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Review

Caspases: an ancient cellular sword of Damocles

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Abstract

Caspases are a family of cysteine proteases homologous to the *Caenorhabditis elegans* programmed cell death gene product CED-3. Caspases and their distant relatives, meta- and paracaspases, have been found in phylogenetically distant nonmetazoan groups, including plants, fungi and prokaryotes. This review summarizes the current information on the mechanisms and functions of non-mammalian caspases and their relatives in apoptotic and nonapoptotic processes, and explores the possible evolutionary origin of the caspase family.

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Abbreviations: PCD, programmed cell death; PARP, poly (ADP-ribose) polymerase; DD, death domain; YCA1, yeast caspase 1; GLC, germline clone; CARD, caspase activation and recruitment domain; DED, death effector domain; TNF, tumor necrosis factor; RHG, repear, grim and hid

Introduction

As the executioners of apoptosis, caspases are a cellular sword of Damocles, lurking inside almost every metazoan cell, poised to kill it in response to intrinsic or extrinsic signals. The literature on caspases and cell death has gone from minimal to mind-boggling in the last 10 years, but there remains much to learn. Rational therapeutic manipulation of apoptotic targets like the caspases holds significant promise for human diseases ranging from cancer to neurodegeneration, but the intricacies of mammalian cell death regulation present a daunting barrier to this long-term goal. As with the study of any complex biological phenomenon, crucial insights can be gained from the study of simpler model systems, including invertebrate caspases and their close cousins in non-metazoans. Here, we review selected research on the caspases and related proteins of these 'lower' organisms and discuss how this work may contribute to our understanding of the functions and evolutionary origins of the mammalian caspases.

Caspases and their relatives in non-metazoans

The idea that cell death might be an evolutionary prerequisite for the development of multicellularity is not new (for example, see Weismann[1]). Indeed, a wealth of evidence now points to an essential role for apoptosis in animal development and homeostasis. Therefore, it is perhaps not surprising that caspases have been found in nearly every metazoan examined thus far, including not only the well-known nematode and arthropod examples (discussed below) but also phylogenetically earlier groups such as cnidarians.[2] The remarkable structural and biochemical similarities among the caspases of diverse phyla underline both the importance of these enzymes and the significance of cell death itself as a fundamental biological process, at least in the metazoans.

No true caspases have been identified thus far outside the metazoan clade, but there is an increasing body of evidence for non-metazoan programmed cell death (PCD) and a possible role of caspase-like enzymes in its execution. For example, caspase-like activities have been implicated in PCD in several plant systems. Cleavage of the canonical caspase tetrapeptide substrates YVAD and DEVD was observed in tobacco tissue undergoing PCD in response to *Pseudomonas* or tobacco mosaic virus infection[3] or treatment with toxic drugs such as menadione,[4] staurosporine and cycloheximide.[5] Similarly, caspase inhibitors (but not inhibitors of other proteases) blocked PCD in the plant hypersensitivity response during infection[3] or toxic drug treatment[5] and suppressed some of the features reminiscent of apoptosis, such as DNA fragmentation and PARP cleavage.[4] Fluorescently tagged, irreversible caspase inhibitors were also found to label proteins in extracts made from plant cells undergoing PCD but not from living cultures,[5] suggesting that the activation of caspase-like proteins occurs only in response to death stimuli. The presumptive proteases responsible for these activities remain to be identified, but it is tempting to speculate that they may be caspases or related molecules, extending the importance of these enzymes into the plant kingdom.

Remarkably, caspase-like activities have been implicated in PCD even in ancient unicellular eukaryotes. For example, treatment of the kinetoplastid protist *Leishmania* with hydrogen peroxide[6] or staurosporine[7] resulted in a form of cell death with some features of caspase-dependent apoptosis, including DNA fragmentation, Poly (ADP-ribose) Polymerase (PARP) cleavage and DEVDase activity. Caspase inhibitors blocked this death, implicating caspase-like enzymes in this presumably active process. Similar observations have been made in related protist systems, such as trypanosomes killed by exposure to human serum.[8] The physiological and evolutionary significance of PCD in unicellular organisms is still a matter of debate, but the possibility that caspase-like enzymes may be involved in such processes could lend

important insights into the ancient origins of the metazoan caspases, as we shall discuss later.

Intriguing candidates for the caspase-like activities described in these plant and protist systems were provided by the recent discovery of metacaspases and paracaspases, two classes of genes with modest but distinct sequence similarity to metazoan caspases.[9] Thus far, metacaspases have been found in plants, fungi, protists and some groups of bacteria, whereas paracaspases have been identified in animals, slime molds and bacteria.[9,10] These two groups of proteins share the universally conserved HG-spacer-C cysteine/histidine catalytic dyad motif found in caspases[9] (see Figure 1), placing all three groups into a large family of related thiol endopeptidases that also includes the legumains, hemoglobinases and the bacterial proteases clostripain and gingipain R[11,12] Currently, little is known about the functions of the metacaspases or paracaspases, but comparative genomic studies suggest that they may play a role in cell death in many contexts. For example, paracaspases from humans, zebrafish and *C. elegans* are predicted to contain death domains (DD), a homotypic protein–protein interaction module found in many molecules involved in metazoan apoptosis,[9] and some metacaspase genes in *Arabidopsis* appear to contain an N-terminal Zn-Pro domain, a motif also found in LSD-1, a protein known to be involved in PCD in plants.[13]

The functional characterization of the metacaspases and paracaspases is still in its early stages and at present it is not clear even whether some or all of these proteins possess protease activity. For example, the human paracaspase did not cause apoptosis or undergo autoprocessing (a hallmark of caspase activation) when transfected in human cells, even when artificially oligomerized.[9] Furthermore, the purified protein itself was unable to cleave any caspase substrate tested thus far.[9] On the other hand, a metacaspase gene recently identified in *Saecharomyces cerevisiae* and dubbed yeast caspase 1 (YCA1) has been implicated in cell death.[14] Overexpression of wild-type YCA1 in yeast led to the appearance of YCA1 cleavage products but the expression of a YCA1 construct with its putative active site cysteine mutated did not, suggesting that YCA1 undergoes true autoprocessing *in vivo*.[14] Although overexpression of YCA1 itself did not kill yeast, it did sensitize them to killing by hydrogen peroxide and resulted in an increase in DEVDase and IETDase activities, all of which could be blocked by a caspase inhibitor.[14] A YCA1 disruptant strain resisted hydrogen peroxide killing better than wild-type yeast at low cell densities, suggesting that endogenous YCA1 may play a role in yeast cell death under some circumstances.[14] Recently, another metacaspase was cloned from the filamentous fungus *Aspergillus* and its overexpression was shown to cause growth inhibition and some morphological changes reminiscent of apoptosis in that system.[15] Therefore, at least in fungi, it may be that metacaspases participate in endogenous cell death regulation.

Comparative genomic analyses of the caspase phylogenetic tree have led to the proposal that metazoan caspases arose late in evolution, as a result of horizontal gene transfer from bacteria.[10] If this hypothesis is correct, it is unlikely that true caspases will be found in non-metazoans. Instead, metacaspases or paracaspases may have evolved to play "caspase-like" roles in the regulation of cell death in these organisms. Indeed, the bacterial protease gingipain K, which belongs to the same clan of cysteine proteases as the caspases,[12] has been found to be inhibited by the baculovirus caspase inhibitory protein p35,[16] suggesting that other proteases related to the caspases may turn out to be responsible for the caspase-like activities observed in non-metazoan systems. Future *in vivo* functional studies of the metacaspases, paracaspases and related enzymes will help to resolve these uncertainties.

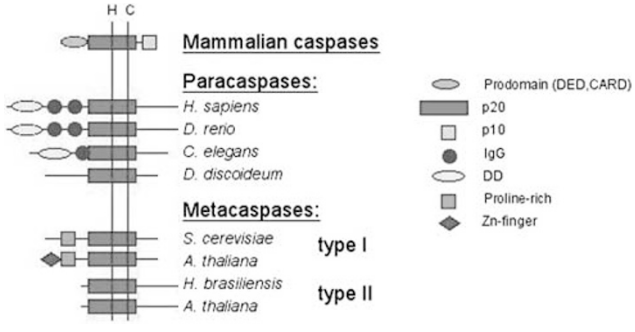

Figure 1 Domain architecture of metacaspases and paracaspases. The domains of the mammalian caspases and meta- and paracaspases from different organisms are shown (see Uren *et al*.[9] for details). DD – death domain; DED – death effector domain; CARD – caspase activation and recruitment domain; IgG – immunoglobulin G-like domain; p20 – caspase large subunit; p10 – caspase small subunit. Sequence alignment resulted in the further subdivision of metacaspases into type I and II.[77] H, C refer to the conserved histidine and cysteine residues of the caspase catalytic triad. Note that only a few of the identified meta- and paracaspases are shown[10]

C. elegans caspases

Of the true metazoan caspases, the first and perhaps the most thoroughly characterized is the CED-3 protein of the nematode *C. elegans*. The *ced-3* gene was initially identified in a genetic screen for mutants with abnormal cell death, where *ced-3* loss-of-function mutations were found to block most, if not all, apoptosis during animal development.[17] Interestingly, *ced-3* mutant animals appeared behaviorally and metabolically normal despite having extra "undead" cells, and at least some of the undead cells themselves were subsequently shown to differentiate and function (e.g., as neurons).[17,18] These observations suggested that the function of *ced-3* was to kill otherwise healthy cells in a genetically programmed and developmentally precise way.

When *ced-3* was cloned, its homology to the murine and human protease interleukin-1 converting enzyme (subsequently renamed caspase-1) was recognized.[19] This key discovery suggested a simple model for *ced-3* function, wherein the CED-3 protease was responsible for cleaving vital substrates in the cell in order to kill it from the inside, consistent with the prior observation that *ced-3* functioned cell autonomously in apoptosis.[20] In support of this hypothesis, *ced-3* was found to induce apoptosis when transfected into Rat-1 fibroblasts[21] or ectopically expressed in *C. elegans*,[22] confirming its role as cell death effector.

CED-3 is expressed throughout *C. elegans* development,[19] probably in most if not all cells within the animal.[22] Like other caspases, CED-3 is expressed as an inactive zymogen and undergoes autocatalytic processing to generate 10 and 20 kDa active subunits and release the N-terminal prodomain.[23,24] The resulting mature, autoprocessed CED-3 protein is a heterodimeric cysteine protease that cleaves its substrates carboxyl to an aspartate residue and recognizes a tetrapeptide motif for cleavage.[24,25] The CED-3 catalytic cysteine is embedded within a QACRG pentapeptide motif, which is the longest sequence conserved between *ced-3* and caspase-1, underlining the importance of protease activity for caspase function.[19] Indeed, a CED-3 mutant in which the catalytic cysteine was changed to serine did not cause apoptosis either in mammalian cells[21] or *C. elegans* animals,[26] and the baculovirus caspase inhibitor protein p35 blocked both CED-3 protease activity *in vitro* and apoptosis in *C. elegans*.[25] Studies with combinatorial peptide libraries showed that the preferred tetrapeptide substrate for CED-3 is DEXD, the same sequence preferred by mammalian effector caspases like caspases-3 and –7.[24] This *in vitro* substrate preference agreed well with the DSVD autocleavage site of CED-3[23] and fit the model that CED-3, as the major or sole caspase involved in *C. elegans* apoptosis, first undergoes autoprocessing and then act as an executioner of cell death.[24]

The autoactivation of the CED-3 zymogen has been shown to require the function of the CED-4 adaptor molecule. The *ced-4* gene was identified in the same screen as *ced-3* and, like *ced-3*, was found to be required for almost all apoptosis during *C. elegans* development.[17] Genetically, *ced-4* was found to act upstream of *ced-3*,[22] providing a possible clue that CED-4 might cause apoptosis by somehow activating quiescent CED-3. On a molecular level, expression of CED-4 was shown to enhance both the processing of CED-3 zymogen and CED-3-induced apoptosis in insect cells.[27] Furthermore, these effects depended on an intact CED-3 prodomain,[27] suggesting that CED-4 acts through CED-3 to cause cell death, perhaps through a direct interaction requiring the CED-3 prodomain. Indeed, CED-4 was found to interact physically with both the CED-3 prodomain and protease domain in cells,[28,29] and purified CED-4 could mediate CED-3 autoprocessing *in vitro* in a prodomain-dependent fashion.[30]

As with certain other caspases, the autoactivation of CED-3 is thought to proceed via an induced-proximity mechanism, wherein CED-3 molecules are brought together to facilitate autocleavage in *trans*. Indeed, enforcing CED-3 dimerization through the use of a chemical dimerizer caused autoprocessing of both *in vitro* translated CED-3 and transfected CED-3 in HeLa cells.[31] The induced proximity of multiple CED-3 zymogens is likely mediated *in vivo* by its association with CED-4, which can oligomerize with itself and CED-3 simultaneously.[31] Therefore, the current model postulates that the CED-3 zymogen autoprocesses in *trans* when CED-3 molecules are brought together through the action of CED-4 oligomerization. This allosteric model for CED-3 activation parallels the apoptosome model for the activation of caspase-9 by the mammalian CED-4 homolog, Apaf-1 (see Degterev *et al*.[32] for a review of mammalian caspase activation). However, a key difference between CED-4-mediated CED-3 activation and Apaf-1-mediated caspase-9 activation is the lack of a demonstrable role for cytochrome *c* in the activation of CED-3.[33,34] Since mitochondrial cytochrome *c* release is an important aspect of mammalian apoptosis, the dearth of evidence for a role for cytochrome *c* in *C. elegans* apoptosis raises the question of whether mitochondrial damage is required for the activation of CED-3. However, factors such as Endo-G and WAH-1 are released from mitochondria during *C. elegans* apoptosis in a *ced-3*-dependent manner and do contribute to DNA degradation and the final demise of the cell.[35,36]

Although the best-characterized role of CED-3 *in vivo* is to mediate protease-dependent apoptosis during *C. elegans* development, the *ced-3* gene may have other functions in the nematode. Recent genetic analyses have indicated that CED-3 may also play a protease-independent role in apoptosis *in vivo*. This possibility was suggested by the identification of the *ced-3* n2452 allele, which eliminates the entire CED-3 protease domain by truncating the *ced-3* mRNA after the third intron.[26] Such an allele might be predicted to function as a genetic null and exhibit a severe *ced-3* phenotype, but in fact the n2452 allele is weaker than other *ced-3* alleles identified previously, as evidenced by the fact that *ced-3*(n2452) animals show fewer undead cells in the pharynx than other animals with loss-of-function mutations in *ced-3* or *ced-4*.[26] This observation suggested that the protease activity of CED-3 might not be required for all apoptosis in *C. elegans*, and that the prodomain of CED-3 might somehow function independently in cell death. Interestingly, expression of a transgene encoding amino acids 1–374 of the CED-3 prodomain alone resulted in the block of some cell deaths in the anterior pharynx of *ced-3*(n2452) animals, suggesting that the CED-3 prodomain can block protease-independent cell deaths *in vivo*.[26] One possible explanation for this potentially puzzling observation is that CED-3 may contain a protease-independent killing activity that is not disrupted by the n2452 allele, implying that n2452 is not a true genetic null. On the other hand, if n2452 is indeed a null, two alternative possibilities exist: either the n2452 mutation may eliminate both a killer and a protective function of CED-3, resulting in its relatively mild phenotype, or the n2452 phenotype may instead indicate that other, stronger *ced-3* alleles in fact act as dominant-negatives that interfere with an unidentified CED-3 protease-independent killing activity. A precise deletion of the entire *ced-3* gene and an examination of the resulting phenotype will probably be required to distinguish among these possibilities.

Aside from its role in developmental cell death, *ced-3* may also function in a primitive form of immune protection in *C. elegans*. A recent study found that infection of *C. elegans* by the pathogenic bacterium *Salmonella typhimurium* induced cell death in the animal's germ line, and that these deaths were blocked in *ced-3* or *ced-4* mutant backgrounds.[37] Interestingly, these mutant animals were also more susceptible to *Salmonella*-mediated killing, even though the mutants' lifespans were not different from wild-type animals in the absence of infection.[37] It may be that the germline cell deaths observed in this infection model (or perhaps somatic cell deaths that remained undetected in the study) provide a measure of bacterial resistance to *C. elegans*. An alternative but perhaps more intriguing possibility is that *ced-3* function

might be required for the activation of disease-resistance pathways in the soma, just as certain caspases are in higher metazoans (see below). A similar pathway in plants, termed the hypersensitive response, may also employ caspase-like proteins for an innate immune function.[13] Therefore, it is possible that the nonapoptotic, immune functions of caspase-like enzymes are at least as evolutionarily ancient as their apoptosis-associated activities. Further investigations of the nonapoptotic functions of *ced-3* will prove interesting and may shed light on this possibility.

In addition to the well-known *ced-3*, the *C. elegans* genome contains three other caspase genes, termed *csp-1*, *-2* and *-3*, which appear to encode three, two and one isoforms of mRNA, respectively.[38] Interestingly, the putative catalytic motifs of these proteins are atypical for the caspase family, with the CSP-1B isoform containing an SACRG pentapeptide and the CSP-2 isoforms containing a VVCRG sequence, as opposed to the typical QACRG pentapeptide found in CED-3 and many other caspases.[38] Since the pentapeptide motif plays a role in determining caspase substrate specificity, the specificities of the CSP proteins may differ substantially from those of other family members. Recombinant CSP-2A and -2B failed to autoprocess and may not be active enzymes, but CSP-1B does appear to function as an autoactivating protease.[38] CSP-1B can cleave itself and CED-3 (although not at CED-3's natural autoprocessing site), suggesting that CSP-1B could potentially act upstream of CED-3 in a proteolytic cascade,[38] akin to the role of apical activator caspases in mammalian systems.[32] Indeed, the existence of additional caspases acting with CED-3 in a partially redundant pathway could perhaps explain the abovementioned *ced-3*(n2452) phenotype. However, the physiological significance of the *csp* genes is not yet clear because they currently have no described *in vivo* function, apoptotic or otherwise. Future experiments characterizing the function(s) of the *csp* genes may provide insight into the broader roles of caspases in *C. elegans* development and homeostasis, and perhaps into analogous mammalian processes as well.

Drosophila caspases

Early evidence that CED-3-like enzymes might be conserved in *Drosophila* came from the observation that expression of the caspase inhibitor p35 protein blocked apoptotic death in flies,[39] although the precise mechanism of p35 activity was not understood at the time. Subsequently, seven predominantly alliterative caspases have been identified in *Drosophila*: *Dcp-1*, *Dronc*, *Drice*, *Dredd/Dcp-2*, *Decay*, *Damm/Daydream* and *Strica/Dream*[40–47] (see Table 1). Of these, the first four have been shown to participate in apoptosis.

Dcp-1

Dcp-1, the first *Drosophila* caspase gene identified, is most similar in sequence to mammalian caspases-3 and -6 and, like these effector caspases, DCP-1 lacks a long prodomain,[40] suggesting that it may play a downstream role in apoptotic pathways. DCP-1 contains the highly conserved QACRG pentapeptide catalytic site and was shown to cleave known caspase substrates such as PARP, p35 and DEVD tetrapeptide *in vitro*, all of which could be inhibited by DEVD-CHO.[40,48,49] Like CED-3, *Dcp-1* induced apoptosis when transfected into mammalian cells.[40]

Table 1 The *Drosophila* caspases

Catalaytic			
Caspase	**Prodomain**	**Site**	**Loss-of-function**
Dcp-1	Short	QACRG	Third instar lethal; nurse cell dumping deffect in GLCs
Dronc	Long, CARD	PFCRG	Rnai is embryonic lethal; protects *vs.* Elger killing
Drice	Short	QACQG	Depletion from s2 extracts abolishes all caspase activity
Dredd	Long, DED	QACQE	Mutants defective in innate immune response *vs.* bacteria
Decay	Short	QACRG	Unknown
Damm	Short	QACKG	Unknown
Strica	long, Ser/Thr-rich	QACKG	Unknown

Information on the features of the prodomains, catalytic site pentapeptides and effects of loss-of-function mutations or manipulations (if available) of the seven *Drosophila* caspases are shown. Caspases with long prodomains may function as apical enzymes in proteolytic cascades (as has been proposed for *Dronc* and *Dredd*), whereas caspases with short prodomains may serve as downstream effector caspases. GLCs – germline clones. See text for complete references.

In situ hybridization showed that *Dcp-1* mRNA is expressed in embryos even at early developmental stages, before zygotic transcription has begun, indicating that it is a maternal contribution to the embryo.[40] Later in embryogenesis, the timing of the reduction in *Dcp-1* message levels correlated well with increased resistance of the embryo to apoptosis induced by X-rays or ectopic expression of the cell death gene *Reaper*, suggesting that DCP-1 might be involved in developmental apoptosis.[40] Indeed, a P-element-generated null mutation showed that *Dcp-1* is an essential gene for normal larval development: *dcp-1* larvae lacked imaginal discs and gonads, had fragile trachea and exhibited melanotic tumors, which are thought to arise from an overproliferation of blood cells or an immune reaction to undead cells, either of which may be consistent with a proapoptotic role of *Dcp-1* in development.[40]

Subsequently, germline clones (GLCs) of *dcp-1*-null mutants were generated, allowing the analysis of females lacking the caspase in the germ line.[50] The *dcp-1* GLCs were sterile, laying eggs that failed to survive beyond the first few mitotic divisions.[50] This defect was caused by a "dumpless" phenotype in the GLC nurse cells, where the nurse cells (which normally transfer their cytoplasm to the developing oocyte in an apoptotic dumping process) failed to die and therefore did not dump properly.[50] Closer examination of the nurse cells in the GLCs showed that they did not exhibit the normal actin cytoskeletal rearrangements associated with dumping, and that their nuclei failed to break down, suggesting that DCP-1 is required for both of these processes *in vivo*.[50] Indeed, DCP-1 was later shown to cleave nuclear lamins from *Drosophila* SL2 cell extracts, supporting this conclusion.[49]

Dronc

DRONC contains a long prodomain with a caspase activation and recruitment domain (CARD), a homodimerization domain found in several mammalian caspases. [41] Unlike DCP-1, the catalytic cysteine of DRONC occurs in a PFCRG pentapeptide motif, which is unusual for caspases and was proposed to affect DRONC's substrate specificity.[41] Consistent with this hypothesis, DRONC was shown to cleave DEVD only poorly, but exhibited about five-fold greater activity against the pentapeptide substrate VDVAD, which is also cleaved by caspase-2, DRONC's closest mammalian relative.[41] *In vitro* studies showed that the preferred DRONC tetrapeptide substrate was TATD, but the enzyme also cleaved VEID and IETD, which are also recognized by mammalian activator caspases.[51] In fact, DRONC cleaved GIETD better than IETD, suggesting that the P5 site in DRONC substrates may play a role in its substrate specificity.[51] Interestingly, DRONC was shown to cleave itself at the tetrapeptide site TQTE, demonstrating that DRONC is able to cleave after glutamate as well as aspartate,[51] an unusual property among the caspases described thus far. Although DRONC cleaved several canonical apoptotic substrates such as lamins and the DNA fragmentation factor DREP-1, it did not cleave p35, making DRONC the first p35-insensitive caspase described, owing perhaps to its unique substrate specificity.[52] The unusual autocleavage site of DRONC and its own unique ability to cleave this sequence may serve to reduce the promiscuous activation of DRONC by other caspases *in vivo*, allowing DRONC-dependent pathways to be insulated and controlled separately from other proteolytic cascades within the cell.[51]

Transfected *Dronc* was shown to kill mammalian and *Drosophila* cells[41,52,53] and endogenous DRONC was cleaved in *Drosophila* BG2 cells induced to die by treatment with cycloheximide,[54] indicating that DRONC could be involved in apoptosis in flies. Indeed, *Dronc* mRNA is expressed at all embryonic stages but was found to be dramatically upregulated from the third larval instar stage in the salivary gland and midgut, two tissues that undergo apoptosis at this developmental point in response to a wave of the hormone ecdysone.[41] Interestingly, *Dronc* mRNA was shown to be upregulated directly both in these tissues and the *Drosophila* l(2)mbn cell line by ectopic ecdysone treatment.[41,55] This transcriptional upregulation was subsequently shown to occur via the orphan nuclear hormone receptor ßFTZ-F1 and its target gene, E93.[56,57] DRONC is probably at least partly responsible for ecdysone-induced apoptosis in both developing flies and cell lines because a large increase in VDVADase activity was demonstrated in extracts from the third instar stage and beyond,[41] and RNAi against *Dronc* protected l(2)mbn cells from ecdysone-mediated killing.[55] This or another function of DRONC is apparently essential for embryogenesis because RNAi against *Dronc* was lethal to the animal.[53]

Its long prodomain and CARD module suggested that DRONC might be an apical caspase in *Drosophila*, acting early to cleave downstream effector caspases. Consistent with this model, DRONC was shown to bind to the effector caspase DRICE[52] (see below) and to cleave DRICE *in* vitro.[51] In BG2 cells, cycloheximide treatment induced rapid cleavage of endogenous DRONC, whereas DRICE processing occurred later and was suppressed by RNAi against *Dronc*.[54,58] Interestingly, DRONC was also found to associate with DARK,[53] a *Drosophila* CED-4 homolog,[59–61] suggesting that DRONC might be activated by an allosteric mechanism reminiscent of the CED-3/CED-4 interaction. In fact, extracts from *dark* mutant flies were less able to process DRONC or DRONC substrates *in vitro* than were wild-type extracts.[53]

Recently, toxic treatment of BG2 cells with cycloheximide was shown to cause a shift in endogenous DRONC from its monomeric form ($\sim$50 kDa) to a larger complex ($>$670 kDa).[54] This shift was associated with the processing of DRONC, implying that DRONC might be activated upon recruitment to the putative complex.[54] Interestingly, cytochrome *c* was also shown to shift to a fraction of similar molecular weight under these conditions, suggesting that DRONC may be activated through a mechanism similar to caspase-9 in the apoptosome, with DRONC, DARK and cytochrome *c* all assembling into an apoptosome-like complex at the mitochondrial membrane.[54] The lack of available antibodies against DARK precluded asking whether DARK is also present in this putative complex, although that will clearly be an important future test of this fly apoptosome model.

Finally, a homolog of mammalian tumor necrosis factor (TNF) family was recently cloned in *Drosophila* and named Eiger.[62] Ectopic *Eiger* expression in the *Drosophila* eye caused apoptosis that could be blocked by coexpression of p35, implying the involvement of caspases.[62] The *Drosophila* death effector domain (DED)-containing caspase *Dredd* (see below) was an obvious candidate for participation in a TNF pathway, based on its homology to mammalian caspases that mediate TNF-induced apoptosis. However, mutations in *Dredd* did not suppress EIGER-mediated killing, whereas a mutation in *Dark* and coexpression of a presumably dominant-negative *Dronc* construct did.[62] Thus, DRONC may be the apical *Drosophila* caspase responsible for responding to an apoptotic signal from EIGER and perhaps other unknown TNF family members.[62] As DRONC is resistant to inhibition by p35, these data may suggest that DRONC necessarily acts through the activation of downstream effector caspases in this pathway.

Drice

The caspase DRICE contains a short prodomain and is most similar to the mammalian caspase-3.[42] As with DRONC, the catalytic cysteine of DRICE appears in a noncanonical pentapeptide motif, QACQG, which is also found in mammalian caspase-8 and -10 and may impact on the substrate specificity of DRICE.[42] DRICE was found to cleave typical caspase substrates such as DEVD, p35, lamins, PARP and the *Drosophila* caspase-activated DNase, dCAD[42,49,63,64] but could not cleave either itself or DCP-1,[49] suggesting that DRICE may function as an effector protease at the end of a caspase cascade *in vivo*. Consistent with the high degree of zymogenicity displayed by other effector caspases,[65] the overexpression of full-length *Drice* in *Drosophila* S2 cells did

not induce apoptosis but did sensitize the cells to apoptotic stimuli such as cycloheximide and etoposide, whereas expression of a truncated *Drice* lacking a prodomain did cause cell death.[42,49] These observations suggested that DRICE may participate in apoptosis at least in cell culture but might require an upstream caspase for its activation. Indeed, endogenous DRICE was shown to be processed in S2 cells induced to die by cycloheximide, etoposide or ectopic *Reaper* expression, and specific immunodepletion of DRICE abolished all detectable DEVDase activity in lysates from these cells, suggesting that DRICE might be the primary executioner caspase activated in this system.[42,63] Finally, ectopic expression of *Drice* in the *Drosophila* eye did not produce any apoptotic phenotype.[49] This observation may be consistent with the cellular data suggesting the need for an upstream activator caspase for DRICE function, but may also call into question the possible role of *Drice* in apoptosis *in vivo*. The generation of specific mutations in *Drice* will help to address this question.

Dredd

Dredd was identified independently by two groups in database searches for *Drosophila* genes homologous to mammalian caspase-8 or *C. elegans ced-3*.[43,44] Little is known about the substrates or cleavage site specificity of DREDD, although its catalytic cysteine appears in a QACQE pentapeptide motif, suggesting that its specificity may differ from QACRG-containing caspases.[44] Like caspase-8, DREDD contains a long prodomain with two putative DED domains, a homotypic protein–protein interaction domain also found in some apical mammalian caspases.[43,44] DREDD was therefore proposed to function as an apical caspase *in vivo*. Consistent with this possibility, DREDD was found to associate with DARK by immunoprecipitation, suggesting that DARK might in some way allosterically activate DREDD.[59] In fact, expression of an active-site (presumably dominant-negative) mutant *Dredd* construct blocked apoptosis induced by *Dark* expression in SL2 cells, whereas the corresponding mutant of the effector caspase *Drice* did not,[59] providing evidence that DREDD may act as an upstream caspase *in vivo*. Similarly, dFADD, a *Drosophila* homolog of the proapoptotic DED-containing mammalian adaptor protein FADD, was shown to interact with DREDD even more strongly than DARK did.[66] Coexpression of dFADD in a cell culture system enhanced apoptosis induced by *Dredd* but not *Dronc*, suggesting that DREDD can be activated by more than one accessory protein.[66] Interestingly, these and the abovementioned results obtained with *Eiger* may suggest a differential regulation of dFADD and TNF-induced apoptosis by DREDD and DRONC, respectively, two separate functions that were unified later in evolution by mammalian caspase-8.[32]

In vivo, *Dredd* mRNA was shown to exist in three alternatively spliced isoforms.[44] Like other caspases, *Dredd* expression was observed in young embryos, indicating a maternal contribution of this mRNA.[44] From embryonic stage 11 and beyond, *Dredd* mRNA was found to accumulate specifically within cells that were fated to die, implying that DREDD may have an important role in these developmental cell deaths.[44] Mutations in *Dredd* also rendered flies resistant to UV irradiation, confirming its role in stress-induced apoptosis.[67] Interestingly, the H99 deletion, which eliminates the prodeath *Reaper*, *Hid* and *Grim* genes (RHG), suppressed the induction *Dredd* mRNA and the ectopic expression of RHG genes led to the induction of *Dredd*,[44] implying that RHG proteins somehow influence *Dredd* expression at the transcriptional level, perhaps during normal development. In addition, *Dredd* expression may be influenced *in vivo* by the recently identified KEP1 protein, which was shown to interact directly with *Dredd* mRNA.[68] *Kep1* function appeared to promote the accumulation of the mRNA isoform of *Dredd* encoding active enzyme.[68] Accordingly, loss-of-function mutations in *Kep1* could suppress apoptosis in the eye induced by ectopic *Reaper* expression,[68] just as deletions spanning the *Dredd* locus had been shown to do.[44]

Reminiscent of recent findings with *ced-3*, *Dredd* has also been shown to participate not only in apoptotic pathways but also in the innate immune system of *Drosophila* (reviewed in Hoffmann and Reichhart [69]). Like many invertebrates, *Drosophila* are highly resistant to microbial infection despite their lack of an adaptive immune system. Resistance to infection derives at least in part from the expression of antimicrobial peptides such as Diptericin, which are produced in the fat body. In independent genetic screens for sensitivity to *E. coli* infection[70] or the loss of Diptericin expression,[71] two groups identified mutations in *Dredd* as compromising the innate immune response. Remarkably, the *dredd* mutants showed very specific defects, losing the ability to upregulate peptides directed against Gram-negative bacteria without any defects in the expression of antimycotic peptides like Drosomycin.[70,71] The *dredd* immune deficiency phenotype resembles the loss-of-function mutants in *Relish*, an NF-κB homolog responsible for the transcriptional upregulation of antibacterial peptides. Indeed, RELISH protein was rapidly activated by proteolytic cleavage when larvae were exposed to LPS or bacteria, and this cleavage event was dependent on *Dredd* function.[72] Direct cleavage of RELISH by DREDD has not been definitively demonstrated *in vivo*, but a putative caspase site in RELISH was shown to be necessary for its cleavage in response to LPS stimulation and the two proteins appear to interact when overexpressed in cultured cells.[73] Furthermore, DREDD's role in the *in vivo* immune response is likely protease-dependent, as ectopic expression of p35 in the fat body abolished Diptericin induction in response to bacterial challenge.[67] In the induction of the innate immune response, DREDD appears to function downstream of the intracellular DD-containing protein IMD (for immune deficient),[67] but how signals are transduced from pathogens outside the cell to IMD inside the cell and on to DREDD is not yet clear. Future studies of the immune functions of DREDD and other caspases in *Drosophila* will prove very interesting and may provide insight into the corresponding nonapoptotic functions of the mammalian caspases.

Decay, Damm/Daydream and Strica/Dream

The remaining three *Drosophila* caspases have been cloned only recently and await thorough *in vivo* functional character-

ization. [45–47] DECAY and DAMM lack long prodomains, suggesting that they may act as effector caspases.[45,46] STRICA contains a long prodomain and therefore may act as an apical caspase, although the prodomain does not contain any canonical protein–protein interaction domains, as are found in DRONC or DREDD.[47] Recombinant STRICA did not cleave any protein or tetrapeptide caspase substrate tested, suggesting that it may require additional proteins for its catalytic activation.[47] In contrast, both DECAY and DAMM cleaved the caspase-2 pentapeptide substrate VDVAD.[45,46] Expression of wild-type *Strica* but not a mutant in the active site cysteine induced apoptosis in SL2 cells,[47] and expression of *Decay* or *Damm* in mammalian cells resulted in modest cell death.[45,46] All three caspases appear to be detectably expressed at the mRNA level during most *Drosophila* developmental stages but did not exhibit the sorts of developmental regulation that were observed for *Dronc* or *Dredd*.[45–47] Mutant alleles of these three caspases will greatly facilitate their *in vivo* functional characterization.

The evolutionary origins of caspases

As we have implied above, genomic analyses show a large increase in the number of caspase genes in metazoan lineages over phylogenetic time, with mammals possessing twice as many as *Drosophila* at the last count. [74] This observation highlights the importance of apoptosis in general – and caspases in particular – to the evolution of metazoan complexity. However, the presence of caspase-related proteins such as metacaspases and paracaspases in evolutionarily distant groups raises the question of how modern caspases arose. In general, it seems likely that the diverse protein domains now found in modern eukaryotic apoptotic proteins originated from a relatively small set of ancient, conserved domains.[74] Since metacaspases and paracaspases are found in animals, plants, fungi, protists and even in bacterial groups such as *Rhizobia*, *Actinomycetes* and cyanobacteria,[74] caspases and related proteins may indeed derive from a common ancient ancestor.

It is especially interesting that paracaspases have been detected in *Rhizobia*, which are α-proteobacteria and are therefore the modern-day relatives of the ancient endosymbiont thought to have given rise to mitochondria.[10] This observation suggested the possibility that an ancient caspase-like gene entered the eukaryotic genome via the original endosymbiont precursor of mitochondria and subsequently underwent a rapid evolutionary divergence into metacaspases, paracaspases and true caspases.[10,74,75] Alternatively, horizontal gene transfer from bacteria to eukaryotes at a later time may account for the origin of some apoptotic proteins, possibly including the caspases.[10] In either case, it may be that these ancestral caspase-like genes shared the ability of modern caspases to kill eukaryotic cells.[10,75,76] According to this hypothesis, ancient caspase-like proteins may have been used by facultative endosymbiotic bacteria to kill and digest their protoeukaryotic hosts, perhaps under conditions of nutrient deprivation.[10,76] Only later were these originally prokaryotic genes co-opted by the eukaryotic genome itself for its own homeostatic purposes. If this model is correct, the evolutionary history of the caspases is almost ironic: having arisen from enzymes used by bacteria to kill their hosts, the modern caspases have evolved into integral components of the apoptotic and innate immune machinery that modern metazoans use to defend themselves against pathogens.

Conclusions

The last 10 years have seen a staggering increase in our knowledge of PCD in general and the caspases in particular. Nevertheless, many important puzzles in the field remain to be solved, including the caspase substrates most critical for executing apoptosis, how survival and death signals are integrated to regulate caspase activation within a single cell, how cell proliferation and cell death are balanced to maintain homeostasis during tissue development (or unbalanced during disease), and what roles the various caspases play outside of apoptosis. Our knowledge of caspases and PCD in the 'lower' organisms described here should provide us with powerful tools for addressing these mysteries in the decades to come.

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