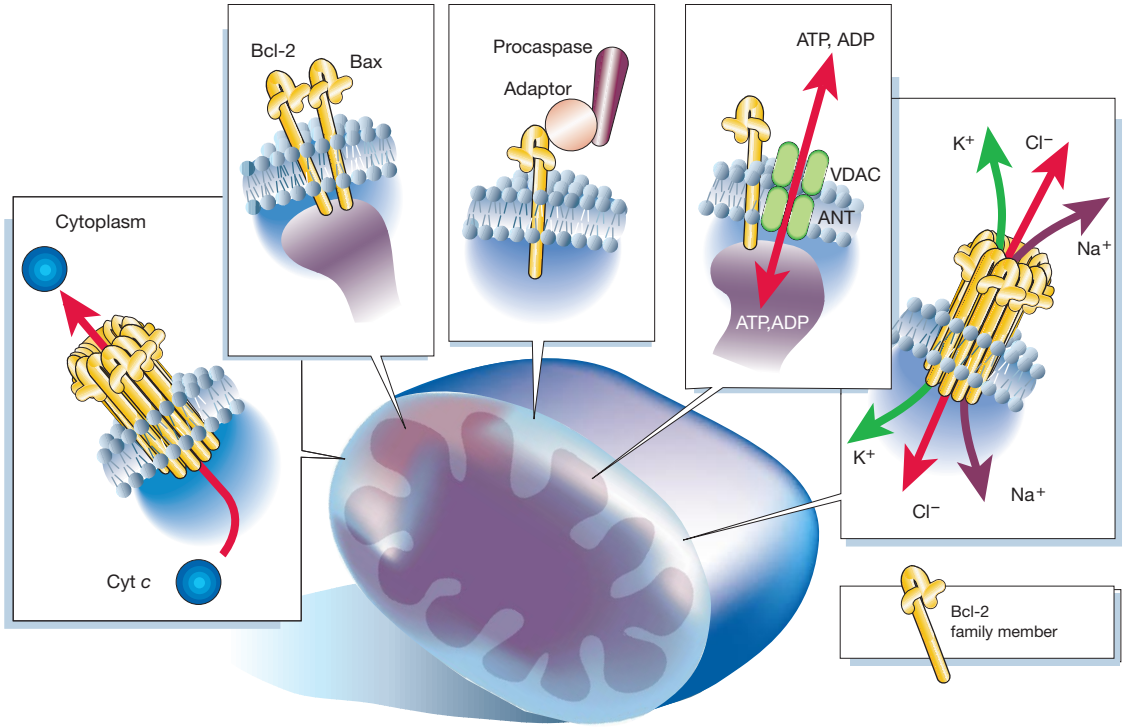
Named after the founding member of the family, which was isolated as a gene involved in B-cell lymphoma (hence the name *bcl*; ref. 69), the Bcl-2 family is comprised of well over a dozen proteins, which have been classified into three functional groups[35,36]. Members of the first group, such as Bcl-2 and Bcl-x_L, are characterized by four short, conserved Bcl-2 homology (BH) domains (BH1–BH4). They also possess a C-terminal hydrophobic tail, which localizes the proteins to the outer surface of mitochondria — and occasionally of the endoplasmic reticulum — with the bulk of the protein facing the cytosol. The key feature of group I members is that they all possess anti-apoptotic activity, and protect cells from death. In contrast, group II consists of Bcl-2 family members with pro-apoptotic activity. Members of this group, which includes Bax and Bak, have a similar overall structure to group I proteins, containing the hydrophobic tail and all but the most N-terminal, BH4 domain[35,36]. Structure/function studies suggest that anti- versus pro-apoptotic activity is determined by relatively large regions of the protein, including two large α-helices that have been proposed to participate in membrane insertion (see text). Group III consists of a large and diverse collection of proteins whose only common feature is the presence of the ~12–16-amino-acid BH3 domain[35]. Although some members of group III, including Bid, are indeed divergent homologues of Bcl-2 and Bax (refs 70, 71), others share little sequence or structural similarity with group I and II, suggesting that the BH3 domain in these proteins has arisen through convergent evolution[41]. Classification of such proteins as Bcl-2 family members is thus more a matter of convenience than a statement of presumed evolutionary relationship.

Box 5

Getting out the vote: possible mechanisms of action of Bcl-2 family members

Bcl-2 family members have been suggested to act through many different mechanisms[34–36,41]. From left to right in the figure below, these include:

- Formation of a pore, through which cytochrome *c* (Cyt *c*) and other intermembrane proteins can escape.
- Heterodimerization between pro- and anti-apoptotic family members. Dimerization is achieved when the BH3 domain of one molecule binds into a hydrophobic pocket formed by the BH1, BH2 and BH3 domains of another family member[72]. Because of structural constraints, both homodimers and heterodimers are asymmetric molecules.
- Direct regulation of caspases via adaptor molecules, as has been described in *C. elegans*. Although the CED-4 homologue Apaf-1 is probably not a Bcl-2 family target, other adaptor proteins, such as BAR (ref. 73), the endoplasmic reticulum-localized protein Bap31 (ref. 74) and Aven (ref. 75), have been described in mammals.
- Interaction with other mitochondrial proteins, such as VDAC and the adenosine nucleotide transporter (ANT), either to generate a pore for cytochrome *c* exit, or to modulate mitochondrial homeostasis (for example, opening of the PTP).
- Oligomerization to form a weakly selective ion channel.



death are caspase-dependent. But the apoptotic programme is much more than just caspases, and in many cell types, activation of the apoptotic programme inevitably leads to death, with or without caspases[57]. Programmed death of cells in which caspases have been blocked often bears little morphological similarity to apoptosis, and can even look suprisingly similar to classical necrotic cell death[58,59].

But that is not all. Physiological forms of cell death with non-apoptotic morphologies have been known for many years[58,60]. How shall we classify such deaths? Atypical apoptosis? Necrosis? Non-apoptotic programmed cell death? Ideally, our final classification will be determined not by morphology, but by what molecular pathways are activated in the dying cell. This will require the development of ever more sophisticated assays for apoptotic proteins.

Conclusions

The field of apoptosis research took off as a result of the careful observations and astute deductions of a group of dedicated pathologists. As Yogi Berra said, "You can observe a lot by watching." Although many of the key apoptotic proteins have been identified, we still are mostly in the dark as to molecular mechanisms of action or activation of these proteins. Events downstream of caspases are murky, and there are kinds of cell death that have not even been touched yet. There is much watching and much deducing left to do. Cell death will continue to be a lively field for the foreseeable future. □

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