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Dying like Flies

Minireview

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Apoptosis is a peculiarly metazoan phenomenon. True, organized death of a kind is observed in some unicellular organisms, but only metazoans possess an elaborate machinery evolved solely to destroy, dismantle, and dispose of cells. Within the past few years, apoptosis has emerged as a key element in homeostasis, which together with cell proliferation and differentiation, regulates the balance between cell loss and cell gain necessary for the establishment and maintenance of metazoan tissues. Insufficient or excessive apoptosis is now recognized as the cause or consequence of many human pathologies and, consequently, there is intense interest in apoptosis as a pharmacological target.

The past few years have seen significant advances in our understanding of both the machinery that executes apoptosis and the mechanisms by which this potentially catastrophic process is controlled. Much of our knowledge stems from studies of the developmentally invariant nematode *C. elegans*. During its ontogeny, 1090 cells are born, of which 131 die by suicide. Genetic analysis has identified three principal genes that are directly involved in the regulation and execution of each cell death: the products of the *ced-3* and *ced-4* genes are required to execute cell death while their action is antagonized by the product of the *ced-9* gene (Ellis and Horowitz, 1986). *ced-3* encodes a caspase, one of an evolutionarily conserved class of cysteine proteases that cleave after aspartate residues in target proteins and that constitute the key destructive engines of cell suicide. The death suppressor *ced-9* encodes a membrane-associated protein functionally and structurally related to Bcl-2, the prototypic anti-apoptotic oncogene of vertebrates (Adams and Cory, 1998). CED-4 has recently been identified as an adaptor protein critical in the activation of the caspase degradative machinery (Chinnaiyan et al., 1997).

Caspases are produced as inactive zymogen precursors which are activated by cleavage at sites that conform to caspase substrate consensus sequences (Thornberry and Lazebnik, 1998). Downstream or "effector" caspases appear to be largely responsible for implementing apoptosis by cleaving their cellular substrates. In a way still imperfectly understood, this precipitates the orchestrated collapse of the cell characteristic of metazoan apoptosis—cell shrinkage and fragmentation, chromatin condensation, and DNA degradation, followed by the rapid phagocytosis of apoptotic debris by neighboring cells.

Effector caspases are themselves activated via cleavage by upstream or "apical" caspases. However, activation of "apical" caspases appears to arise through an autocatalytic process in which multiple procaspase molecules are brought into close proximity in a multiprotein complex: the low intrinsic protease activity of the procaspase is then sufficient to trigger intermolecular cleavage and subsequent activation. Formation of such procaspase complexes is mediated by the prodomains of the apical caspases that interact with adaptor molecules to form the "apoptosome." The adaptor molecules serve as protein platforms that both mediate procaspase aggregation and couple caspase activation to various signaling pathways. In *C. elegans* for example, the adaptor molecule CED-4 is required to activate the CED-3 caspase and binds the prodomain of CED-3 via a conserved interaction domain christened CARD (caspase activation and recruitment domain). The interaction of CED-9 with CED-4 quells CED-4's ability to activate CED-3.

The closest known human homolog of CED-4 is Apaf1. Apaf1 activates caspase 9 via the proenzyme's CARD domain. However, this activation requires holo-cytochrome C, which is released from the intermembrane space of mitochrondria by an as-yet-unexplained mechanism and appears to be an important trigger of apoptosis in vertebrates (Green and Reed, 1998). It is not known whether cytochrome c fulfills a similar pivotal role in fomenting apoptosis in the nematode. However, even if it does, it is unlikely that any genetic analysis would uncover a role for cytochrome c in apoptosis, given the essential role of cytochrome c in electron transport. Nonetheless, the remarkable homology in basal apoptotic machineries of nematodes and mammals argues for its analogous proapoptotic role in the worm. In vertebrates the Bcl2 protector proteins appear, like CED-9, to suppress caspase 9 activation through direct interaction with Apaf1 (Hu et al., 1998; Pan et al., 1998). However, they also have a key role in suppressing cytochrome c release from mitochondria, thereby thwarting caspase 9 activation. In vertebrates, the protective action of Bcl2 proteins is antagonized by a family of proteins, all of which share homology with the BH3 (Bcl2 homology region 3) domain of Bcl2 (Kelekar and Thompson, 1998) and are subject to modulation by a variety of upstream signals such as phosphorylation or caspase cleavage. In *C. elegans*, the BH3 protein EGL-1 has recently been identified as essential for cell death (Conradt and Horvitz, 1998).

One important way in which vertebrate cell death appears to differ from that of its invertebrate counterparts is in the existence of so-called death receptors, a family of transmembrane proteins of which the prototypes are Fas/CD95 and TNFR1. When activated by their ligands, these receptors recruit a discrete intracellular death complex mediated by a novel family of adaptor molecules. The prototypic adaptor is FADD/MORT1, which engages and activates a discrete class of apical caspases, most notably caspase 8, via a unique prodomain moiety called the death effector domain (DED) (Ashkenazi and Dixit, 1998). In effect, the CD95–FADD–caspase 8 complex is another type of "apoptosome" that results in autocatalytic *trans*-cleavage of caspase 8 and subsequent activation of downstream effector caspases. Thus far, no counterparts of the CD95/TNFR1 receptors or their ligands have been identified in invertebrates, and

it is conceivable that such "death receptors" do not exist in invertebrates. CD95, in particular, is specifically utilized as a means to trigger apoptosis in infected or "foreign" cells by the vertebrate immune system and is involved in the self-deletion of potentially malignant cells (Evan and Littlewood, 1998), neither of which is required in invertebrates that lack complex immune systems and do not suffer the continuous onslaught of oncogenic mutations within their soma.

Aside from the nematode, the darling of the geneticist is the fruit fly *D. melanogaster*. Substantial genetic analysis in this organism has, likewise, exposed a whole paraphernalia regulating cell death. Elegant studies from the laboratory of Hermann Steller identified a region of chromosome 3, 75C1-2, that is essential for all the extensive developmental cell deaths during *Drosophila* embryogenesis (White et al., 1994). Subsequently, three genes were identified within this region—*reaper*, *hid*, and *grim*, each of which is a key mediator of developmental cell death. Expression of *reaper* and *grim* is confined to those cells doomed to die and is induced in response to a variety of lethal insults such as X-irradiation. In contrast, *hid* is expressed not only in most doomed cells but also in many cells that survive. Intriguing though all this is, it presents something of a mystery because there are as yet no known counterparts of any of these genes in either nematode or mammal. Conversely, genetic analysis has failed to uncover evidence for Bcl2/CED-9 proteins in the fly. Nor is it known whether cytochrome c plays a central role in the fly analogous to that in vertebrates. Nonetheless, *Drosophila* shares features of the apoptosis machinery with worm and vertebrate. For example, flies possess caspases, which did not emerge from genetic analysis but from brute force searching on the basis of presumed sequence homology. Moreover, one of these caspases (DCP-2/Dredd) shares a DED motif with vertebrate caspase 8, the classical effector of the Fas pathway. This may indicate that analogous signaling pathways exist in the fly. Finally, *Drosophila*, like worms and vertebrates, possesses IAPs, evolutionarily conserved proteins of unclear function originally identified as "inhibitors of apoptosis" in baculovirus.

At present, the significant apparent differences between worm, fly, and man make it difficult to determine any coherent and universal architecture for the control and execution of apoptosis. Indeed, the fly is something of an outlier (Table 1). It remains to be seen whether these differences are real, perhaps reflecting differing requirements in each type of organism, or are merely indications of our limited state of knowledge.

Table 1. Comparison of Known Effectors and Regulators of Apoptosis in *C. elegans*, *Drosophila*, and Man

Component	*C. elegans*	*H. sapiens*	*D. melangogaster*
Caspase	CED-3	Caspases 1–14	DCP-1, drICE, etc.
Adaptor	CED-4	Apaf1	?
Protector	CED-9	Bcl2, $BclX_L$	?
Cofactor	?	Cytochrome c	?
BH3 modulator[a]	EGL-1	Bax, Bak, Bid, Bad, etc.	?
Death receptor	?	CD95/Fas, TNFR1, etc	?
Apoptotic effector	?	?	Hid, Reaper, Grim
IAP[b]	CeBIR-1, CeBIR-2	cIAP1, cIAP2, XIAP, etc.	DIAP-1, DIAP-2

[a] Proapoptotic members of the Bcl2 family.
[b] Homologs of baculovirus inhibitor of apoptosis proteins.

Control of Apoptosis

Self-evidently, apoptosis is a potentially catastrophic process that must be tightly controlled. Some years ago, Raff (1992) posited the notion that cell death is a default state of metazoan cells that must be continuously gainsaid by environmental signals. Such "survival signals" are normally provided by neighboring cells that, often being of a differing lineage, limit the survival of a metazoan cell to a precise trophic environment within the soma. Cells that become misplaced, perhaps through injury, developmental error, or malignancy, are deprived of their requisite trophic support and so spontaneously delete themselves. Such "default" death is an essential building block of many complex tissues, most notably the immune and central nervous systems, both of which arise through overproduction of cells followed by spontaneous suicide of those cells that fail to establish productive antigenic specificities or functional synaptic connections, respectively. In addition, the hard-wiring of each somatic cell with an inbuilt autodestruct mechanism renders the elimination of infected cells simple: a cytotoxic T cell needs only to activate the destruct sequence to remove the source of infection.

The "default" death pathway is suppressed by extracellular survival factors such as cytokines and hormones, direct cellular interactions with neighbors or extracellular matrix (ECM), and synaptic connections. For example, many cell types require integrin-mediated adhesion, either to each other or to extracellular matrix proteins, for their survival. This important mechanism restricts the location and mobility of cells whose viability depends upon direct physical contact with their source of survival signal. Thus, epithelial cells undergo anoikis—detachment-induced apoptosis—when deprived of ECM attachment: tubular structures form during embryogenesis because, during cavitation, cells in the interior of a cylinder lose contact with basement membrane and consequently die. Indeed, cell death through adhesion deprivation is an essential component of development in the immune system, in neural tube closure, and in tissue regression and wound healing. Soluble survival factors also play key roles in the establishment and maintenance of metazoan tissues. Well-characterized examples in vertebrates include neurotrophic factors, such as NGF, required for maintenance of cell viability in the central and peripheral nervous systems (Raff et al., 1993), and the insulin-like growth factors, promiscuous survival factors active in many tissues. In vertebrates, survival factors are critical determinants of tissue viability and degeneration and their action is implicated in carcinogenesis.

There is substantial evidence for the importance of survival factors in invertebrates. In particular, *Drosophila* offers many examples in which trophic signals are required for the establishment and maintenance of various

tissues. Perhaps the best characterized is the *Drosophila* eye, an organ that, at least within the redoubt of the laboratory, is dispensable for the survival of the organism and can therefore be subjected to the most intense genetic scrutiny (Freeman, 1997). Each of the ~800 ommatidia within the eye shares the same organization of photoreceptors, an organization disrupted in a legion of categorized mutants. The eponymous *sevenless* mutation deletes the R7 photoreceptor from each ommatidium: the affected gene encodes a receptor tyrosine kinase whose downstream effectors have delineated an archetypal Ras-Raf-MAPK signaling pathway common to flies, nematodes, and mammals (and, hence, presumably to all metazoans). Sevenless activates Dras1, which triggers the MAP kinase cascade: the MAP kinase kinase kinase Draf activates the MAPK2 Dsor, which activates the MAPK Rolled, which then regulates the activity of the mutually antagonistic transcription factors Pointed P2 and Yan. Pointed P2 promotes R7 establishment whereas Yan inhibits it. Activation of this pathway in the presumptive R7 cell, through ligation of Sevenless by its cognate ligand Boss on the neighboring R8 photoreceptor, is required to trigger terminal R7 differentiation. However, Sevenless signaling is, by itself, insufficient for R7 formation. In addition, signaling through the *Drosophila* EGF receptor (DER) is needed, amongst other things, to maintain the viability of the emerging R7 cells. Thus, the DER ligands (e.g., Spitz, Gurken, and Vein) act as survival factors in certain tissues. In contrast Argos, an antagonist ligand that inhibits DER activation, promotes cell death in such tissues.

How Does the DER/Ras Signaling Pathway Act to Suppress Cell Death?

Two papers in this issue of *Cell* (Bergmann et al., 1998; Karuda and White, 1998) indicate that a critical target of this pathway is the death-inducing protein Hid. Karuda and White used a genetic screen for mutations that ameliorate the lethality of the proapoptotic Reaper protein transgenically targeted to the *Drosophila* eye using the glass multimer reporter (GMR). Mutations that interfere with any component of the DER-Ras-MAPK pathway exacerbate Reaper-mediated killing whilst corresponding gain-of-function mutations suppress it. The anti-apoptotic effect of the Ras/MAPK pathway is not confined to the eye: disseminated expression of a constitutively active Ras mutant (Q13) greatly suppresses the widespread cell death that accompanies normal embryonic development in the fly. This developmental death requires genes within the 75C1-2 region of *Drosophila* chromosome 3 that contains the apoptotic effectors *reaper*, *hid*, and *grim*. Thus, the product of one or more of these genes is the initiator of cell death triggered by interfering with Ras/MAPK signaling.

Subsequent analysis showed that expression of *hid*, but not *reaper* or *grim*, is directly modulated by Ras/MAPK signaling. Activating mutations of either Dras1 or Draf suppress *hid* expression, an effect mimicked by ectopic expression of Pointed P2, an Ets domain transcription factor that is functionally activated by MAPK. Conversely, both a dominant interfering mutant of the DER receptor and ectopic expression of Yan, a transcription factor that is inactivated by MAPK and which antagonizes Pointed P2, induce accumulation of *hid* mRNA and promote apoptosis. An important role for *hid* as a target for Ras/MAPK signaling is confirmed by the fact that such ectopic apoptosis is, at least partially, inhibited in hid^{null} mutants. hid^{null} mutants are also resistant to killing by ectopically expressed Reaper, which might suggest that Hid is a necessary effector of Reaper action. Curiously, however, hid^{null} mutants also exhibit resistance to killing by ectopically expressed *hid*, an observation that may indicate that Hid is lethal only above a critical threshold level, one never reached in the absence of expression of the endogenous gene.

An alternative explanation might be that Hid action is also modulated by posttranscriptional mechanisms. This notion is validated by the study of Bergmann et al., who used a genetic screen designed to look directly for modifiers of eye ablation induced by GMR-directed expression of *hid*. Again, the initial trawl exposed a clutch of genes that implicate the DER/Ras/MAPK pathway in regulating Hid-mediated cell death: *gap1*, encoding a GTPase-activating protein, and *sprouty* and *argos*, which encode secreted inhibitors of DER activation. Again, loss-of-function alleles of *DER*, *Dras1*, *Draf*, or *rolled* exacerbate Hid killing, whereas the corresponding gain-of-function mutations inhibit it. In contrast to Karuda and White, Bergmann et al. find no evidence for suppression of Reaper-induced apoptosis by Ras, although both agree that Grim-induced cell death is independent of Ras signaling.

In all organisms, Ras has multiple downstream effectors whose relative contribution to any Ras-dependent process may be dissected using Ras effector mutants that activate only one effector pathway at a time (Downward, 1998). Bergmann et al. use effector mutants to confirm that the Raf/MAPK pathway is the major antiapoptotic pathway in the fly eye, although a weaker ancillary survival signal is mediated by the phosphatidylinositol 3-kinase (PI3-K)/Dakt pathway; the arcane Ral GDS pathway does not appear to be involved. Bergmann et al. acknowledge that the Ras/MAPK pathway affects *hid* expression, but concentrate on the possibility that MAPK inhibits Hid by directly phosphorylating the Hid protein. They confirm this genetically by showing that mutation of key MAPK target residues in Hid generates a protein with enhanced lethality over the wild type and that is unaffected by Ras/MAPK signaling. Thus, the DER/Ras/MAPK pathway is a critical pathway promoting cell survival that has the Hid protein as its principal target.

Does this discovery have wider implications for our understanding of the regulation of apoptosis? In principle, the answer should be yes. However, there are two potential "flies in this ointment." The first has already been alluded to—no counterpart of Hid is known in the nematode or vertebrate. There are two possibilities: either Hid is present in these organisms but has yet to reveal itself in any genetic or biochemical analysis, or Hid (and maybe Grim and Reaper too) are arthropod inventions. The remarkable overall similarities in metazoan biology makes the latter possibility unpalatable, but without any understanding of Hid function at the molecular level, it is difficult to search for analogous functions in other species. In order to place Hid, and the pathways that regulate it, into a larger context, elucidation of Hid functions is essential.

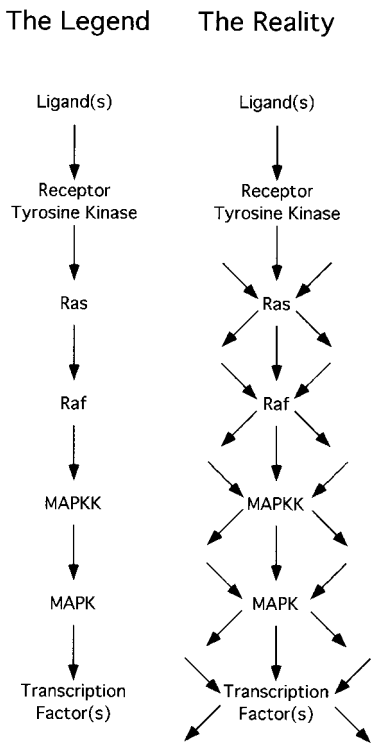

Figure 1. The Ras/MAP Kinase Signaling Pathway: The Legend and the Reality
See text for details.

The second problem lies in any interpretation of signal transduction as a linear sequence of affectors and effectors. Although initial genetic studies in the fly eye indicated a comfortingly linear Ras→Raf→MAPKK→MAPK signaling sequence, an increasing amount of data indicates that all is not so simple. Both the receptors at the apex of this pathway and Ras itself are linked to a variety of other signaling pathways, and the same may well be true of the MAPKK Dsor and the MAPK Rolled. Furthermore, flow down the Ras→Raf→MAPK pathway is subject to extensive modulation by other intersecting signaling pathways within each cell type and through control of the intensity and timing of activation (Freeman, 1997).

As in the fly, the well-characterized mammalian survival signaling pathways activated by the high affinity NGF receptor TrkA and by IGF-IR also utilize Ras as a key nexus. However, although the Raf/MAPK signaling pathway suppresses apoptosis in some neuronal and myeloid cells, studies with Ras effector mutants (identical to those used in the study of Bergmann et al.) indicate that the predominant survival signal appears to route through the Ras effector PI3-K and the serine/threonine protein kinase Akt (PKB) (Downward, 1998). One critical target of Akt is Bad, a BH3 protein and antagonist of Bcl2 that is inactivated by Akt phosphorylation (Franke and Cantley, 1997). Although in *Drosophila*, Akt exerts appreciable antiapoptotic activity (Staveley et al., 1998), Bergmann et al. find that it provides only a weak ancillary survival signal in the fly eye. So does this indicate a different use of pathways in fly and vertebrate? Probably not. It is clear that Ras sits at the apex of multiple effectors, each of which presides over its own suite of diverging and multifunctional signaling pathways. In such a network of pleiotropic effectors, no single cell fate is ever really controlled by any single effector (Figure 1). Rather, the predominant outcome of Ras signaling is a result of a network of interactions between differing signaling pathways wherein some signals are reinforced while others are gated. Fate, in cells as in man, is a tangled web.

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