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Phil. Trans. R. Soc. Lond. B 1994 **345**, 247-250
doi: 10.1098/rstb.1994.0101

Programmed cell death in *Drosophila*

H. STELLER, J. M. ABRAMS†, M. E. GRETHER AND K. WHITE‡

Howard Hughes Medical Institute, Department of Brain and Cognitive Sciences and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, U.S.A.

SUMMARY

During *Drosophila* development, large numbers of cells undergo natural cell death. Even though the onset of these deaths is controlled by many different signals, most of the dying cells undergo common morphological and biochemical changes that are characteristic of apoptosis in vertebrates. We have surveyed a large fraction of the *Drosophila* genome for genes that are required for programmed cell death by examining the pattern of apoptosis in embryos homozygous for previously identified chromosomal deletions. A single region on the third chromosome (in position 75C1,2) was found to be essential for all cell deaths that normally occur during *Drosophila* embryogenesis. We have cloned the corresponding genomic DNA and isolated a gene, *reaper*, which is capable of restoring apoptosis when reintroduced into cell death defective deletions. The *reaper* gene is specifically expressed in cells that are doomed to die, and its expression precedes the first morphological signs of apoptosis by 1–2 h. This gene is also rapidly induced upon X-ray irradiation, and *reaper* deletions offer significant protection against radiation-induced apoptosis. Our results suggest that *reaper* represents a key regulatory switch for the activation of apoptosis in response to a variety of distinct signals.

In *Drosophila*, naturally occurring or programmed cell death (PCD) is a prominent phenomenon throughout most of the organism's life cycle. Large numbers of cells die both during embryonic development (Abrams *et al.* 1993; White *et al.* 1994) and later during metamorphosis (Kimura & Truman 1990), but some PCD continues into adult life (see, for example, Giorgi & Deri 1976). The decision about which cells will die is often not predetermined by lineage but, like in vertebrates, can be controlled by a variety of distinct epigenetic factors. This plasticity is particularly evident in the developing insect nervous system, where the onset of PCD can be regulated by cell–cell interactions (Cagan & Ready 1989; Wolff & Ready 1991; Ramos *et al.* 1993), trophic control (Steller *et al.* 1987; Campos *et al.* 1992) and the steroid hormone ecdysone (reviewed in Truman 1984). PCD in *Drosophila* appears to be important as a sculpting force during morphogenesis, to eliminate cells that have been damaged or are unable to complete their differentiation programme (for examples, see Fristrom 1969; Bryant 1988; Magrassi & Lawrence 1988; Dura *et al.* 1987; Smouse & Perrimon 1990; Abrams *et al.* 1993), and for establishing and maintaining the appropriate ratios among different cell types in organs that naturally vary in size. For example, the size of the *Drosophila* optic ganglia is always perfectly matched to the variable size of the eye (Power 1943). This matching in the number of cells is accomplished by adjusting the rate of both cell death as well as cell proliferation and differentiation through competitive interactions between retinal neurons and their targets (Fischbach & Technau 1984; Selleck & Steller 1991; Campos *et al.* 1991; Winberg *et al.* 1992).

Naturally occurring cell deaths in *Drosophila* typically display the characteristic morphological and biochemical changes associated with apoptosis in vertebrates (Kerr *et al.* 1972), but subtle morphological differences have been noted as well in some instances (see, for example, Giorgi & Deri 1976; Bryant 1988; Abrams *et al.* 1993; Wolff & Ready 1991). These similarities indicate that at least some of the molecular components of the basic cell death programme have been evolutionary conserved, a notion that has received direct support from recent molecular studies in other species (e.g. Vaux & Weissmann 1992; Rabizadeh *et al.* 1993; Yuan *et al.* 1993; Hengartner & Horvitz; 1994, this volume). The powerful genetic and molecular biology techniques available in *Drosophila* make it an ideal system for elucidating the mechanism by which cells undergo apoptosis. The *Drosophila* embryo is particularly well suited for a genetic analysis of PCD. First, a substantial amount of apoptosis occurs during embryogenesis in a rather predictable pattern, and these deaths can be rapidly and reliably visualized in live preparations using the vital dye acridine orange (Abrams *et al.* 1993). Second, by screening embryos as opposed to advanced developmental stages for the lack of PCD, one does not have to make any assumptions about the

† Present address: Deptartment of Cell Biology & Neuroscience, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235, U.S.A.
‡ Present address: Cutaneous Biology Research Center, Massachusetts General Hospital, Harvard Medical School, MGH East, Building 149, Charlestown, Massachusetts 02129, U.S.A.

Phil. Trans. R. Soc. Lond. B (1994) **345,** 247–250
Printed in Great Britain

viability of cell death defective mutants. Finally, a large fraction of the *Drosophila* genome can be quickly surveyed by examining the pattern of cell death in embryos homozygous for previously characterized chromosomal deletions. Although these deletions typically include genes essential for viability, the large maternal supply of household functions (Garcia-Bellido *et al.* 1983) permits development well beyond the stage at which cell death begins.

By making use of an extensive collection of chromosomal deletions, we were able to screen approximately 50% of the *Drosophila* genome for functions globally required for PCD. The majority of deletions did not significantly affect the amount of cell death in the embryo, and many other deletions were associated with excessive AO staining, presumably as the result of developmental defects. A few deletions produced markedly reduced levels of PCD. These may affect the process by which cells are selected to die in response to specific signals. In contrast to all other deletions, we found only a single region on the third chromosome (position 75C1,2) to be required for all cell deaths that normally occur in the *Drosophila* embryo (White *et al.* 1994). Embryos homozygous for Df(3L)H99, the smallest cell death defective deletion available in the 75C1,2 interval, contained many extra cells and did not undergo certain morphogenetic movements, but developed a segmented cuticle and began to move. However, these embryos failed to hatch into larvae, apparently due to a strong defect in head involution. Genetic mosaic analysis with H99 indicates that this deletion does not contain any genes that are generally required for cell proliferation, differentiation or survival. Homozygous H99 mutant eye clones contain the full repertoire of morphologically normal, terminally differentiated cell types. The only abnormality we have detected in these clones is the apparent presence of extra cells, consistent with a block of PCD that normally occurs during eye development (K. White & H. Steller, unpublished results). Interestingly, H99 mutants were not only deficient in normal cell death, but also offered significant protection against ectopic cell deaths, such as those induced by X-irradiation and in developmental mutants. Such a global blockade of PCD in response to many distinct death signals indicates that this deletion removes a component of central importance to the cell death programme.

We have cloned all the genomic DNA corresponding to the H99 interval and identified a gene, *reaper*, which appears to play a central control function for the initiation of PCD (White *et al.* 1994). Significant levels of apoptosis can be restored in H99 mutants upon re-introducing a genomic *reaper* clone by germ line transformation experiments. Furthermore, expression of a *reaper* cDNA also leads to the induction of apoptosis (see below). Sequence analysis indicates that *reaper* encodes a small peptide of only 65 amino acids which shares no significant homologies to other known proteins (White *et al.* 1994). The open reading frame (ORF) of *reaper* has been highly conserved in *D. simulans*, a close relative of *D. melanogaster*, indicating that this sequence functions indeed as a protein coding region. The *reaper* ORF has also been significantly conserved in *D. virilis* (M. Grether, A. F. Lamblin, R. Jespersen & H. Steller, unpublished results), which is separated from *D. melanogaster* by 60 million years of evolution. Due to its novel sequence, the biochemical function of this peptide is currently unknown. However, we have good reasons to believe that *reaper* is not a cell death effector protein, i.e. that it is not part of the cell death machinery itself. This conclusion is derived from the observation that some cell death can be induced in *reaper* deletions upon radiation with high doses of X-rays. Significantly, the few cells that die under these circumstances have all the morphological and biochemical characteristics of apoptosis. This indicates that the basic cell death programme is intact, but cannot be readily activated in *reaper* deletions. Consistent with the idea that *reaper* plays a central control function for the activation of PCD, we find that *reaper* mRNA is specifically expressed in cells that are doomed to die, and that expression of *reaper* precedes the first morphological signs of apoptosis by 1–2 h. In addition, X-irradiation of embryos leads to rapid and massive ectopic expression of *reaper*, followed by widespread cell death (J. Abrams & H. Steller, unpublished observations). Finally, *reaper* expression appears to be activated in cells that fail to differentiate due to genetic defects, and *reaper* deletions protect against cell death under these conditions (J. Abrams & H. Steller, unpublished observations). Our results suggest that most, if not all PCDs in *Drosophila* occur by one common mechanism, and that multiple signaling pathways for the induction of cell death converge onto the *reaper* gene. According to our model, *reaper* expression would lead to the selective activation of cell death effector proteins that may be present but inactive in most, if not all cells (see Raff 1992; Raff *et al.* 1993, this volume).

Our model predicts that expression of *reaper* should be sufficient for the induction of apoptosis. We have tested this model by generating transgenic fly strains that express a *reaper* cDNA clone under the control of the heat inducible hsp70 heat shock promoter. Upon heat shock induction, high levels of ectopic cell death were induced in transgenic embryos, and cells which would normally live initiated PCD (K. White & H. Steller, unpublished observations). We conclude that *reaper* expression is sufficient for the induction of apoptosis, and we propose that *reaper* represents a key regulatory function to activate a ubiquitous cell death effector pathway (figure 1). In principle, *reaper* could exert such a function by directly activating cell death effector proteins. Alternatively, *reaper* may inactivate proteins that protect cells from PCD, such as bcl-2 and/or related genes (e.g. Vaux *et al.* 1988; Hockenberry *et al.* 1988; Hengartner & Horvitz 1994). In the nematode *C. elegans*, inactivation of ced-9, a member of the bcl-2 family, is sufficient for the activation of PCD (Hengartner *et al.* 1992). A resolution between these possibility will require the identification of target proteins for *reaper*.

Another unresolved question is how multiple signalling pathways converge onto the *reaper* gene. It

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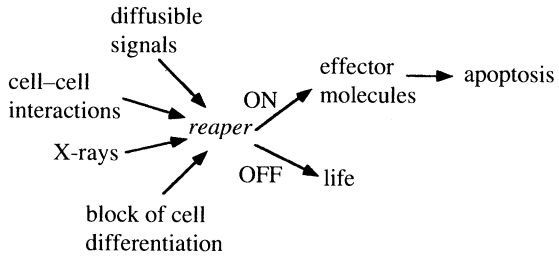

Figure 1. Model for the role of *reaper* during apoptosis. The induction of *reaper* appears to represent a key regulatory switch for the activation of a global cell death programme. Multiple distinct signalling pathways lead to the induction of *reaper* mRNA expression, and deletions that include *reaper* offer protection against apoptosis that is normally induced by these different signals. The expression of *reaper* is normally restricted to cells that will die, and ectopic expression of *reaper* is sufficient for the induction of apoptosis. Our model proposes that *reaper* acts upstream of cell death effector molecules, as the few cell deaths that occur in *reaper* deletions upon X-irradiation are morphologically indistinguishable from those in wild-type.

is conceivable that the integration of these pathways occurs at the level of the *reaper* promoter, by activation of transcription through the binding of distinct transcription factors. For example, in analogy to vertebrates (see Lane *et al.*, this volume), radiation may induce a p53 like protein in *Drosophila* which conceivably could bind to and activate *reaper* transcription. Alternatively, different signalling pathways could converge upstream of *reaper* and, in an extreme case, lead to the activation of *reaper* transcription through binding of a transcription factor to a single regulatory site. Finally, the specificity of *reaper* expression could be controlled at the post-transcriptional level, e.g. through selective RNA stabilization in cells that are doomed to die. Each of these models makes specific predictions that can be tested by standard promoter analyses.

A final point that warrants some discussion is the paucity of mutations that result in a global block of PCD in the *Drosophila* embryo. From screening a set of deletions covering approximately half of the *Drosophila* genome, we found only a single region to be required for all apoptotic deaths, and deleting this region did not even appear to affect the basic cell death machinery. On the other hand, there are several reasons to suspect that a number of genes are required for the onset and early stages of apoptosis, prior to the induction of acridine orange staining which was used as our initial assay. Acridine orange stains apoptotic cells only after nuclear and cytoplasmic condensation have become apparent, and actually appears to be a slightly later marker than TUNEL. In the nematode *C. elegans*, at least two genes, ced-3 and ced-4, are required for the onset of all PCDs in this organism (Ellis & Horvitz 1986, Ellis *et al.* 1991). There are several possible reasons why no other cell death genes have been identified in our screen. First, genes whose products are maternally supplied to the embryo would not have been picked up by us. However, it is not clear whether maternal products make a significant contribution to apoptosis in *Drosophila*, because cell death begins only about 7 h after fertilization (Abrams *et al.* 1993), long after zygotic transcription has started. In our mind, a more likely explanation for the inability to identify deletions of cell death effector genes is the possibility that multiple genes may contribute to cell killing ('death from multiple causes'). If the absence of a cell death gene had simply resulted in slower deaths or deaths with a somewhat abnormal morphology, it would not have been picked up in our screen. However, it is precisely this kind of phenotype that may be expected from the removal of a cell death effector protein, and it should be possible to identify appropriate *Drosophila* mutants by more refined screens in the future. Alternatively, access to the elusive PCD effector proteins may be provided by isolating and characterizing proteins that interact with *reaper*. We expect that the combination of both genetic and biochemical approaches will make a major contribution to our understanding of the molecular basis of PCD. As many basic aspects of apoptosis and at least some of the proteins that regulate it have been evolutionary conserved, we expect that this work will also provide a better insight into the mechanisms of apoptosis in mammals, and its role in human pathogenesis.

We thank K. Farrell, R. Jespersen and L. Young for excellent technical assistance, and A. F. Lamblin for comments on this manuscript. This work was supported in part by a Pew Scholars Award (H.S.), and postdoctoral fellowships from the NIH (K.W.) and the ACS (J.M.A.). H.S. is an Associate Investigator with the Howard Hughes Medical Institute.

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