Steroid regulation of autophagic programmed cell death during development

Cheng-Yu Lee\(^1,2\) and Eric H. Baehrecke\(^1, *\)

\(^1\)Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, Maryland 20742, USA
\(^2\)Department of Biology, University of Maryland, College Park, Maryland 20742, USA

*Author for correspondence (e-mail: baehreck@umbi.umd.edu)

Accepted 19 January; published on WWW 22 March 2001

SUMMARY

Apoptosis and autophagy are morphologically distinct forms of programmed cell death. While autophagy occurs during the development of diverse organisms and has been implicated in tumorigenesis, little is known about the molecular mechanisms that regulate this type of cell death. Here we show that steroid-activated programmed cell death of Drosophila salivary glands occurs by autophagy. Expression of p35 prevents DNA fragmentation and partially inhibits changes in the cytosol and plasma membranes of dying salivary glands, suggesting that caspases are involved in autophagy. The steroid-regulated BR-C, E74A and E93 genes are required for salivary gland cell death. BR-C and E74A mutant salivary glands exhibit vacuole and plasma membrane breakdown, but E93 mutant salivary glands fail to exhibit these changes, indicating that E93 regulates early autophagic events. Expression of E93 in embryos is sufficient to induce cell death with many characteristics of apoptosis, but requires the H99 genetic interval that contains the rpr, hid and grim proapoptotic genes to induce nuclear changes diagnostic of apoptosis. In contrast, E93 expression is sufficient to induce the removal of cells by phagocytes in the absence of the H99 genes. These studies indicate that apoptosis and autophagy utilize some common regulatory mechanisms.

Key words: Steroid, Ecdysone, Autophagy, Apoptosis, Drosophila melanogaster

INTRODUCTION

Programmed cell death plays a critical role during animal development by functioning in the destruction of unneeded cells and tissues (Jacobson et al., 1997; Vaux and Korsmeyer, 1999). The term programmed cell death was established to distinguish physiological or genetic-regulated cell death from necrotic cell destruction (Lockshin and Zakeri, 1991). Genetically regulated cell death is an integral component of normal development, and is used in processes such as limb formation and nervous system remodeling (Robinow et al., 1993; Saunders, 1966). Cell death is also involved in removal of abnormal cells during development, including those that form during tumorigenesis (Thompson, 1995).

Morphological studies of developing vertebrate embryos resulted in the definition of three types of physiological cell death (Schweichel and Merker, 1973). The first type, widely known as apoptosis, is found in isolated dying cells that exhibit condensation of the nucleus and cytoplasm, followed by fragmentation and phagocytosis by cells that degrade their contents (Kerr et al., 1972). The second type, known as autophagy, is observed when groups of associated cells or entire tissues are destroyed. These dying cells contain autophagic vacuoles in the cytoplasm that function in the degeneration of cell components. The third type, known as non-lysosomal cell death, is least common, and is characterized by swelling of cavities with membrane borders followed by degeneration without lysosomal activity. While autophagy fulfills the definition of programmed cell death (Lockshin and Zakeri, 1991), occurs during development of diverse organisms (Clarke, 1990), and has been implicated in tumorigenesis (Bursch et al., 1996; Liang et al., 1999; Schulte-Hermann et al., 1997), little is known about the molecular genetic mechanisms underlying this type of programmed cell death.

The morphological characteristics that distinguish apoptosis and autophagy suggest that these cell deaths are regulated by independent mechanisms (Clarke, 1990). Comparison of biochemical changes during lymphocyte apoptosis and insect intersegmental muscle autophagy also indicate that these physiological cell deaths occur by distinct mechanisms (Schwartz et al., 1993). However, recent studies of steroid-triggered cell death of Drosophila larval salivary glands suggest that these cells utilize genes that are part of the conserved apoptosis pathway (Jiang et al., 1997; Lee et al., 2000), even though these cells exhibit characteristics of autophagy (von Gaudecker and Schmale, 1974). Specifically, the caspase drone and homolog of ced4/Apaf-1 ark, two components of the core apoptotic machinery, increase in transcription immediately prior to salivary gland cell death (Lee et al., 2000). Thus, characterization of the mechanisms governing the regulation of autophagy will identify how these cell deaths differ from those that occur by apoptosis.

Steroid hormones activate programmed cell death during animal development (Evans-Storm and Cidlowski, 1995). During insect metamorphosis, the steroid 20-hydroxyecdysone (ecdyson) activates programmed cell death to eliminate...
unneeded larval cells (Robinow et al., 1993; Truman et al., 1994). *Drosophila* larval salivary glands are an excellent system for studying the genetic hierarchy that is activated by steroids during programmed cell death. A pulse of ecdysone 10–12 hours after puparium formation (APF) triggers caspase-mediated programmed cell death of *Drosophila* larval salivary glands (Jiang et al., 1997). Within 4 hours of this rise in hormone titer, salivary glands exhibit several features of programmed cell death including nuclear staining by Acridine Orange, DNA fragmentation, and exposure of phosphatidylserine on the outer leaflet of the plasma membrane (Jiang et al., 1997; S. van den Einde and E.H.B., unpublished).

The mechanisms of steroid signaling have been extensively studied in *Drosophila* larval salivary glands because of the advantages of the giant polytene chromosomes, which form steroid-induced puffs reflecting a transcriptional regulatory hierarchy (Andres and Thummel, 1992; Ashburner et al., 1974; Becker, 1959; Clever, 1964). Previous studies have implicated the ecdysone-regulated genes *EcR, usp* (ultraspiracle), βFTZ-F1, BR-C, E74 and E93 in larval salivary gland programmed cell death (Broadus et al., 1999; Hall and Thummel, 1998; Jiang et al., 2000; Lee et al., 2000; Restifo and White, 1992). *EcR, usp, βFTZ-F1, BR-C* and *E74* function in processes other than cell death including the differentiation of adult cells (Bender et al., 1997; Broadus et al., 1999; Fletcher et al., 1995; Hall and Thummel, 1998; Restifo and White, 1992). In contrast, *E93* appears to function more specifically in destruction of larval tissues (Lee et al., 2000). *EcR, usp* and βFTZ-F1 act at the top of this signaling pathway and regulate BR-C, E74 and E93 (Broadus et al., 1999; Woodard et al., 1994). BR-C, E74 and E93 impact on the transcription of programmed cell death genes including rpr (reaper), hid (head involution defective/v; wrinkled), crq (croquemort), Ark and dronc (Nc; Nedd2 like caspase) during larval tissue destruction (Jiang et al., 2000; Lee et al., 2000), suggesting a potential mechanism for steroid-triggered cell death. However, the relationship between the primary steroid response genes BR-C, E74 and E93 remains unclear. Although salivary gland programmed cell death appears to occur by autophagy (von Gaudecker and Schmale, 1974), little is known about how these cells change in structure during cell death, and how genes that function in steroid signaling impact autophagic cell death.

*Drosophila* possesses the programmed cell death pathway components that have been conserved in organisms that are as different as nematodes and humans (Abrams, 1999). Homologs of caspases including DCP-1, DREDD, diICE, DRONC and DECAY (Chen et al., 1998; Dorstyn et al., 1999a; Dorstyn et al., 1999b; Fraser and Evan, 1997; Inohara et al., 1997; Song et al., 1997), the CED4/APAF-1 homolog ARK (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999), the CED-9/Bcl-2 family members DROB-1/DEBCL/dBORG-1 and dBORG-2 (Baker Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000), and the inhibitors of apoptosis DIAP1 and DIAP2 (Hay et al., 1995) have been identified. In addition, the novel rpr, hid and grim cell death genes have been isolated and molecularly characterized (Chen et al., 1996; Grether et al., 1995; White et al., 1994). While rpr, hid and grim do not exhibit extensive homology with vertebrate genes, expression of these genes is sufficient to induce mammalian cell death (Haining et al., 1999; McCarthy and Dixit, 1998). This suggests that these novel genes utilize targets that are conserved in vertebrate cells, and interactions between hid and *diap1* support this conclusion (Goyal et al., 2000; Lisi et al., 2000; Wang et al., 1999). Ectopic expression of either rpr, hid, or grim is sufficient to induce caspase-dependent programmed cell death (Chen et al., 1996; Grether et al., 1995; White et al., 1996). These three novel genes reside in a single genetic interval named Df(3L)H99 (abbreviation H99) that is required for virtually all programmed cell death during embryogenesis (Chen et al., 1996; Grether et al., 1995; White et al., 1994). The conservation of the core components that are utilized during cell death of diverse organisms indicates that fruit flies are an excellent system for genetic studies of programmed cell death.

Here we show that steroid-regulated programmed cell death of salivary glands involves genes that function in apoptosis including caspases. However, these dying cells possess morphological characteristics that are distinct from apoptotic cells and that occur during autophagic cell death. Expression of the inhibitor of caspases p35 prevents DNA fragmentation and partially inhibits changes in vacuole and plasma membrane breakdown that normally accompany autophagic cell death of salivary glands. The steroid-regulated BR-C, E74A and E93 genes all function in this salivary gland cell death, but mutations in these genes result in different cell death defects. E93 mutant salivary glands do not exhibit changes in vacuole and plasma membrane breakdown, while these changes occur in BR-C and E74A mutant salivary glands. Expression of E93 is sufficient to induce apoptosis in different cell types. Furthermore, E93-induced cell death requires the function of the H99 genetic interval that contains the rpr, hid and grim cell death genes to induce nuclear apoptotic changes. In contrast, expression of E93 is sufficient to induce the removal of cells and engulfment by phagocytes that express the CD36-related protein Croquemort (CRQ) in the absence of the H99 genes. These studies indicate that E93 plays an important role in activation of autophagic cell death, and that autophagy and apoptosis utilize some common regulatory mechanisms.

**MATERIALS AND METHODS**

**Salivary gland histology**

Canton S wild-type, βFTZ-F1 (βFTZ-F1<sup>17/βFTZ-F1<sup>19</sub></sup>, BR-C (rpr<sup>5</sup>/Y), E74A (E74A<sup>P(neo)/Df(3L)JST-81k19</sup>, or E93 (E93<sup>Df(3R)F2</sup> and E93<sup>Df(3R)F3</sup>) mutant salivary glands were dissected from animals at various stages of development (hours after puparium formation) at 25°C. For semi-thin sections and light microscopy, salivary glands were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 16 hours at 4°C, embedded in LR white resin, sectioned, stained in Gill’s Haemotoxylin and Eosin Y, and analyzed using a Zeiss Axioshot II microscope. For electron microscopy, salivary glands were dissected in 0.1 M Mops buffer (pH 7.0) for 8 hours at room temperature, 3% glutaraldehyde/1% paraformaldehyde in 0.1 M Mops buffer (pH 7.0) for 16 hours at 4°C, post-fixed in 1% osmium tetroxide for 1 hour, embedded in Spurr’s resin, sectioned, and analyzed using a Zeiss EM 10 transmission electron microscope.

**p35 inhibition of salivary gland cell death**

To assess p35 inhibition of salivary gland programmed cell death, females of the genotype *y; w; fkh-GAL4/y; w; fkh-GAL4* were crossed with males of the genotype *y; w; UAS-GFP/UAS-GFP; UAS-p35/UAS-p35*. Controls consisted of embryos that were collected from the
individual parental genotypes. Flies were allowed to lay eggs for 24 hours, and progeny were raised at 25°C, staged at puparium formation, aged for 24 hours, and analyzed for the presence or absence of salivary glands using GFP as a marker. To assess p35 inhibition of cellular changes during salivary gland cell death, females of the genotype y,w; fkh-GAL4/w, fkh-GAL4 were crossed with males of the genotype y; w; UAS-p35/UAS-p35. Flies were allowed to lay eggs for 24 hours, progeny were raised at 25°C, staged at puparium formation, and aged for 24 hours. Animals were fixed, embedded in paraffin, and sectioned as previously described (Restifo and White, 1992). The TUNEL method was performed as described by Wang et al. (Wang et al., 1999), and the sections were then stained with Eosin Y. Negative and positive controls for the TUNEL procedure included wild-type Canton S staged 4-6 and 12 hours APF. Semi-thin sections of dissected salivary glands were prepared and analyzed as described above.

**E93-induction of cell death**

Transgenic UAS-E93(1) Drosophila have been described previously (Lee et al., 2000). Initially, E93 was expressed during embryogenesis utilizing a heat-inducible GAL4 gene. Embryos were collected for 8 hours from a cross of females of the genotype y,w;UAS-E93(1)/y,w;UAS-E93(1); CyO;ScO; TM2, Ubx;Sh and males of the genotype y, w; hs-GAL4/hs-GAL4, and aged overnight at 18°C. Controls consisted of embryos that were collected from the individual parental genotypes. Embryos were heat shocked for 1 hour at 37°C, allowed to recover for 1 hour at 25°C, and analyzed for the activation of programmed cell death.

To express E93 in a specific pattern during embryogenesis, females of the genotype y,w;UAS-E93(1)/y,w;UAS-E93(1); CyO;ScO; TM2, Ubx;Sh and males of the genotype y, w; h-GAL4/h-GAL4 (Brand and Perrimon, 1993) were crossed, embryos were collected for 18 hours, and analyzed for E93 expression, cell death and cuticle phenotypes. Controls consisted of embryos that were collected from the individual parental genotypes. Programmed cell death was assayed by nuclear Acidine Orange staining, or the TUNEL method to detect fragmented DNA, following previously described protocols (Abrams et al., 1993; White et al., 1994). E93 protein was detected using an affinity purified rabbit polyclonal antibody (Franc et al., 1996) following standard techniques (Baehrecke, 1997). Embryonic cuticle preparation and X-gal staining was done as previously described (Baehrecke, 1997). For analyses of paraffin sections, dechorionated embryos were fixed in 2.5% glutaraldehyde/1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0), X-gal stained, embedded and sectioned as described previously (Restifo and White, 1992). Embryos were processed for electron microscopy as described previously (Abrams et al., 1993; White et al., 1994).

**Influence of E93 expression on transcription of crq**

To determine if expression of E93 influences the transcription of crq, embryos were collected for 5 hours and aged for 18 hours at 25°C from a cross of females of the genotype y,w;UAS-E93(1)/y,w;UAS-E93(1); CyO;ScO; TM2, Ubx;Sh and males of the genotype y, w; h-GAL4/h-GAL4. Controls consisted of embryos that were collected from wild-type (Canton S), homozygous UAS-E93(1) alone, or h-GAL4 alone. Embryonic RNA was extracted, electrophoresed, transferred to nylon membranes, and hybridized with radiolabeled E93, crq and rp49 following previously described methods (Baehrecke and Thummel, 1995).

**RESULTS**

**Salivary gland programmed cell death occurs by autophagy**

Salivary glands exhibit several markers of apoptosis immediately before programmed cell death (Jiang et al., 1997). However, morphological studies indicate that salivary glands undergo autophagy (von Gaudecker and Schmale, 1974). These studies prompted us to conduct detailed analyses of cell structure during steroid-regulated programmed cell death of salivary glands, to gain insight into the timing of cellular changes that can be used for genetic analyses of autophagy. Salivary glands were dissected from wild-type animals 12, 13, 14 and 14.5 hours APF, and semi-thin and thin sections were examined by light and transmission electron microscopy. Salivary gland cells of animals 12 hours APF are cube-shaped, and possess intact plasma membranes, large Eosin-positive vacuoles, and banded polytene chromosomes (Fig. 1A,B; n=many cells from 10 salivary glands). One hour later, salivary gland cells become more round in shape, large Eosin-positive vacuoles appear to fragment, and an Eosin-negative class of vacuoles appears that is associated with the plasma membrane (Fig. 1C,D; n=many cells from 10 salivary glands). Analyses

Df(3L)H99/TM2, Ubx and males of the genotype y,w;CyO/ScO; Df(3L)H99, h-GAL4/TM2, Ubx, aged for 12 hours at 25°C, and were stained with Acidine Orange or Nile Blue to assess programmed cell death (Abrams et al., 1993). Embryos lacking nuclear Acidine Orange staining, but showing the phenotype that occurs when E93 is expressed under the control of h-GAL4, were selected for transmission electron microscopy (identical to the method used in previous studies of H99 and rpr; White et al., 1994). Embryos were also collected for 3 hours from a cross of females of the genotype y,w;UAS-E93(1)/y,w,UAS-E93(1); CyO;ScO; Df(3L)H99/TM6B, Ubx-lacZ2 and males of the genotype y,w; CyO;ScO; Df(3L)H99, h-GAL4/TM6B, Ubx-lacZ2 and aged for 12 hours at 25°C. These embryos were then analysed for either DNA fragmentation, CRQ expression, or embedded in paraffin wax for sectioning.

Programmed cell death was assayed by nuclear Acidine Orange and Nile Blue staining, or TUNEL-labeling to detect fragmented DNA, following previously described methods (Abrams et al., 1993; White et al., 1994). CRQ protein was detected using a rabbit polyclonal antibody (Franc et al., 1996) following standard techniques (Baehrecke, 1997). Embryonic cuticle preparation and X-gal staining was done as previously described (Baehrecke, 1997). For analyses of paraffin sections, dechorionated embryos were fixed in 2.5% glutaraldehyde/1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0), X-gal stained, embedded and sectioned as described previously (Restifo and White, 1992). Embryos were processed for electron microscopy as described previously (Abrams et al., 1993; White et al., 1994).

**Influence of E93 expression on transcription of crq**

To determine if expression of E93 influences the transcription of crq, embryos were collected for 5 hours and aged for 18 hours at 25°C from a cross of females of the genotype y,w;UAS-E93(1)/y,w;UAS-E93(1); CyO;ScO; TM2, Ubx;Sh and males of the genotype y, w; h-GAL4/h-GAL4. Controls consisted of embryos that were collected from wild-type (Canton S), homozygous UAS-E93(1) alone, or h-GAL4 alone. Embryonic RNA was extracted, electrophoresed, transferred to nylon membranes, and hybridized with radiolabeled E93, crq and rp49 following previously described methods (Baehrecke and Thummel, 1995).
of salivary glands 14 hours APF reveals the presence of autophagic vacuoles that contain cellular structures including mitochondria (Fig. 1E,F). The larger Eosin-positive vacuoles that are present in 12- and 13-hour salivary glands do not contain such cellular structures, and the apparent fragmentation of these structures immediately precedes the formation of autophagic vacuoles. By 14.5 hours APF, plasma membranes are destroyed, few vacuoles are present, and chromosomes are not banded (Fig. 1G,H; \(n\)=many cells from 10 salivary glands). Salivary glands are destroyed 15-15.5 hours APF. The association of dynamic changes in vacuole structure with this cell death process indicates that salivary glands undergo autophagy, and the presence of markers of apoptosis suggests similarities between these two types of programmed cell death.

**Caspases function in salivary gland cell death**

Transcription of the apoptosis regulators *rpr*, *hid*, *ark* and *drone* increases immediately prior to steroid-regulated programmed cell death of salivary glands (Lee et al., 2000). Furthermore, expression of the baculovirus inhibitor of caspases p35 prevents destruction of salivary glands (Jiang et al., 1997). To gain a better understanding of the role that caspases play in salivary gland destruction by autophagy, we expressed green fluorescent protein (GFP) in salivary glands in the presence or absence of the caspase inhibitor p35. This was accomplished by placing the GFP and p35 genes under control of the yeast GAL4 upstream activation sequence (Brand and Perrimon, 1993), and combining them with the *fkh*-GAL4 activator that drives expression in salivary glands. Analyses of control animals lacking p35 expression indicated that their salivary glands are always destroyed (Fig. 2A; \(n\)=100). In contrast, all of the animals expressing p35 had persistent salivary glands 24 hours APF (Fig. 2B; \(n\)=100). Animals that express p35 in salivary glands exhibit no developmental delay, and form adult structures even though salivary glands persist long after they would normally be destroyed.

To assess the role of caspases in the cellular changes observed during autophagy, salivary glands of animals that express p35 were analyzed for DNA fragmentation and cell morphology. Wild-type Canton S 4- to 6-hour prepupae served as a negative control; salivary glands of these animals never exhibit DNA fragmentation and always contain vacuoles in the
Autophagic programmed cell death

Following the rise in ecdysone titer that triggers salivary gland cell death 12 hours APF, salivary glands of the positive control Canton S exhibit DNA fragmentation even though they still have large vacuoles in the cytosol (Fig. 2D; \( n \)=many cells from 12 salivary glands). Salivary glands of animals that express p35 did not exhibit DNA fragmentation 24 hours APF, even though many of these salivary glands have progressed to a late stage in autophagy as indicated by the absence of vacuoles in the cytosol (Fig. 2E; \( n \)=many cells from 12 salivary glands). These p35-expressing salivary glands exhibit two distinct morphologies – 89% lacked vacuoles, while 11% possessed vacuoles (\( n \)=18). Salivary glands that express p35 and do not possess the large eosin-positive vacuoles always lack plasma membranes (Fig. 2F), while those that contain the large eosin-positive vacuoles always have plasma membranes (Fig. 2G). The morphology of cells within a given salivary gland was extremely similar, and the observed cytological variation was dependent on the animal selected for analysis. The lack of DNA fragmentation and partial persistence of vacuoles and plasma membranes in cells expressing p35 indicates that caspase function is required for the cellular changes that precede autophagy of salivary glands. The variation of the changes in the cytosol suggests that caspase inhibition was incomplete in these cells. However, expression of a second copy of p35 did not significantly increase the persistence of vacuoles and plasma membranes. While caspase function is required for DNA fragmentation (Fig. 2E) and proper destruction of salivary glands (Fig. 2B), the partial inhibition of cell death changes by p35 suggests that caspas may not be solely responsible for changes in the cytosol during destruction of this tissue. In contrast to p35, ectopic expression of \( diap2 \) did not have any impact on salivary gland cell death (data not shown), suggesting no role for this inhibitor of apoptosis in this process.

**Steroid-regulated genes function in salivary gland cell death**

Studies of the steroid-activated genetic regulatory hierarchy in *Drosophila* indicate that the \( \beta \)FTZ-F1, BR-C, E74A, and E93 genes function in destruction of salivary glands (Broadus et al., 1999; Jiang et al., 2000; Lee et al., 2000; Restifo and White, 1992). To determine the function of these steroid-regulated genes in the cellular changes that occur during cell death, salivary glands were dissected from homozygous mutant animals 24 hours APF, fixed, embedded, and sectioned. Homozygous \( \beta \)FTZ-F1 mutant salivary glands are arrested prior to the onset of cell death and possess large eosin-positive vacuoles, intact plasma membranes, and banded polytene chromosomes (Fig. 3A,B; \( n \)=many cells from 10

---

**Fig. 2.** p35 inhibits changes in DNA, vacuole and membrane structure that accompany salivary gland programmed cell death. (A) Control animals (\( fkh \)-GAL4, UAS-GFP) never possess salivary glands 24 hours APF (\( n \)=100). (B) Animals that contain \( fkh \)-GAL4, UAS-p35, and UAS-GFP all possess salivary glands (arrow) 24 hours APF (\( n \)=100). (C) Salivary glands of 4- to 6-hour prepupae contain large vacuoles (asterisks) and do not show fragmented DNA as indicated by the light TUNEL-negative nuclei (arrows). (D) Salivary glands of animals 12 hours APF contain large vacuoles (asterisks) and contain fragmented DNA as indicated by the dark TUNEL-positive nuclei (arrows). (E) Salivary glands of p35-expressing animals 24 hours APF do not possess fragmented DNA as indicated by the light TUNEL-negative nuclei (arrows), even though these salivary glands have progressed to a late stage in cell death as indicated by the absence of vacuoles. (F,G) Salivary glands dissected from animals 24 hours APF that express p35 either lack (F) or possess (G) vacuoles (asterisk) and plasma membranes (arrows). Scale bar in F, 10 \( \mu \)m, F and G are the same magnification.
salivary glands). This morphology is similar to that of wild-type salivary glands 12 hours APF (Fig. 1). E93 mutant salivary gland cells also contain large eosin-positive vacuoles and plasma membranes, and have variable chromosome banding (Fig. 3C,D; n=many cells from 10 salivary glands). This morphology is similar to that of \(\beta\)FTZ-F1 mutant salivary glands, and was observed in both E93\(^1\) and E93\(^2\) mutant alleles. In contrast, most BR-C and E74A mutant salivary gland cells lack vacuoles and plasma membranes, and show variable chromosome banding (Fig. 3E-H; n=many cells from 10 salivary glands/genotype). Although some BR-C and E74A mutant salivary gland cells possess small vacuoles (Fig. 3F for example), they are extremely different from the cells of E93 mutant salivary glands that invariably contain large eosin-positive vacuoles.

Examination of BR-C and E74A mutant salivary glands cells revealed cells in the process of autophagy, while these changes were not observed in \(\beta\)FTZ-F1 and E93 mutant salivary gland cells. E74A mutant salivary glands cells contained, for example, nascent autophagic vacuoles composed of rough endoplasmic reticulum that appeared to be enclosing cellular components including mitochondria and smaller vacuoles (Fig. 3I). In addition, E74A mutant salivary glands cells contained myelin-like figures (Fig. 3J), which have been reported in numerous autophagic cells that are thought to be partially degraded cellular components. Therefore, \(\beta\)FTZ-F1 and E93 appear to be required for the onset of autophagic cell death changes, while BR-C and E74A salivary glands initiate autophagy but are not completely destroyed.

Expression of E93 is sufficient to induce cell death during embryogenesis

Previous studies indicated that E93 functions more specifically than other steroid-regulated genes in the regulation of programmed cell death (Lee et al., 2000). Expression of E93 in wing imaginal discs is sufficient to induce cell death when these tissues normally undergo steroid-induced morphogenesis (Lee et al., 2000). To test if E93 is sufficient to induce cell death in cells that appear to undergo hormone-independent development, we expressed E93 in embryos, a stage when this gene is not normally expressed (Baehrecke and Thummel, 1995). This was accomplished by placing the E93 gene under control of the yeast GAL4 upstream activation sequence (Brand and Perrimon, 1993). Initially, these E93 transformants

---

**Fig. 3.** Mutations in steroid-regulated genes prevent changes in vacuole and plasma membrane structures that accompany salivary gland programmed cell death. Salivary glands were dissected from mutant animals 24 hours APF and analyzed by light (A,C,E,G) and transmission electron (B,D,F,H-J) microscopy. (A,B) Salivary glands from \(\beta\)FTZ-F1 mutants (\(\beta\)FTZ-F1\(^1\)/\(\beta\)FTZ-F1\(^2\)) possess intact plasma membranes (arrow), large vacuoles in the cytoplasm (V), and banded polytene chromosomes in the nuclei (N). (C,D) Salivary glands from E93 mutants (E93\(^1\)/Df(3R)93F\(^x\)) possess intact plasma membranes (arrow), large vacuoles in the cytoplasm (V), and variable banding of the polytene chromosomes in the nuclei (N). (E,F) Salivary glands from BR-C mutants (rhp\(^7\)/rhp\(^7\)) lack plasma membranes and large vacuoles in the cytoplasm, and possess variable banding of the polytene chromosomes in the nuclei (N). (G,H) Salivary glands from E74A mutants (E74A\(^{P\{neo\}}\)/Df(3L)ist-81k19) lack plasma membranes and vacuoles in the cytoplasm, and possess variable banding of the polytene chromosomes in the nuclei (N). (I,J) The cytoplasm of BR-C and E74A mutants is destroyed by autophagy (E74A\(^{P\{neo\}}\)/Df(3L)ist-81k19 mutants are shown). (I) Salivary glands of E74A mutants contain nascent autophagic vacuoles composed of rough endoplasmic reticulum enclosing cytoplasmic components including mitochondria (arrows) and smaller vacuoles (*). (J) Salivary glands of E74A mutants also contain myelin-like figures (arrows) that have been reported in numerous cells that undergo autophagy and are presumably residual partially digested cellular structures. Scale bars in A and B, 10 \(\mu\)m; I and J, 1 \(\mu\)m; A,C,E and G are the same magnification, and B,D,F and H are the same magnification.
(UAS-E93) were crossed with flies carrying a heat-inducible GAL4 transgene. Embryos collected from this cross were heat shocked and assayed for the induction of cell death. We observed rapid and widespread cell death within 1 hour of heat shock, while controls exhibited slightly greater than wild-type levels of cell death (data not shown). UAS-E93 transformants were then crossed to flies that express GAL4 in a spatially restricted manner, enabling us to test if E93 is sufficient to induce cell death in a specific pattern. UAS-E93 flies were mated with Drosophila carrying a GAL4 transcriptional activator driven by the hairy (h) enhancer, that is expressed in stripes during embryogenesis. In control embryos (UAS-E93 alone or h-GAL4 alone), no E93 protein was detected (Fig. 4A). Control embryos also exhibited a wild-type pattern of cell death in the central nervous system using Acridine Orange staining (data not shown) and TUNEL to detect fragmented DNA (Fig. 4C), and almost no cell death was detected in the epidermis. When h-GAL4 is combined with UAS-E93, E93 protein is expressed in stripes (Fig. 4B). A striped-pattern of ectopic cell death is observed in h-GAL4 UAS-E93 embryos using both Acridine Orange staining (data not shown) and TUNEL (Fig. 4D, note that the embryos in C and D are in a different orientation to those in A and B). The development of these E93-expressing progeny is arrested before the completion of embryogenesis, and they exhibit defects in germ band retraction and cuticular patterning. Specifically, h-GAL4 UAS-E93 embryos possess severe head defects and fusion and partial deletion of alternating denticle belts (Fig. 4F), while control embryos display wild-type cuticular structures (Fig. 4E). The lethal phenotype of E93-expressing embryos is similar to that observed in hairy mutant embryos, as well as other pair rule segmentation mutants in Drosophila (Nüsslein-Volhard and Wieschaus, 1980). These data indicate that the ectopic expression of E93 is sufficient to induce highly patterned cell death in embryos.

To test if E93-induced cell death and lethality are dependent on caspase activity, we coexpressed E93 with the caspase inhibitor p35 in Drosophila embryos, future adult wings and salivary glands. Flies containing the UAS-E93 and UAS-p35 transgenes were mated with flies containing either the embryonic h-GAL4, the wing vg-GAL4, or the salivary gland fkh-GAL4 drivers. Coexpression of p35 did not inhibit the lethality or cell death that is induced by E93 under the control of each of the GAL4 drivers (data not shown).

**E93 induces aspects of cell death in the absence of the rpr, hid and grim cell death genes**

We tested the functional requirement of rpr, hid and grim for E93-induced cell death. Control embryos that ectopically express E93 exhibit extensive cell death as assayed by nuclear Acridine Orange staining (Fig. 5A; n=300), and deletion of cuticle structures (Fig. 5B; n=300). As previously reported, embryos with the H99 deletion, and thus lacking the rpr, hid and grim cell death genes, show no nuclear Acridine Orange staining (Fig. 5C), and have cuticles that are largely normal with the exception of head structures (Fig. 5D; Abbott and Lengyel, 1991; White et al., 1994). We crossed flies containing UAS-E93 and the H99 deletion to flies with h-GAL4 and the H99 deletion. Progeny of this cross were examined for cell death and cuticle defects. Embryos that express E93 in the absence of H99 do not show nuclear Acridine Orange staining, which is close to the predicted Mendelian ratio of 25%, indicating that E93 does not induce Acridine Orange staining in the absence of the rpr, hid and grim genes. A similar ratio was observed in H99 controls that do not express E93. To our surprise, embryos that express E93 and lack H99 exhibit severe cuticle defects (Fig. 5E; n=300). The frequency and severity of cuticle phenotypes achieved is extremely similar to that observed when UAS-E93 and h-GAL4 are combined in a wild-type genetic background (Fig. 5B). This indicated that epidermal cells had been deleted in embryos lacking H99 even though they do not exhibit typical nuclear Acridine Orange staining.

The apparent conflict between a lack of nuclear Acridine Orange staining and loss of cells led us to analyze other programmed cell death markers in animals that are deficient for H99 and ectopically express E93. Initially, we analyzed DNA fragmentation by the TUNEL method. This experiment allowed accurate genotyping of embryos, as homozygous H99 mutants could be distinguished by the absence of a lacZ transgene on the balancer chromosome. As expected, control embryos that contained the lacZ transgene reliably exhibited...
cell death as indicated by the presence of fragmented DNA (Fig. 5G; n=300). Similar to studies using Acridine Orange as a marker of cell death, homozygous H99 embryos that express E93 exhibited almost no DNA fragmentation (Fig. 5H; n=300). This result prompted detailed cellular analyses of H99 mutants that express E93. We used Nile Blue staining to identify dying cells, as this is a rapid and reliable marker of programmed cell death in Drosophila (Abrams et al., 1993), and allows examination of dying cell morphology by light microscopy. The results using Nile Blue were similar to those using Acridine Orange – 76% of embryos exhibited Nile Blue staining, while 24% were not stained (Fig. 5LJ; n=300). In addition, both Nile Blue-stained and unstained embryos contained large cells with the appearance of apoptotic corpses (Abrams et al., 1993) (Fig. 5-L). To verify the presence of apoptotic corpses, homozygous H99 embryos expressing E93 were genotyped based on the absence of β-galactosidase activity (G14), Nile Blue staining (I-L), and histological analysis of sections (M,N) were also undertaken. (A) Embryos that contain both h-GAL4 and UAS-E93 always show extensive cell death as indicated by nuclear Acridine Orange staining (green spots, the diffuse staining is the result of autofluorescence of the yolk). (B) Cuticles of embryos that contain both h-GAL4 and UAS-E93 exhibit severe deletions of both denticle and head structures. (C) Embryos lacking the H99 genetic interval show no programmed cell death as assayed by nuclear Acridine Orange staining. (D) Cuticles of H99 mutant embryos are wild-type with the exception of head structures. (E) Embryos that contain both h-GAL4 and UAS-E93, but lack the H99 locus, exhibit no nuclear staining with Acridine Orange. (F) Cuticles of H99 mutant embryos that contain both h-GAL4 and UAS-E93 exhibit severe defects indicating that cell death occurs, even though no nuclear Acridine Orange staining occurs in this genotype. (G,LK) Embryos that contain both h-GAL4 and UAS-E93, and contain the H99 genetic interval, exhibit nuclear hallmarks of programmed cell death. (H,JL) Embryos expressing E93 under the control of h-GAL4, but lacking the H99 genetic interval, do not exhibit the nuclear hallmarks of programmed cell death. Note that E93-expressing embryos lacking H99 function contain clusters of unstained cells reminiscent of apoptotic corpses (J and L, L is an enlargement of the posterior region of the embryo in J). Cells of similar morphology in E93-expressing embryos that possess H99 function reliably stain with Nile Blue (I and K; K is an enlargement of the posterior region of the embryo in I). (M) Section of an embryo containing h-GAL4 and UAS-E93, but lacking the H99 region, possesses large cells with the morphological characteristics of apoptotic corpses (arrow). Scale bar, 10 μm. (N) Transmission electron microscopic analyses of embryos that contain both h-GAL4 and UAS-E93 and lack H99 reveals phagocytes engulfing cells, even though these embryos lack the nuclear hallmarks indicative of apoptosis. Scale bar, 1 μm.

Fig. 5. Expression of E93 in animals lacking the H99 genetic interval induces removal of cells without nuclear cell death markers being detected. E93 was expressed in the presence and absence of the H99 genetic interval, and programmed cell death was assayed by Acridine Orange staining (A,C,E). Examination of cuticle phenotype (B,D,F), TUNEL-labeling and β-galactosidase activity (G,IH), Nile Blue staining (I-L), and histological analysis of sections (M,N) were also undertaken. (A) Embryos that contain both h-GAL4 and UAS-E93 always show extensive cell death as indicated by nuclear Acridine Orange staining (green spots, the diffuse staining is the result of autofluorescence of the yolk). (B) Cuticles of embryos that contain both h-GAL4 and UAS-E93 exhibit severe deletions of both denticle and head structures. (C) Embryos lacking the H99 genetic interval show no programmed cell death as assayed by nuclear Acridine Orange staining. (D) Cuticles of H99 mutant embryos are wild-type with the exception of head structures. (E) Embryos that contain both h-GAL4 and UAS-E93, but lack the H99 locus, exhibit no nuclear staining with Acridine Orange. (F) Cuticles of H99 mutant embryos that contain both h-GAL4 and UAS-E93 exhibit severe defects indicating that cell death occurs, even though no nuclear Acridine Orange staining occurs in this genotype. (G,LK) Embryos that contain both h-GAL4 and UAS-E93, and contain the H99 genetic interval, exhibit nuclear hallmarks of programmed cell death. (H,JL) Embryos expressing E93 under the control of h-GAL4, but lacking the H99 genetic interval, do not exhibit the nuclear hallmarks of programmed cell death. Note that E93-expressing embryos lacking H99 function contain clusters of unstained cells reminiscent of apoptotic corpses (J and L, L is an enlargement of the posterior region of the embryo in J). Cells of similar morphology in E93-expressing embryos that possess H99 function reliably stain with Nile Blue (I and K; K is an enlargement of the posterior region of the embryo in I). (M) Section of an embryo containing h-GAL4 and UAS-E93, but lacking the H99 region, possesses large cells with the morphological characteristics of apoptotic corpses (arrow). Scale bar, 10 μm. (N) Transmission electron microscopic analyses of embryos that contain both h-GAL4 and UAS-E93 and lack H99 reveals phagocytes engulfing cells, even though these embryos lack the nuclear hallmarks indicative of apoptosis. Scale bar, 1 μm.

CRQ expression is associated with E93-induced apoptosis and autophagy of salivary glands

The CD36 relative crq functions in phagocytosis of apoptotic cells during embryogenesis, and the level of CRQ protein is sensitive to the amount of apoptosis (Franc et al., 1999). Expression of E93 activates cell death with many characteristics of apoptosis, and removal of these dying cells does not require rpr, hid and grim function (Fig. 4.5). Since CRQ is a useful marker of phagocytes that remove apoptotic cells, we tested if expression of E93 is sufficient to induce CRQ expression in H99 mutants. As shown previously (Franc et al., 1999), H99 mutant embryos exhibit a low level of CRQ expression (Fig. 6A,B). Flies containing UAS-E93 and the H99 deletion were crossed to flies with h-GAL4 and the H99 deletion. Expression of E93 is sufficient to induce CRQ expression, and the level of CRQ appears similar in control embryos that are not homozygous mutant for the H99 genes and sibling homozygous H99 mutant embryos as indicated by the presence or absence of β-galactosidase activity (Fig. 6C-F). We never observed stripes of CRQ expression in the
hundreds of embryos analyzed, suggesting that CRQ expression is induced in phagocytic macrophages and not the dying cells as would be expected during apoptosis.

**E93** mutants do not exhibit increased levels of *crq* RNA that occur in wild-type salivary glands immediately prior to autophagy (Lee et al., 2000). To determine if ectopic expression of E93 is sufficient to induce *crq* transcription, we expressed E93 during embryogenesis. **UAS-E93** flies were mated with flies carrying **h-GAL4**, and RNA was isolated from 18- to 23-hour old embryonic progeny as well as three control embryonic genotypes (wild-type, **UAS-E93** alone, or **h-GAL4** alone). Northern blot hybridization indicated that *crq* transcript levels are elevated in embryos that express E93 (Fig. 6G).

**E93** mutant salivary gland cells do not exhibit changes in cytoplasmic vacuoles and plasma membranes, as well as *crq* transcription, that occur during autophagy (Fig. 3; Lee et al., 2000). These data suggest that both phagocytes and autophagic cells may utilize *crq* for removal of dying cells, but these results could also be explained by the association of phagocytic macrophages with dying salivary glands. Therefore, we tested if CRQ is expressed in dying salivary gland cells at the stage we observe autophagy. Salivary glands were dissected from wild-type animals 12 and 14 hours APF, and stained with CRQ antibodies. Salivary glands dissected from animals 12 hours APF do not express CRQ protein. (IJ) Salivary glands dissected from animals 14 hours APF express CRQ protein (arrow). (J) CRQ protein (arrow) appears to be localized in the cytoplasm of salivary gland cells, based on the location of the plasma membrane (arrowhead).

**DISCUSSION**

The synchronized nature of programmed cell death of larval tissues at the onset of *Drosophila* metamorphosis provides advantages for the study of dying cells in the context of animal development. Larval tissue destruction is triggered by the steroid ecysone, and this signal activates a transcriptional regulatory hierarchy that includes the **BR-C**, **E74** and **E93** genes. These steroid-regulated genes then activate genes that play a more direct role in programmed cell death. While genes that are associated with apoptosis, including caspases, appear to function in salivary gland cell death, these cells exhibit an autophagic morphology. Thus, the destruction of salivary glands is not only useful for understanding how steroid hormones activate programmed cell death, but provides insights into the similarities and differences between apoptosis and autophagy.

**Steroid-activated programmed cell death of salivary glands occurs by autophagy**

Studies of physiological cell death in developing vertebrate embryos resulted in characterization of three forms of cell destruction; apoptosis, autophagy and non-lysosomal
Steroid-regulated genes impact distinct cellular changes in dying cells

Ecdysone impacts on the transcription of the cell death genes rpr, hid and diap2 (Jiang et al., 1997). This regulation is mediated by the ecdysone receptor, and a group of ecdysone-activated factors that include the BR-C, E74 and E93 genes (Jiang et al., 2000; Lee et al., 2000). We have analyzed the function of the steroid-regulated BR-C, E74 and E93 genes in salivary gland cell death. E93 mutant salivary glands exhibit persistence of large vacuoles and plasma membranes, while these structures are destroyed in BR-C and E74A mutants. Two possible explanations exist for the differences in BR-C, E74A and E93 mutant salivary gland cell morphology. E93 mutant salivary glands could be arrested at an earlier stage of cell destruction that is similar to that of 12-hour wild-type cells, while BR-C and E74A mutants are arrested at a stage that is similar to 14.5-hour salivary gland cells (Fig. 1). This model is supported by previous studies indicating that E93 function is required for proper regulation of BR-C and E74A transcription (Lee et al., 2000). Alternatively, E93 could function to regulate autophagy that results in destruction of vacuoles and plasma membranes, while BR-C and E74A do not function in the regulation of these cellular changes even though these genes are required for salivary gland cell death. The latter interpretation is intriguing when one considers that expression of E93 is sufficient to induce characteristics of apoptosis (Fig. 4), and can induce the removal of cells even in the absence of the rpr, hid and grim cell death genes and nuclear apoptotic changes (Figs 5, 6).

The separation of plasma membrane and nuclear apoptotic responses has been elicited by inclusion of specific caspase inhibitors in cultured mammalian cells (McCarthy et al., 1997; Mills et al., 1998). Furthermore, certain mouse cells that lack caspase3 function fail to exhibit chromatin condensation and DNA degradation, even though they exhibit other hallmarks of apoptosis (Woo et al., 1998). Although distinct caspases appear to be activated in Drosophila cells that express either RPR or FAS (Kondo et al., 1997), no subcellular differences were noted in association with these specific caspase activities. Drosophila with mutations in the dep1 caspase gene exhibit defects in nuclear breakdown (McCall and Steller, 1998), indicating that a specific caspase is required for this distinct cellular change during development. Interestingly, the H99 genetic interval is required for E93 induction of nuclear cell death markers during embryogenesis, but genes in this region are not essential for similar nuclear changes during oogenesis (Foley and Cooley, 1998). While accumulating data indicates that distinct genetic pathways exist for the regulation of the morphological changes that occur during programmed cell death, future studies are needed to help us understand the common and unique components that ultimately result in the hallmarks of cell death.

Autophagy and apoptosis appear to utilize some similar genetic mechanisms

Studies of programmed cell death have emphasized the relationship between the evolutionarily conserved ced-3/caspase, ced-4/Apaf-1 and ced-9/Bcl-2 genes. Genetic studies of Drosophila programmed cell death resulted in the identification of the novel rpr, hid and grim genes (Chen et al., 1996; Grether et al., 1995; White et al., 1994; White et al., 1996), which function in apoptosis that is mediated by caspases. Thus, these novel Drosophila genes exploit components of what is considered to be a universal death signaling pathway (Abrams, 1999). rpr, hid and grim appear to activate cell death by interacting with DIAP1, which inhibits caspase activation (Goyal et al., 2000; Lisi et al., 2000; Wang et al., 1999).

Several factors indicate that salivary gland autophagy is regulated by genes that also function in apoptosis. First, caspases function in salivary gland cell death. Expression of the baculovirus inhibitor of caspases, p35, inhibits destruction of this tissue (Fig. 2). Furthermore, p35 expression prevents DNA fragmentation and partially inhibits morphological changes in vacuoles that are associated with autophagy (Fig. 2), indicating that caspases are utilized during autophagy. Transcription of the Apaf1 homologue ark and the caspase, drcn increases immediately preceding salivary gland cell death, and this transcription is blocked in E93 mutants (Lee et al., 2000), further supporting that caspases function in salivary gland autophagy. Second, transcription of the proapoptotic genes, rpr and hid increases immediately prior to salivary gland autophagy (Jiang et al., 1997), and the transcription of these genes is blocked by mutations in steroid-regulated genes that are involved in this process (Lee et al., 2000) (Fig. 3). Ectopic expression of E93, a critical determinant of salivary gland autophagy, in salivary gland cell death in response to ecdysone (Jiang et al., 1997). This cell death can be detected by markers that are typically associated with apoptosis including nuclear staining by Acridine Orange, TUNEL to detect DNA fragmentation, and exposure of phosphatidylserine on the outer leaflet of the plasma membrane (Jiang et al., 1997; S. van den Eijnde and E. H. B., unpublished). The changes in vacuolar structure that immediately precede the synchronous destruction of larval salivary gland cells are clearly more similar to autophagy than heterochrony (apoptosis) as described by Scweich and Merker (Schweich and Merker, 1973). Large vacuoles increase in number in prepupal salivary glands (Lane et al., 1972), and rearrangement of the cytoskeleton and an increase in acid phosphatase activity are associated with these structures (Jochova et al., 1997). Dynamic changes in salivary gland structure may reflect important biochemical changes during programmed cell death. Large Eosin-positive vacuoles appear to fragment, a distinct class of Eosin-negative vacuoles are formed that are closely associated with the plasma membrane, and vacuoles containing organelles are observed in the cytoplasm immediately preceding destruction of salivary glands (Fig. 1). An increase in transcription of the caspase drcn occurs at this stage (Lee et al., 2000), and inhibition of caspase activity blocks DNA fragmentation and partially prevents changes in vacuoles and plasma membranes (Fig. 2), suggesting that these morphological changes may be attributed in part to the activity of enzymes typically associated with apoptosis. While morphological analyses of apoptosis and autophagy suggest different mechanisms for these forms of cell death, our studies indicate that some genes that function in apoptosis also function during autophagy.
Autophagic programmed cell death

Autophagy and apoptosis are morphologically distinct, suggesting that the mechanisms underlying the regulation of these forms of programmed cell death are different. Nearly all of the large polytenized larval cells die during *Drosophila* metamorphosis (Robertson, 1936). The synchrony and volume of these cell deaths suggests that engulfment of each dying cell may be limited by the number of available phagocytes. One obvious distinction between autophagy and apoptosis is the location of the lysosomal machinery that degrades the dying cell. Autophagic cells destroy their own contents, while apoptotic cells depend on phagocytes to accomplish terminal degradation. This distinction may account for much of the differences in the morphological appearance of these two forms of dying cells, but do not exclude the possibility that a single autophagic cell utilizes the mechanisms that exist in distinct apoptotic and phagocytic cells. The specific expression of CRQ during autophagy supports this possibility (Fig. 6), but genetic studies of *crq* function are needed to test this hypothesis. Future studies of autophagy, and its relationship to apoptosis, will illustrate the similarities and differences between these forms of programmed cell death.

We thank I. Madanat, S. Klinedinst, B. Cooksey, T. Maugel, H. Steller, K. White, J. Abrams, J. Agapite, N. Franc, A. Rodriguez, B. Hay, C. Woodard, F. Karim, J. Wing, J. Nambu, D. Sandstrom, Z. Liu, S. Mount, S. Tanda, D. Wasserman, the Laboratory of Biological Ultrastructure, and the Bloomington *Drosophila* Stock Center for reagents, flies, and helpful comments. This work was supported by NIH grant GM59136 to E. H. B.

REFERENCES


Lisi, S., Mazon, I. and White, K. (2000). Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in Drosophila. Genetics 154, 669-678.


Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during Drosophila and Manduca development. Development 120, 219-234.


