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Death by design: mechanism and control of apoptosis

Zhiwei Song and Hermann Steller

Active cellular suicide by apoptosis plays important roles in animal development, tissue homeostasis and a wide variety of diseases, including cancer, AIDS, stroke and many neurodegenerative disorders. A central step in the execution of apoptosis is the activation of an unusual class of cysteine proteases, termed caspases, that are widely expressed as inactive zymogens. Originally, the mechanisms for regulating the caspase-based cell death programme seemed to be different in *Caenorhabditis elegans*, mammals and insects. However, recent results suggest that these apparent differences in the control of cell death reflect our incomplete knowledge, rather than genuine mechanistic differences between different organisms.

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Apoptosis is a morphologically distinct form of cell death that is designed to rapidly remove unwanted and potentially dangerous cells[1,2]. During the development of most metazoan animals, many more cells are produced than are eventually needed, and apoptosis plays a key role in removing surplus cells and sculpting the developing embryo[3] (Fig. 1). In addition, the inappropriate regulation of apoptosis is associated with a variety of diseases, including cancer, AIDS, neurodegenerative diseases and ischaemic stroke[4]. Because apoptosis represents an active, gene-directed mechanism, it should eventually be possible to control this process for therapeutic purposes.

During the past few years, rapid progress has been made in identifying some of the molecules that are responsible for the regulation and execution of apoptosis. The existence of a cell-suicide programme was originally proposed on the basis of the stereotyped, morphological changes associated with natural cell death, but definitive evidence for the existence of a designated death programme came from genetic studies of cell death in *Caenorhabditis*

TCB, Vol. 9, N° 12 (0962-8924) PII: S0962-8924(99)01670-0
TIBS, Vol. 24, N° 12 (0968-0004) PII: S0968-0004(99)01485-1
TIG, Vol. 15, N° 12 (0168-9525) PII: S0168-9525(99)01899-5

elegans[5,6]. In particular, this work revealed the importance of an unusual class of cysteine proteases, now termed caspases (for 'cysteine aspartase'), for cell death[7]. Caspases are synthesized initially as inactive pro-enzymes but are converted to the active protease when cells are selected to die. A central goal of apoptosis research is to understand how the caspase-based death programme is only activated in cells that are destined to die. The initiation of apoptosis is carefully regulated by many different signals, which can originate either from within the doomed cell or from its extracellular environment. For example, cell-lineage information, damage inflicted by ionizing radiation or viral infection, extracellular survival factors, cell–cell interactions and hormones can all have a profound effect on the decision between life and death of a cell[8]. Given this complexity, it is not surprising that multiple mechanisms are used to control the conversion of the caspase zymogen to the active protease.

Caspases: the loaded gun

Caspases are a family of cysteine proteases that function in apoptosis or cytokine processing, or both. They are synthesized as pro-enzymes (or zymogens) and remain inactive in most healthy cells. Upon activation by different death signals, the single-chain pro-caspases are cleaved at specific aspartic acid residues to remove an inhibitory N-terminal pro-domain and to generate two distinct subunits. These subunits assemble into a heterotetramer to form the active protease[9]. Once activated, caspases are thought to cleave many important cellular proteins and thereby bring about the characteristic apoptotic morphology. The first evidence that caspases play important roles in cell death came from the discovery that the *C. elegans* cell-death gene *ced-3* encodes a protein that is homologous to the caspase interleukin-1β-converting enzyme (ICE)[10]. Subsequently, the use of specific inhibitors and the analysis of caspase-deficient animals have firmly established their role during apoptosis in insect and vertebrate systems. How are caspase zymogens converted to the active enzyme? Because the processing of the pro-enzyme involves cleavages after aspartate residues, one caspase can be activated by another caspase in a proteolytic cascade. Caspases with long pro-domains are thought to be involved in the initial activation of such a cascade, and those with short pro-domains appear to function downstream in the actual cell killing[9]. The key to triggering such a proteolytic cascade is that initiator pro-caspases possess weak protease activity and, therefore, when adaptor molecules bring zymogens close together, they can cleave each other and generate mature caspases (see below).

Death receptors, the direct physical activators of caspases

Possibly the best-understood pathway for activating caspases through induced proximity is the CD-95/Fas/Apo-1 system[11,12]. CD-95 belongs to the tumour-necrosis factor receptor (TNFR) family and functions in the removal of activated T cells at the end of the immune response. Binding of extracellular ligands, such as the Fas ligand, to these receptors induces trimerization of the receptor. This trimerization, in turn, recruits the adaptor molecule FADD and pro-caspase-8 into a multimeric complex termed DISC (death-inducing signal complex) in which caspase-8 is activated. Activated caspase-8 goes on to cleave downstream caspase zymogens, such as caspase-3. In addition to activating caspase-3, caspase-8 also cleaves a pro-apoptotic member of the Bcl-2 family, BID (see below), and this amplifies the death signal received from the cell surface. In addition to Fas and TNFR, several other death receptors that trigger apoptosis have been identified. Finally, several decoy receptors, which share homologous extracellular domains with the death receptors but lack the cytoplasmic domain, can compete with specific death receptors for ligand binding to reduce the effectiveness of specific death ligands[13,14].

Caspase activation by CED-4/Apaf-1 and cytochrome *c*, the unexpected internal death signal

Another important class of caspase-activating molecules is represented by *C. elegans* CED-4 and mammalian Apaf-1. The *ced-4* gene acts genetically upstream of *ced-3* in *C. elegans*, and CED-4 can physically interact with pro-CED-3 and certain mammalian pro-caspases[15–17]. Apaf-1, which shares significant amino acid homology with CED-4, can bind to the pro-domain of pro-caspase-9 and activate it in the presence of cytochrome *c* and dATP in a cell-free system[18]. These observations suggest that CED-4/Apaf-1 regulates the activation of a caspase cascade by complex formation, possibly in a manner similar to the 'proximity model' described for CD-95.

Surprisingly, the activation of caspases by Apaf-1 also requires the presence of cytochrome *c* (Ref. 19). Cytochrome *c* normally is located in the space between the inner and outer membranes of the mitochondria, where it serves an essential function in the respiratory chain. Therefore, it has been proposed that the release of cytochrome *c* from mitochondria is a crucial step in the initiation of apoptosis. However, the precise circumstances during which cytochrome *c* is released *in situ*, and its role in the regulation of apoptosis *in vivo*, remain to be determined. For example, it is not clear whether the presumed pro-apoptotic role of cytochrome *c* is restricted to stress-induced cell death or whether it plays a more general role during apoptosis in normal development and tissue homeostasis. Nevertheless, it appears that CED-4/Apaf-1 can bring pro-caspase molecules into close proximity, so that they can activate one another.

Crystal-structure analysis of the complex formed between the N-terminus of Apaf-1 (residues 1–97) and the pro-domain of caspase-9 (residues 1–112) revealed that the two domains share strikingly similar globular structures. Each consists of a highly positively charged and a highly negatively charged surface. The two domains can form a complex through two surfaces that are not only complementary in shape but also opposite in charge[20]. The authors predicted that the interaction between CED-3 and CED-4 could be similar overall. Further oligomerization of Apaf-1–caspase-9 complexes might be mediated by the CED-4 homology domain of Apaf-1.

Double-edged swords in the regulation of caspase activation: the Bcl-2 protein family

In *C. elegans*, the activity of CED-9 is required to prevent inappropriate cell deaths, and *ced-9* acts genetically upstream of *ced-4* and *ced-3* (Ref. 21). Significantly, *ced-9* encodes a protein homologous to mammalian Bcl-2-like molecules that regulates apoptosis in mammals[22,23]. The human *BCL-2* gene was identified initially at a translocation breakpoint that is common in many B-cell lymphomas. As a result of this translocation, *BCL-2* comes under the control of the immunoglobulin heavy chain enhancer and is constitutively expressed in B cells. The resulting protection against apoptosis apparently permits the survival and accumulation of aberrant B cells that ultimately give rise to malignancies. In recent years, a large number of additional *Bcl-2*-like genes have been identified[22,23]. Like *Bcl-2*, many of these new genes have anti-apoptotic activities, but some members, such as *Bax*, can actually stimulate apoptosis. Despite intense efforts, the precise mechanism by which Bcl-2-like proteins regulate cell death remains controversial. Over the years, several different biochemical functions have been proposed[24]. A model that currently enjoys considerable popularity is based on the observation that Bcl-2-like proteins can have pore-forming activities in synthetic lipid membranes[25,26]. Because Bcl-2 is present in the outer mitochondrial membrane, it has been suggested that it blocks apoptosis by inhibiting the release of apoptosis-inducing factors, such

as cytochrome *c*, from mitochondria[27–29]. An alternative model for Bcl-2 function is based on the observation that CED-9 can form a complex with CED-3 and CED-4 upon overexpression in mammalian cells[17]. In this model, CED-9 inhibits CED-4 from activating CED-3 by direct physical association with CED-4. However, it is not clear that the observed physical interactions are relevant under physiological conditions *in vivo*.

One important property of Bcl-2-like proteins is their ability to form homo- and heterodimers with other family members to modulate their activity[22,23,30]. The *egl-1* gene of *C. elegans* promotes apoptosis by inhibiting the anti-apoptotic activity of CED-9 (Ref. 31). Likewise, pro-apoptotic Bcl-2 family proteins in mammals, such as BAX and BID, can antagonize the life-saving activities of protective family members. Therefore, the battle between these antagonistic Bcl-2 family members could provide a mechanism to fine-tune the level of protection against cell death.

The other brake on death: inhibitor of apoptosis proteins (IAPs)

Inhibitor of apoptosis proteins (IAPs) were first discovered in baculovirus through their ability to inhibit apoptosis of insect cells upon viral infections[32,33]. The characteristic structural motif of all IAP family members is the baculovirus IAP-repeat (BIR) of ~70 amino acids. Different IAPs can have between one and three BIRs. Besides the BIR domain, some IAP members also contain additional structural motifs that have been described in other molecules, such as a RING and a CARD domain. To date, homologues of IAPs have been identified in mammals, insects, *C. elegans* and even in yeast. As it does not appear that yeast cells can undergo apoptosis, protection against cell death might not be the sole function of BIR-containing proteins. Indeed, recent studies indicate that some IAPs could play important roles in cytokinesis[34].

IAPs appear to inhibit cell death through direct interactions with caspases. Three human IAPs – XIAP, cIAP1 and cIAP2 – can specifically bind to and inhibit caspase-3 and -7 *in vitro*. One of the *Drosophila* IAPs, DIAP1, can inhibit the Dcp1 caspase in yeast[35]. However, it remains to be seen whether IAPs normally act to inhibit active caspases, or whether their physiological function is to prevent caspase activation by blocking zymogen processing.

Harbingers of death from *Drosophila*: REAPER, HID and GRIM

The induction of apoptosis in *Drosophila* requires the activities of three closely linked genes, *reaper* (*rpr*), *grim* and *head involution defective* (*hid*), whose gene products kill by activating a caspase pathway[36]. *reaper*, *hid* and *grim* are all transcriptionally regulated by a variety of death-inducing stimuli, and the *hid* gene is repressed by active Ras signalling[37,38]. Therefore, it appears that these genes act as integrators for relaying different death-inducing signals to the core death programme.

RPR, HID and GRIM proteins appear to induce cell death by binding to and inhibiting the anti-apoptotic activity of DIAP1 (Ref. 39). It has been proposed that IAPs act at multiple steps in the apoptotic pathway, both upstream and downstream of RPR, HID and GRIM[40–42]. However, these models are based on results obtained under somewhat unphysiological conditions. In particular, high-level overexpression of IAPs might protect against coexpression of RPR, HID and GRIM through complex formation and sequestration of the pro-apoptotic proteins. However, we think that IAPs are most likely to be the downstream targets for RPR, HID and GRIM during the induction of apoptosis.

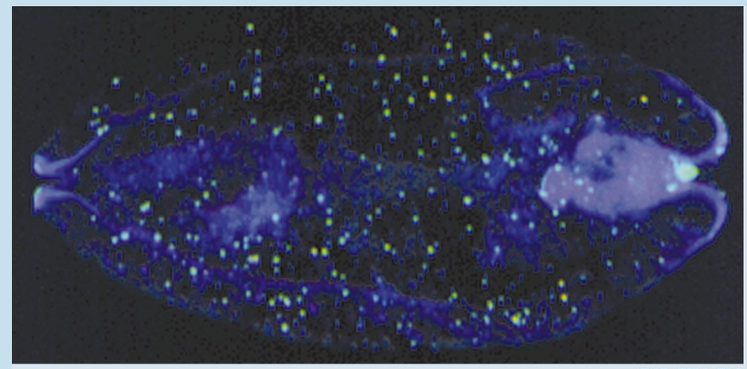

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FIGURE 1. Confocal image showing LacZ immunoreactivity in a *Drosophila* embryo from an enhancer trap strain that directs LacZ to nuclei of macrophages. These cells (also called haemocytes) are 'professional' phagocytes that engulf apoptotic corpses during embryonic development. Image kindly provided by J.M. Abrams and D. Smith.

Have insects evolved a distinct mechanism for the control of apoptosis?

The apparent absence of Bcl-2- and CED-4/Apaf-1-family proteins in *Drosophila*, and the lack of mammalian homologues for RPR, HID and GRIM, led to the impression that the regulation of programmed cell death in *Drosophila* might be

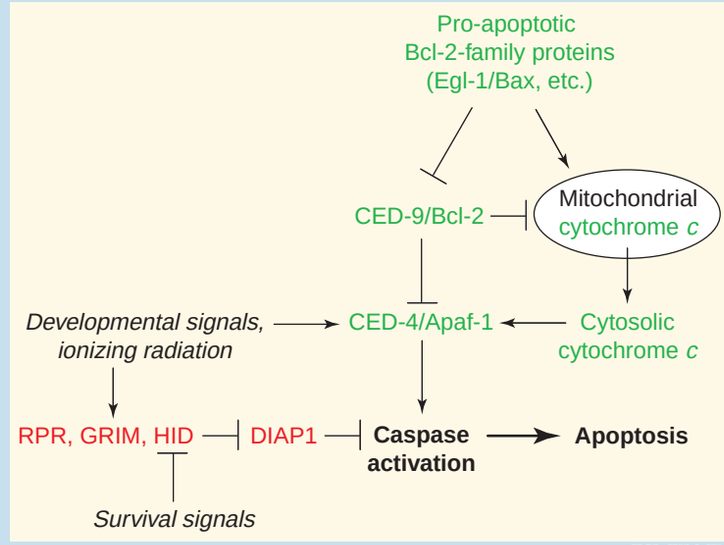

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FIGURE 2. Two distinct pathways for the control of caspases during apoptosis were discovered originally in *Caenorhabditis elegans* and mammals, and insects, respectively. In the 'classic pathway' derived from work in *C. elegans* and mammalian systems (green components), the conversion of zymogen pro-caspases is stimulated by CED-4/Apaf-1-like proteins, which appear to act by promoting pro-caspase aggregation. CED-4/Apaf-1 are negatively regulated by anti-apoptotic members of the Bcl-2 family, which, in turn, are inhibited by pro-apoptotic family members. Apaf-1 requires cytochrome *c* as a cofactor, and it is thought that Bcl-2-family proteins have opposing roles to regulate the release of cytochrome *c* from mitochondria. Studies of apoptosis in insects have identified an inhibitory pathway that is negatively regulated by apoptotic activators (red components). Inhibitors of apoptosis proteins (IAPs), such as *Drosophila* IAP1 (DIAP1), are required to prevent inappropriate caspase activation and apoptosis, and the anti-apoptotic activity of DIAP1 is blocked by RPR, HID and GRIM. Recent studies suggest that both pathways are used simultaneously to regulate apoptosis in *Drosophila* and, presumably, also in other systems (see text for details). This highly simplified model does not attempt to incorporate the complexities introduced by the presence of multiple caspases, which can act in a proteolytic cascade, and the activation of caspases through aggregation induced by death receptor systems, such as CD95.

distinct from that in *C. elegans* and vertebrates[43,44]. However, the recent discovery of *Drosophila* homologues of *ced-4*/*Apaf-1* and *Bcl-2*-like sequences indicates that these apparent differences might be of historical, but not of functional, significance[45,46]. Rather, it appears that insects use two distinct mechanisms simultaneously to control the activation of a caspase-based death programme (Fig. 2). On the one hand, factors such as CED-4/Apaf-1 promote caspase activation, and this step is simultaneously inhibited by IAPs. Both regulatory inputs are controlled by upstream factors (Bcl-2 family versus RPR, HID and GRIM), which, in turn, are the targets for complex control by signalling pathways. It is very likely that a similar dual control of caspase activation operates during mammalian apoptosis. Although no mammalian homologues of RPR, HID and GRIM have been reported to date, there are good reasons to believe that homologous molecules function in the control of apoptosis in vertebrates. First, the expression of RPR, GRIM or HID can induce apoptosis in mammalian cells, and recombinant RPR can activate caspases and apoptosis-like events in a *Xenopus* cell-free system[47–49]. Second, RPR, GRIM and HID interact physically and genetically with IAPs[40–42,50]. Finally, the pro-apoptotic activity of HID is inactivated upon direct phosphorylation by mitogen-activated protein (MAP) kinase[37]; therefore, HID interacts directly with at least two highly conserved protein families. Thus, it is likely that homologous molecules function in the control of apoptosis in vertebrates.

The proposed dual control of caspase activation would offer increased security and flexibility for selecting cells that are fated to die. For example, conditions in which the CED-4/Apaf-1 pathway is only weakly active are insufficient to overcome protection by IAPs. On the other hand, modest levels or RPR/HID/GRIM might be inadequate to kill in the absence of active CED-4/Apaf-1-like proteins. Therefore, under physiological circumstances, successful induction of cell death might require that either both pathways are coordinately regulated or that one of them is induced very strongly. At least some death-inducing signals, such as ionizing radiation, can indeed activate both pathways simultaneously[46]. The analysis of how these pathways are interconnected and how they are utilized *in situ* will remain an interesting challenge during the coming years.

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