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Review

Serial killers: ordering caspase activation events in apoptosis

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Abstract

Caspases participate in the molecular control of apoptosis in several guises; as triggers of the death machinery, as regulatory elements within it, and ultimately as a subset of the effector elements of the machinery itself. The mammalian caspase family is steadily growing and currently contains 14 members. At present, it is unclear whether all of these proteases participate in apoptosis. Thus, current research in this area is focused upon establishing the repertoire and order of caspase activation events that occur during the signalling and demolition phases of cell death. Evidence is accumulating to suggest that proximal caspase activation events are typically initiated by molecules that promote caspase aggregation. As expected, distal caspase activation events are likely to be controlled by caspases activated earlier in the cascade. However, recent data has cast doubt upon the functional demarcation of caspases into signalling (upstream) and effector (downstream) roles based upon their prodomain lengths. In particular, caspase-3 may perform an important role in propagating the caspase cascade, in addition to its role as an effector caspase within the death programme. Here, we discuss the apoptosis-associated caspase cascade and the hierarchy of caspase activation events within it.

Keywords: apoptosis; apoptosome; cascade; caspase; protease

Abbreviations: AIF, apoptosis-inducing factor; Apaf-1, apoptotic protease activating factor; CAD, caspase-activated deoxyribonuclease; CARD, caspase recruitment domain; DR, death receptor; DFF45; DNA fragmentation factor-45; FADD, Fas-associated protein with a death domain; IAP, inhibitor of apoptosis protein; ICE, interleukin-1β converting enzyme; IL-1β, interleukin-1β; NK, natural killer; PARP, poly (ADP-ribose) polymerase; RAIDD, RIP and Ich-1 homologous protein with a death domain; TNF, tumour necrosis factor; TRADD, TNF receptor associated protein with a death domain; TRAIL, TNF-related apoptosis-inducing ligand

Introduction

The hydrolysis of a peptide bond is a simple yet powerful means of altering the activity of a protein. It is hardly surprising then that proteases – enzymes that catalyse the cleavage of peptide bonds – are used to modify protein activities in numerous biological contexts. At their most primitive, proteases serve to degrade proteins at multiple sites during protein digestion and metabolism. However, with the acquisition of greater specificity, proteases can be harnessed to perform more subtle alterations to proteins that modify rather than destroy activity.[1]

Proteases participate in protein destruction and the regulation of protein activities in numerous cellular contexts. Proteases act as protein blenders outside of the cell (digestive proteases), and in a similar but more discriminating manner within the cell (proteasomes). They participate in protein maturation (e.g. caspase-1 in the context of pro-IL-1β maturation), and export (e.g. the trypsin-like proteases of the Spätzle/Toll ligand pathway). Proteases also act as regulatory molecules that amplify and feed forward reactions that culminate in explosive end-points (e.g. the clotting and complement cascades) and finally, as demolition experts that allow cells to migrate (proteases of the extracellular matrix) or die (proteases of the cell death machinery).

In the context of cell death, proteases participate in several guises; as triggers of the death programme, as regulatory elements within it, and ultimately as a subset of the effector elements of the machinery itself. Numerous reviews have detailed how genetic screens for proteins that regulate programmed cell death in *C. elegans* yielded CED-3 – a cysteine protease with an unusual substrate specificity for Asp residues – which soon led to the discovery of its mammalian relatives (the caspases).[2] Since then, a large number of CED-3-related proteases have been cloned – 14 in mammals – but it is unlikely that they all participate in apoptosis. This embarrassment of riches has revealed that the cell death machinery is wonderfully complex, but we still have a good way to go before we understand how all of the components interact. In this review, we will restrict ourselves to a discussion of the role that caspases play in the death machinery and in particular we will summarize what is known concerning the hierarchical activation of caspases within the context of different pro-apoptotic stimuli.

Regulation of proteolysis

Although it is clear that proteases play very constructive roles both inside as well as outside of the cell, their destructive tendencies need to be carefully controlled to minimize the

threat of damage to proteins other than their intended targets. For this reason, the potentially destructive forces of proteases are typically muzzled within the cell until their services are required. This is usually achieved in two basic ways: (1) many proteases are synthesized as inactive pro-enzymes (zymogens) that require removal of a pro-peptide by limited proteolysis, and (2) cells typically synthesise proteins that can complex with proteases and inhibit their activities (protease inhibitors). Both strategies are utilized in the control of cell death-related proteases.

For example, it is well established that the majority of the caspases are constitutively expressed as inactive pro-forms that require limited proteolysis to become active. Because the proteolytic cleavage events that convert caspase pro-forms to their active forms occur at Asp residues within the caspase, this suggests that caspases must either cleave themselves or become activated by other caspases. It is this important fact which establishes the potential for a caspase cascade, but it also poses the question of how the cascade can be instigated. As we shall see, proteins that promote aggregation of caspases play a particularly important role in proximal caspase activation events.

Protease inhibitors are also heavily exploited within the cell death machinery to regulate protease activities. The IAP family (X-IAP, c-IAP-1, c-IAP-2, N-AIP) appear to negatively regulate caspase activities by acting as pseudosubstrates for the caspases, binding to active caspases and neutralizing their activity.[3,4] A different strategy is utilized by another class of inhibitor – the pseudocaspases – which have evolved by gene duplication events but have lost enzymatic activity. These pseudocaspases can integrate into caspase activation complexes (e.g. c-FLIP competes with caspase-8 for binding to FADD at the cytoplasmic face of the CD95/Fas/APO-1 receptor) and attenuate caspase activation as a consequence.[5]

The caspases

To date, 14 caspases have been identified in mammals.[6] Of these, eleven have been cloned in humans, with caspases-11, -12 and -14 only conclusively identified in the mouse thus far.[7,8] Multiple caspases are also present in other organisms; four have been identified in *Drosophila melanogaster*,[9–13] at least two active caspases have been found in *Caenorhabditis elegans*,[2,14] two have been found in *Xenopus laevis*,[15] and one in the insect *Spodoptera frugiperda*.[16] The presence of more than one caspase in organisms like *C. elegans*, and the fact that they are able to process each other,[14] suggests that caspase cascades are unlikely to be confined to mammalian cells.

Functionally, the mammalian caspases can be divided into two broad families; those that are thought to be centrally involved in cell death (caspases -2, -3, -6, -7, -8, -9, and -10) and those most closely-related to caspase-1 (also called ICE) whose primary role seems to be in cytokine processing (caspases -1, -4, -5 and -11). At present, there is insufficient data available to enable caspases -12, -3 and -14 to be categorized, although in terms of their primary structure, caspases -12 and -13 appear to be related to the cytokine processing subfamily,[8,17] whereas caspase-14 is more related to the cell death family caspases -2 and -9.[18] Comparatively little is known about the caspases that are involved in cytokine processing and the manner in which they are activated beyond the fact that caspase-11-deficient mice are unable to activate caspase-1 and that caspase-1 may be activated by oligomerization.[19,20] However, our knowledge of the manner in which activation of the cell death-related caspases is achieved is more advanced and will form the basis for much of the subsequent discussion.

The phases of death: initiation, commitment, amplification and demolition

For convenience, we have divided the death programme into separate phases (Figure 1). The 'initiation' phase, during which cells receive signals that *may* result in the activation of the death programme. The 'commitment' phase, the point after which death signals become irreversible. The 'amplification' phase, where multiple caspases are recruited to co-operate in the destruction of the cell, and finally, the 'demolition' phase, where a panoply of active caspases dismantle cellular structures, either directly, or via activation of other enzymes (such as CAD/DFF45). We will consider each of these phases in turn.

Initiation: activation of apical caspases

Initiators of the apoptosis machinery can be broadly grouped into three different categories: (1) death receptors, (2) the contents of cytotoxic T and NK cell granules, and (3) stimuli that provoke generalized cellular damage.

Death receptor-initiated cell death signals

The death receptors (CD95/Fas/APO-1, TNFR1, DR3/WSL-1/TRAMP, DR4/TRAIL-R1, DR5/TRAIL-R2, DR6) are a subset of the TNF/NGF receptor family of cell surface molecules that possess a common motif within their cytoplasmic tails, called the death domain.[21,22] The death domains of these receptors are responsible for recruiting adaptor molecules that, in turn, recruit caspases to the receptor complex. This simple strategy greatly increases the probability of caspase activation by facilitating cross processing of caspases within the cluster. This proximity-induced processing is thought to drive apical caspase activation events that are initiated by all of the death receptors.[23,24] Thus, death receptors can drive caspase activation in a very direct manner. The best characterized receptors in this regard are the CD95 and TNFR1 receptors that use the FADD and TRADD adaptors to recruit caspase-8.[21] It has also been suggested that TNFR1 recruits caspase-2 via the RAIDD/CRADD adaptor molecule,[25] however the biological significance of this is unclear since TNF and CD95L-induced apoptosis are completely absent in cells derived from *CASP-8* null mice.[26] Moreover, *CASP-2* null mice do not display defects in the CD95/Fas or TNFR1-initiated cell death pathways.[27] The adaptors and the caspases that are recruited into the DR4/TRAIL-R1, DR5/TRAIL-R2, and DR6 signalling complexes are currently unknown.

So what happens upon activation of caspase-8 in the context of CD95 or TNFR1 receptor signalling? A simple and appealing model is that caspase-8 then propagates the death signal by direct processing of other caspases. Caspase-3 in particular appears to be a very good substrate for caspase-8.[28] This has been endorsed using

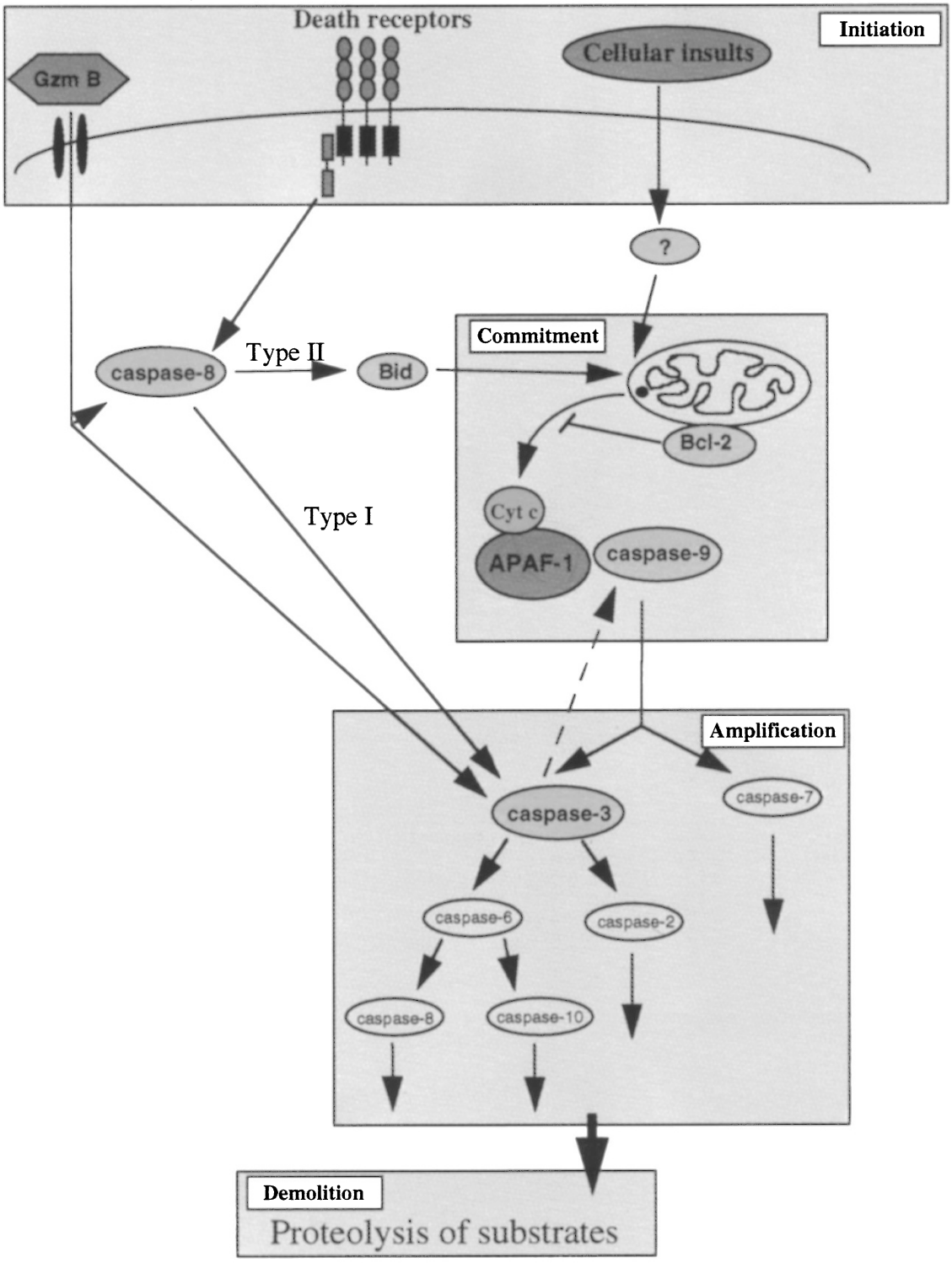

Figure 1 Schematic representation of the routes to caspase activation within the cell death programme. See main text for details of each of the four phases (initiation, commitment, amplification and demolition)

peptide inhibitors and affinity labelling techniques which indicate that caspase -3 and -7 are simultaneously activated by caspase-8.[29] Once activated, caspase-3 can then propagate the cascade further by activation of other caspases such as caspase-6 and caspase-2 (Figure 1).[29,30] This model seems to fit the majority of CD95L and TNF-initiated cell deaths and in this context death repressor proteins such as Bcl-2 are unable to intervene. However, a subset of CD95L and TNF-initiated cell deaths do not fit this model since Bcl-2 and Bcl-x do protect from FasL or TNF in certain cell types.[31] Because several members of the Bcl-2 family, including Bcl-2 itself, appear to exert their effects on the cell death pathway by regulating mitochondrial events, this implicates the mitochondrial pathway in a subset of receptor-initiated cell deaths. This had led to the proposal that two distinct routes to apoptosis initiated by the TNF and CD95 death receptors exist.[31] In type I cells, the direct pathway is engaged and Bcl-2 fails to protect in this context. In type II cells, Bcl-2 and Bcl-x can confer protection because the death signal is routed through the mitochondria.

So how does caspase-8 engage the mitochondrial pathway? Recent data suggests that BID, a death promoting member of the Bcl-2 family, is the intermediary in this context.[32–34] Unlike most other members of the Bcl-2 family, BID is normally localized to the cytoplasm. However, caspase-8 can catalyze the cleavage of BID, the C-terminal portion of which then translocates to the mitochondria where it integrates into the outer membrane and provokes the release of cytochrome c – an important accessory molecule for downstream caspase activation events. How BID triggers cytochrome c release is currently unknown, but it is well established that Bcl-2 and Bcl-x can regulate this event,[35,36] thereby explaining how TNF and CD95L-induced death signals can be regulated in type II cells.

Cytotoxic lymphocyte-initiated death signals

Cytotoxic lymphocytes (T and NK cells) contain granules that can be discharged onto the surface of target cells, delivering what has been called 'the kiss of death' (Figure 1). These granules contain, amongst other things, granzyme B (also called fragmentin-2) a serine protease which cleaves after Asp residues, and a pore-forming protein called perforin (or cytolysin) that is likely to permit entry of the other granule components into the target cell. This immediately suggests a mechanism for cytotoxic lymphocyte-initiated apoptosis where granzyme B enters the cell and triggers the caspase cascade by directly cleaving and activating caspases.[37,38] While it has been demonstrated that granzyme B can cleave most of the caspases *in vitro*, it appears that in cells its preferred target is caspase-3, which then proceeds to activate caspases -7, -8 and -9.[39]

Generalized cellular damage-initiated death signals

All other stimuli that can provoke apoptosis have been grouped into this category, mainly because it is still far from clear how these stimuli engage the caspase component of the death machinery. This group includes diverse apoptosis-promoting stimuli such as cytotoxic drugs, radiation, heat shock, survival factor deprivation and other cellular stresses. The signals that these stimuli evoke within the cell are disparate, but the death pathways engaged by the vast bulk of these stresses seem to converge on the mitochondria.[35] Furthermore, Bcl-2 and Bcl-x protect in the majority of these cases, again implicating the mitochondrial pathway as an important conduit for death signals triggered by stimuli within this group.[35] Thus, although cytotoxic drugs, radiation and heat shock may injure cells in very different ways, all of these pathways appear to engage the mitochondrial apoptosome by triggering the release of certain mitochondrial components (Figure 1).

Commitment: mitochondrial damage?

Recently, considerable effort has focused upon the role of the mitochondrion in transducing pro-apoptotic stimuli.[40–44] A discussion of all of the evidence that has implicated the mitochondrion as an important sensor of cellular damage is outside of the scope of the present review. However, to summarize, it appears that numerous pro-apoptotic stimuli provoke changes in the permeability of the mitochondrial outer membrane that permits escape of certain proteins – such as AIF and cytochrome c – that are normally confined to the mitochondrial intermembrane space. How these proteins escape is the subject of much debate, but it is clear that this can be achieved in a caspase-dependent (via caspase-8 cleavage of BID) or caspase-independent (unknown effectors) manner, depending on the nature of the pro-apoptotic stimulus. Thus, the death receptor and cytotoxic T/NK cell granule-initiated pathways can provoke the caspase-dependent route to mitochondrial damage (via caspase-8) whereas the majority of other death-promoting stimuli seem to achieve this via as yet unidentified factors (Figure 1). Irrespective of the way in which mitochondrial damage is achieved, the consequences of cytochrome c and AIF leakage are likely to be the same in both contexts.

AIF appears to exert its effects in a caspase-independent manner by translocating to the nucleus and triggering the chromatin collapse and digestion into high molecular weight fragments that is commonly observed during apoptosis.[41] In contrast, cytochrome c exerts its effects by regulating the activities of Apaf-1, a molecule that can promote clustering of caspase-9.[40,45–49] Cytochrome c achieves this by binding to Apaf-1 – probably in the WD-40 repeat region of the molecule – which, in association with ATP, facilitates the assembly of a high molecular weight complex (the mitochondrial apoptosome) composed of several Apaf-1 molecules, each of which has bound a molecule of caspase-9 via its N-terminal CARD domain (Figure 2). This process bears a lot of similarity to the aggregation of caspases that is achieved by the cell surface death receptors. Thus, Apaf-1 promotes caspase-9 activation via a proximity-induced processing strategy which drives the next phase of the caspase cascade.

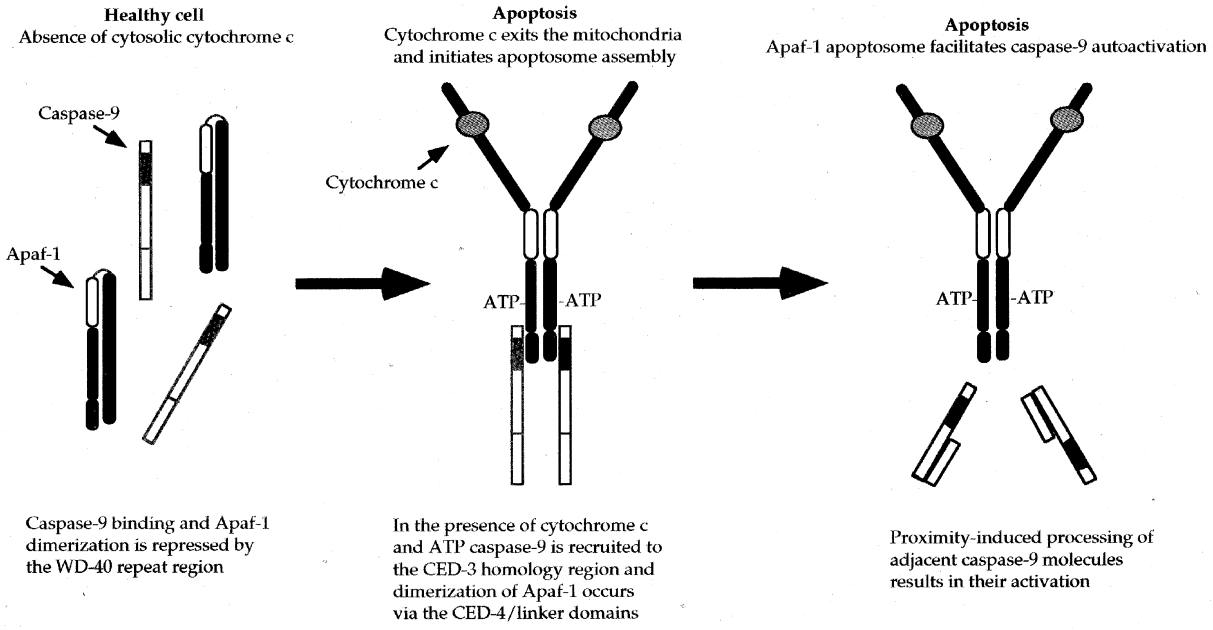

Figure 2 Apaf-1 promotes caspase-9 activation by oligomerization. Cytochrome c and ATP regulate the ability of Apaf-1 to recruit caspase-9 and form multimers

Amplification: activation of 'downstream' caspases

Upon activation of caspase-9 by Apaf-1, this caspase can then propagate the death signal by activating other caspases. The study of this phase of the death programme has been greatly facilitated by the observation that cytochrome c (in conjunction with dATP) can trigger apoptosis in cell-free extracts, an unusual property that is not displayed by the vast majority of agents that can promote apoptosis in intact cells.[30,40,46] Studies using cell-free systems have revealed that cytochrome c activates caspase-9 (via Apaf-1) and that caspase-9 then initiates a series of other caspase activation events that represent a *bona fide* caspase cascade, since removal of certain members of the cascade blocks subsequent events (see Figure 1).[30]

The caspase activation events driven by caspase-9 appear to be the simultaneous activation of caspases -3 and -7.[30,47,50] Caspase-3 then drives the activation of caspases -2 and -6, followed by the activation of caspases -8 and -10.[30] In the absence of caspase-3, caspases -7 and -9 are still activated but activation of other caspases downstream of this point (see Figure 1) is arrested.[30] Removal of caspase-6 blocked the activation of caspases -8 and -10 in this context, suggesting that these activation events are, somewhat surprisingly, driven by caspase-6.[30] The activation of long-prodomain caspases late in this cascade may be unexpected, but these observations are bolstered by similar observations in other systems. In addition, it seems quite plausible that caspases that are apical (initiator) caspases in the context of certain pro-apoptotic stimuli may participate in an amplification role in other contexts. This phase of the death programme may serve as an amplification step that activates the full complement of caspases that are required to dismantle the cell in the appropriate manner.

It is important to note that not all of the caspases that are activated during this phase may be necessary for the cell to die *per se*. However, they are likely to be required for the cell to adopt the typical apoptotic phenotype. For example, cells deficient in caspase-3 (either from *CASP-3* null mice, or due to a frameshift mutation, i.e. MCF-7 cells) clearly die in response to many pro-apoptotic stimuli.[51–53] However, caspase-3-deficient cells undergo an aberrant form of apoptosis (restricted blebbing, delayed DNA fragmentation), suggesting that the downstream amplification events and non-caspase cleavage events that are mediated by caspase-3 cannot be carried out by caspases activated earlier in the pathway. In a similar but more dramatic way, if all caspase activity is blocked using broad spectrum caspase inhibitors, cells that progress beyond the mitochondrial commitment point typically exhibit features of necrosis rather than apoptosis.[54,55]

Demolition: substrate attack and cellular collapse

When the full complement of caspases that are necessary for the proper execution of the death programme have become activated, the final demolition phase can begin. We will not deal with the substrates themselves, as this topic is the subject of several recent reviews. The distinction between the amplification and demolition phase is obviously an artificial one, since it is presumed that substrates will become cleaved as soon as the caspase responsible for particular cleavage events are activated. However, it should be noted that whereas many of the caspases have a cytosolic localization, many of their known substrates are contained within the

nucleus or other cellular compartments. Thus, there is likely to be some delay between activation of a caspase and the subsequent proteolytic attack of its preferred substrate(s). Movement of caspases between sub-cellular compartments in response to pro-apoptotic stimuli is an area of intensive investigation at present.

'Initiator' and 'effector' caspases: a simple demarcation?

Caspases have been grouped into either upstream/signalling/initiator caspases and downstream/effector caspases, largely based upon their respective prodomain lengths. The rationale for this grouping derives primarily from the observation that long prodomain caspases (such as caspase-8) are typically recruited into membrane receptor complexes by means of adaptor proteins, and consequently, initiate the caspase cascade. Thus, the upstream caspases (those with long prodomains whose presumed function is to activate other caspases) would appear to be caspases -2, -8, -9 and -10, and the effector caspases (those with short prodomains that are presumed to dismantle the cell) would appear to be caspases -3, -6 and -7.

However, although this model is a very useful one, closer scrutiny of the available data indicates that this is an over-simplification. While caspases -8 and -9 have been shown to initiate caspase cascades in particular instances, there is scant evidence that caspase -2 and -10 function likewise. Although an adaptor molecule, RAIDD, has been identified that interacts with the prodomain of caspase-2 and is thought to link caspase-2 to TNFR1,[25] it was subsequently shown that mice with targeted deletions in the *CASP-2* gene have an intact TNFα-induced death pathway.[27] Furthermore, in studies employing *in vitro* systems to study the events in the cytochrome c initiated caspase cascade, not only are the long-prodomain caspases -2, -8 and -10 activated in response to the Apaf-1-mediated activation of caspase-9, but their activation is dependent upon the actions of caspase-3, a short prodomain 'effector' caspase.[30,56]

These instances of the downstream activation of 'upstream' caspases have been further supported by the finding that the activation of caspases -2 and -8 is impaired in dexamethasone-induced apoptosis of thymocytes from *APAF-1* or *CASP-9* null mice.[57,58] It has also been demonstrated that following its activation by caspase-9, caspase-3 feeds back on caspase-9 thereby amplifying the cascade.[30,47] Thus, it would seem that in a caspase cascade where caspase-9 is apical, caspase-3 does not function merely to cleave substrates but also acts to propagate and amplify the protease cascade by activating other caspases, including the long-prodomain caspases.

A further indication of the limitations of inferring the function of caspases upon the basis of their prodomains arose from studies undertaken using a positional scanning synthetic combinatorial library.[59] This study placed the caspases into three groups based upon the preferred amino acid sequences adjacent to the site at which the caspases cleave. Group I caspases, whose preferred cleavage sequence was found to be (W/L)EHD, consisted of caspases -1, -4 and -5, all of which are likely to be cytokine processing caspases. However, group II caspases, which display the sequence specificity of DEXD which is akin to the sequence at which many caspase substrates such as PARP are found to be cleaved, contain the short pro-domain caspases -3 and -7 but also the long pro-domain caspase, caspase-2 (although there is no evidence that they cleave the same set of substrates). Also, the group III caspases, whose favoured substrate sequence of ((I/L/V)EXD) is similar to the sites at which caspases are cleaved upon their activation, include the short pro-domain effector caspase, caspase-6, in addition to the long pro-domain caspases, -8 and -9.

Thus, it is probably incorrect to employ a simple demarcation of caspases into signalling or effector categories. The role that a particular caspase occupies within the activation cascade is likely to be partially context-dependent. Moreover, although the prodomains of caspases -3, -6 and -7 are only a few kilodaltons in size, it is not inconceivable that these caspases may interact with caspase clustering molecules through their prodomains, or other parts of the molecule. Conversely, it is also possible that some of the long-prodomain caspases, in addition to processing other caspases, may cleave cellular substrates during apoptosis. While studies from knockout mice have demonstrated the importance of caspases -8 and -9 in development,[26,58,60] the defects exhibited by *CASP-3* null mice may not be solely attributable to a lack of substrate cleavage.[51] Although some apoptotic events such as the change in nuclear morphology are altered in mice lacking caspase-3, other events such as the exposure of phosphatidylserine and the cleavage of the caspase substrates fodrin, DFF45, lamin and gelsolin are delayed but not entirely abolished.[61,62] Thus, it is possible that some of the defects in *CASP-3* knockout mice arise as a result of improper dissemination of the caspase cascade and that this is a function of caspase-3 that is as important as its role as an effector caspase.

Future directions

Although our understanding of caspase activation and effector function has improved dramatically over the past few years, there is much that remains to be explored. Little is known concerning the role of several members of the caspase family (caspases -4, -5, -10, -12, -13, -14) and whether they participate in apoptosis. It is likely that a number of these are involved in cytokine processing but this remains to be clarified. In this context, it is interesting to ask how cells are able to activate these caspases for the purpose of cytokine processing without triggering the death programme. Compartmentalization, rapid destruction of active caspases, and selective caspase inhibitors are likely to play important roles in this situation. It is also very likely that caspases have functions outside of the cell death or cytokine processing pathways. Interestingly, recent studies have implicated caspases in the denucleation that occurs during lens fibre differentiation and in cell attachment and migration.[63,64] One thing is certain, figuring out the *modus operandi* of the serial killers of the cell is likely to keep us busy for some time to come.

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