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Steroid regulation of autophagic programmed cell death during development

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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 *dronc* 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 (ecdysone) 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*/*w*; *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, drICE, 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*^{17}/*βFTZ-F1*^{19}), *BR-C* (*rbp*^{5}/*Y*), *E74A* (*E74A*$^{P[neo]}$/*Df(3L)st-81k19*), or *E93* (*E93*^{1}/*Df(3R)93F*^{x2} and *E93*^{2}/*Df(3R)93F*^{x2}) 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 Axiophot II microscope. For electron microscopy, salivary glands were fixed in 3% glutaraldehyde/0.2% tannic acid 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*/*y,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*/*Sb* 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*/*Sb* 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 Acridine 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 rat polyclonal antibody (Lee et al., 2000) following standard techniques (Baehrecke, 1997). Embryonic cuticles were prepared as described previously (Baehrecke, 1997).

To assess p35 inhibition of *E93*-induced lethality, females of the genotypes *y,w/y,w*; *UAS-p35/UAS-p35*; *TM2*, *Ubx/Sb*, or *y,w,UAS-E93(1)/y,w,UAS-E93(1)*; *CyO/Sco*; *TM2*, *Ubx/Sb*, or *y,w,UAS-E93(1)/y,w,UAS-E93(1)*; *UAS-p35/UAS-p35*; *TM2*, *Ubx/Sb*, or *y,w,UAS-E93(1)/y,w,UAS-E93(1)*; *UAS-p35/UAS-p35*; *UAS-p35/UAS-p35* were crossed with males of the genotypes *y, w*; *h-GAL4/h-GAL4*, *y, w*; *vg-GAL4/CyO*, or *y, w, fkh-GAL4*. For *h-GAL4* experiments, embryos were collected overnight, counted, and hatching larvae were counted and collected every 2 hours. For *vg-GAL4* experiments, flies were allowed to lay eggs for 24 hours, and eclosing adults were genotyped using dominant markers and counted. For *fkh-GAL4* experiments, flies were allowed to lay eggs for 24 hours, progeny were allowed to develop, and third instar larvae were sexed, dissected, and assayed for the presence of salivary glands. To analyze p35 inhibition of *E93*-induced cell death in wing imaginal discs, *y,w,UAS-E93(1)/y,w,UAS-E93(1)*; *UAS-p35/UAS-p35*; *TM2*, *Ubx/Sb*, females were crossed with *y, w; vg-GAL4/vg-GAL4* males and allowed to lay eggs for 24 hours. Wing discs of progeny were dissected from 2-hour prepupae and analyzed for programmed cell death. Analyses of *diap2* inhibition of *E93*-induced cell death were conducted in a similar manner to studies of *p35*.

E93-induction of cell death in H99 mutants

For analyses of the impact of expressing *E93* in cell death mutants, embryos were 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*/*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 Acridine Orange or Nile Blue to assess programmed cell death (Abrams et al., 1993). Embryos lacking nuclear Acridine 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-lacZ* and males of the genotype *y,w*; *CyO*/*Sco*; *Df(3L)H99*, *h-GAL4*/*TM6B*, *Ubx-lacZ* 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 Acridine 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 were 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*/*Sb* 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

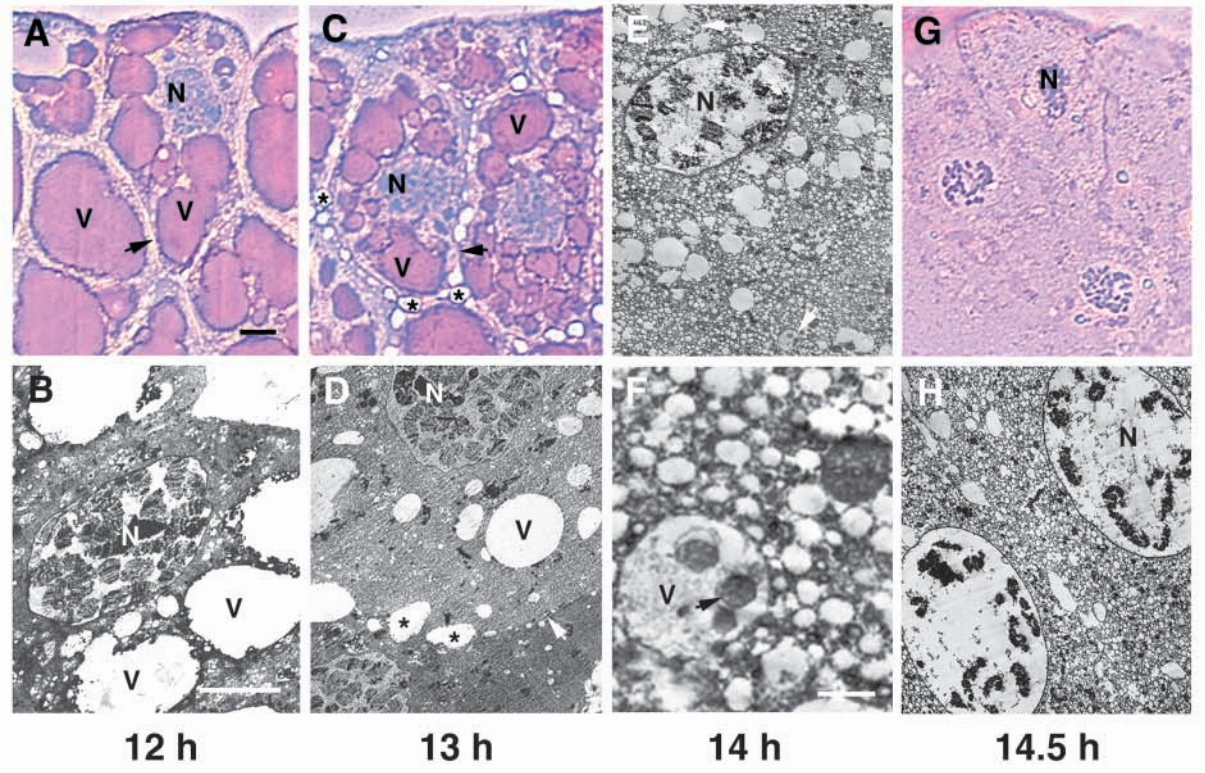

Fig. 1. Salivary glands exhibit dynamic morphological changes during programmed cell death. Salivary glands of wild-type Canton S animals were staged relative to puparium formation and analyzed by light (A,C,G) and transmission electron (B,D-F,H) microscopy. (A,B) Salivary glands dissected 12 hours APF possess cube-shaped cells, intact plasma membranes (arrow), large vacuoles in the cytoplasm that stain with Eosin Y (V), and banded polytene chromosomes in the nucleus (N). (C,D) Salivary glands dissected 13 hours APF possess round-shaped cells, intact plasma membranes (arrow), the large vacuoles in the cytoplasm appear to fragment (V), a second class of smaller vacuoles that fail to be stained with eosin-Y are observed near the plasma membrane (*), and banded polytene chromosomes in the nucleus (N). (E) Autophagic vacuoles that contain cytoplasmic structures (arrows) are most abundant in salivary glands 14 hours APF when no large vacuoles are detected, but the polytene chromosomes in the nucleus (N) are still banded. (F) Analyses of autophagic vacuoles (V) reveal that they contain mitochondria (arrow). (G,H) Salivary glands dissected 14.5 hours APF lack, or contain remnants of, plasma membranes and vacuoles, and their polytene chromosomes have lost their banding pattern, but they still contain a basement membrane-like structure that contains the remnants of the cytosol and nucleus (N). The salivary gland is completely removed within an hour of this stage. Scale bars in A and B, 10 μm; ; F, 1 μm. A, C and G are the same magnification, while B, D, E, and H are the same magnification.

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 *dronc* 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

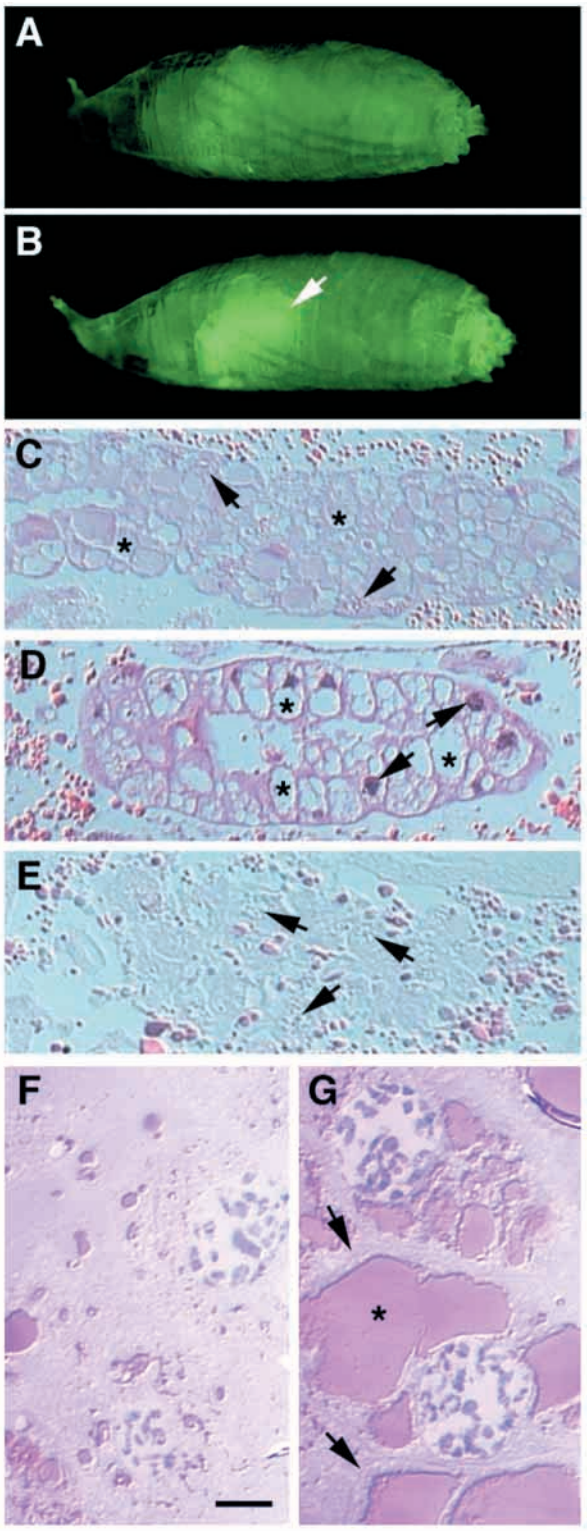

Fig. 2. p35 inhibits changes in DNA, vacuole and membrane structure that accompany salivary gland programmed cell death. (A) Control animals (*fkh-GAL4, UAS-GFP*) never possess salivary glands 24 hours APF (*n*=100). (B) Animals that contain *fkh-GAL4, UAS-p35*, and *UAS-GFP* all possess salivary glands (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 μm, F and G are the same magnification.

cytosol (Fig. 2C, *n*=many cells from 10 salivary glands). 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 caspases 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 *βFTZ-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 *βFTZ-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

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 *βFTZ-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 *βFTZ-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, *βFTZ-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

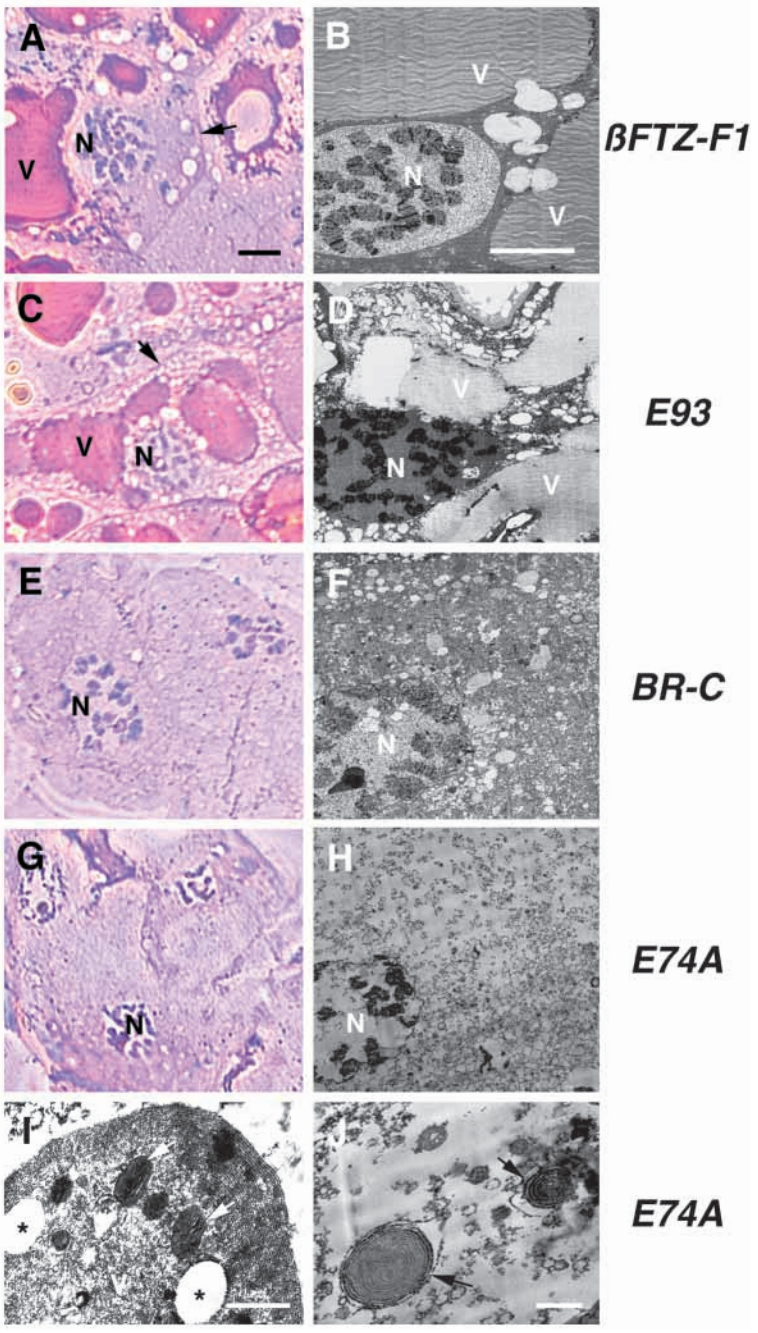

Fig. 3. Mutations in steroid-regulated genes prevent changes in vacuole and plasma membrane structures that accompany salivary gland programmed cell death. Salivary glands were dissected from mutant animals 24 hours APF and analyzed by light (A,C,E,G) and transmission electron (B,D,F,H-J) microscopy. (A,B) Salivary glands from *βFTZ-F1* mutants ($\beta FTZ\text{-}F1^{17}/\beta FTZ\text{-}F1^{19}$) 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 ($E93^{1}/Df(3R)93F^{x2}$) 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 (rbp^{5}/Y) 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 ($E74A^{P[neo]}/Df(3L)st\text{-}81k19$) 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 ($E74A^{P[neo]}/Df(3L)st\text{-}81k19$ 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 μm; I and J, 1 μm; 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.

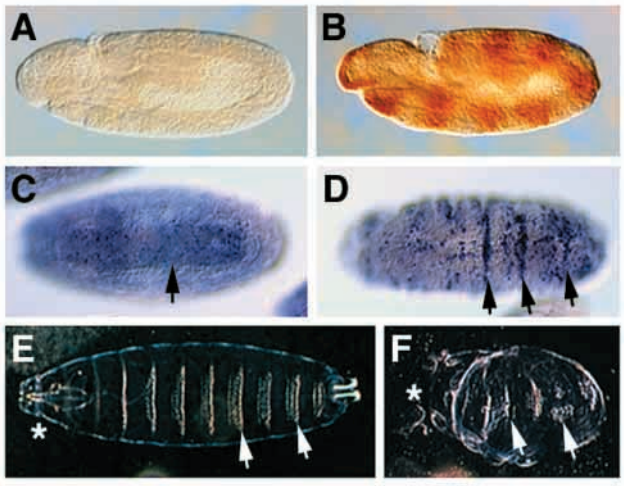

Fig. 4. Ectopic expression of E93 is sufficient to induce programmed cell death during embryogenesis. E93 was expressed in embryos that were then assayed for programmed cell death using the TUNEL procedure to detect DNA fragmentation (C, D), or cuticle phenotype (E, F). (A) Control embryos (either *h-GAL4* or *UAS-E93*) never express E93 protein. (B) Embryos that contain both *h-GAL4* and *UAS-E93* express E93 in a striped pattern. (C) Control embryos exhibit a wild-type pattern of programmed cell death (dark spots) in the ventral nerve cord (arrow). (D) Embryos containing both *h-GAL4* and *UAS-E93* exhibit large amounts of ectopic programmed cell death in a striped-pattern (arrows). Not all cells that express E93 die, as the extent of cell death appears to vary between embryos. (E) Control progeny possess a wild-type cuticular phenotype including normal head structures (asterisk) and denticle belts (arrows). (F) In contrast, embryos that contain both *h-GAL4* and *UAS-E93* exhibit cuticle defects in the head (asterisk) and denticle belts (arrows). Although all embryos expressing *E93* possess cuticle defects, the severity of denticle belt deletion varies with at least 60% of the progeny exhibiting defects as severe as those presented. Embryos are oriented with the anterior end to the left. A and B, lateral views; C-F ventral views.

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 (Fig. 5E; *n*=300). We observed that 21% of the progeny of the cross in this experiment lacked 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. 5F; *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

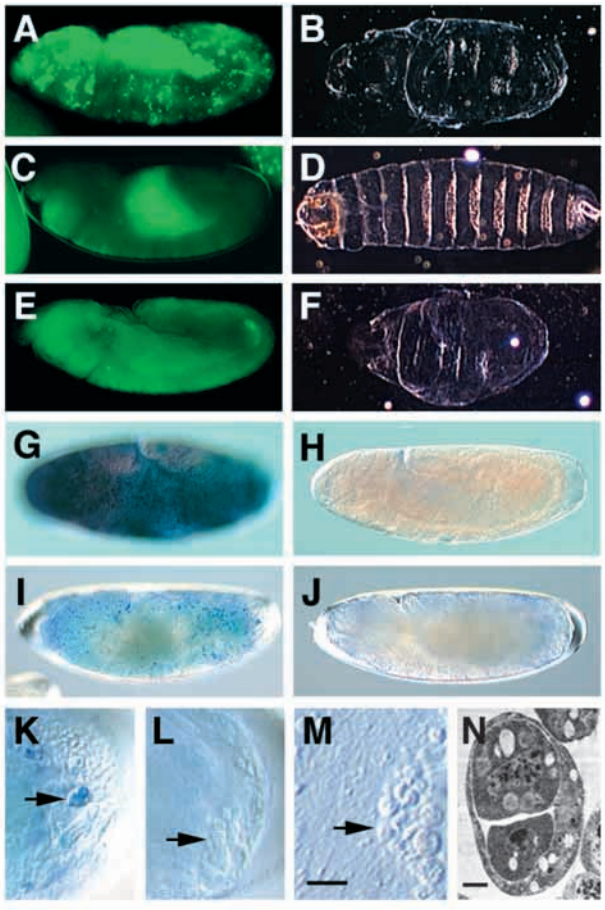

Fig. 5. Expression of *E93* in animals lacking the H99 genetic interval induces removal of cells without nuclear cell death markers being detected. E93 was expressed in the presence and absence of the H99 genetic interval, and programmed cell death was assayed by Acridine Orange staining (A,C,E). Examination of cuticle phenotype (B,D,F), TUNEL-labeling and β-galactosidase activity (G,H), 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,I,K) Embryos that contain both *h-GAL4* and *UAS-E93*, and contain the H99 genetic interval, exhibit nuclear hallmarks of programmed cell death. (H,J,L) 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.

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. 5I,J; *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. 5I-L). To verify the presence of apoptotic corpses, homozygous H99 embryos expressing E93 were genotyped based on the absence of β-galactosidase activity that is associated with the insertion of a *lacZ* transgene on balancer chromosome, and analyzed in thick paraffin sections. Many of the homozygous H99 embryos expressing *E93* contained large round cells that are similar in morphology to the apoptotic corpses that are one of the hallmarks of programmed cell death (Abrams et al., 1993) (Fig. 5M). Furthermore, ultrastructural analyses by transmission electron microscopy revealed that *E93* is sufficient to induce engulfment by phagocytes in embryos lacking the H99 interval (Fig. 5N). These data indicate that *E93* is sufficient to trigger cellular changes that result in engulfment by phagocytes, and that coordination of the complete apoptotic response requires genes in the H99 deletion.

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

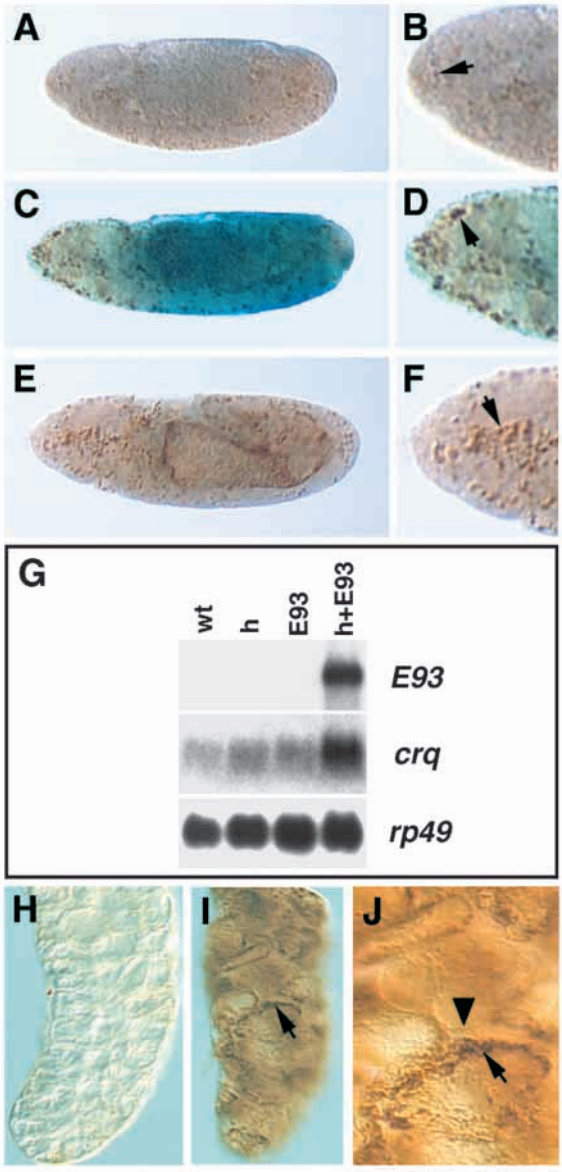

Fig. 6. CRQ expression is associated with E93-induced cell removal in H99 mutants, and salivary gland autophagy. E93 was expressed in the presence and absence of the H99 genetic interval, and the resulting embryos were assayed for expression of CRQ protein and β-galactosidase activity (A-F) and *crq* transcription (G). (A,B) Embryos that lack the H99 genetic interval possess few cells that express CRQ (brown stained cells, arrow). (C,D) Embryos that express E93 and contain the H99 genetic interval, as indicated by the presence of β-galactosidase activity, possess many cells that express CRQ (arrow). (E,F) Embryos that express E93 and lack the H99 genetic interval also possess a large number of cells that express CRQ (arrow). (G) Northern blot analysis indicates that *crq* transcript levels are increased by expression of E93 during embryogenesis. Canton S (wt), *h-GAL4* (h), *UAS-E93* (E93), or *h-GAL4* and *UAS-E93* (h + E93) flies were allowed to lay eggs for 5 hours, and were aged for 18 hours at 25°C, RNA was extracted, and analyzed by northern blot hybridization with *E93* and *crq*. Hybridization with *rp49* was used as a control for loading and transfer. (H) Salivary glands of 12-hour wild-type prepupae that contain large vacuoles do not express CRQ protein. (I,J) Salivary glands of wild-type animals 14 hours APF possess rings of punctate CRQ protein expression (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).

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 (Fig. 6H), while salivary glands dissected 14 hours APF express CRQ (Fig. 6I). Closer examination indicates that CRQ is expressed in the cytoplasm of degenerating salivary gland cells (Fig. 6J), suggesting a possible role for *crq* in autophagy. Combined, these data suggest that *E93* regulates *crq* during salivary gland autophagy.

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 ecdysone, 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

(Schweichel and Merker, 1973). Larval salivary glands of *Drosophila* undergo rapid programmed cell death in response to ecdysone (Jiang et al., 1997). This cell destruction can be detected using 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 heterophagy (apoptosis) as described by Scweichel and Merker (Schweichel 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 *dronc* 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.

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 *dcp1* 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, *dronc* 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, is sufficient to induce cell death with numerous characteristics of apoptosis (Figs 4, 5). In addition, the association of CRQ expression with E93-induced removal of apoptotic cells and autophagy of salivary glands provides yet another link between these morphologically distinct forms of programmed cell death (Fig. 6). Combined, these factors indicate that autophagy and apoptosis utilize at least some similar mechanisms.

The location and type of cell appears to be an important determinant for the type of programmed cell death that occurs in the context of animal development. Autophagy occurs when groups of cells or entire tissues die, while apoptosis occurs in isolated dying cells (Schweichel and Merker, 1973). Our studies are consistent with these criteria; salivary gland destruction occurs by autophagy and requires *E93* function, while ectopic induction of cell death by expression of E93 during embryogenesis has the characteristics of apoptosis. We hypothesize that this is due to similarities between autophagy and apoptosis as discussed above. Alternatively, autophagy and apoptosis may be mechanistically distinct, and our ability to induce ectopic cell death by expression of E93 is simply due to activating a death program in different cell types. This explanation is supported by our data demonstrating that p35 inhibits salivary gland cell death, but that p35 is not capable of inhibiting E93-induced cell death in embryos. However, several possibilities exist to explain the disparity of these data. First, ectopic expression of E93 during embryogenesis may lead to higher than normal levels of this protein. In side-by-side comparisons with the proapoptotic genes *rpr* and *hid*, expression of E93 resulted in greater cell death and lethality (C.-Y. L. and E. H. B., data not shown). Thus, the strong killing potential of E93 may be sufficient to overcome inhibition of cell death by p35. Second, other cell death genes are not inhibited by expression of p35, including cell death that is induced by ectopic expression of the caspase DRONC (Hawkins et al., 2000; Meier et al., 2000). Third, inhibition of vacuolar changes by expression of p35 during salivary gland cell death is incomplete, even though DNA fragmentation is inhibited in this tissue (Fig. 2). Thus, caspases may play a role in salivary gland cell death, and both p35 experiments and the transcription of *dronc* during salivary gland autophagy support this conclusion. However, it is possible that other proteolytic mechanisms act in concert with caspases in the bulk degradation of salivary gland cells. If multiple proteolytic mechanisms are implemented during cell death, then inhibition of a single pathway may not be sufficient to completely block cellular changes. Our data suggests that such complexity may exist in salivary glands, and that *E93* may not directly regulate caspases. In support of this, ectopic expression of E93 during embryogenesis does not lead to higher levels of *dronc*, although this caspase is highly expressed at this developmental stage (C.-Y. L. and E. H. B., data not shown). Accumulating evidence suggests that proteolysis during apoptosis may be mediated by more than caspases, as ubiquitin-mediated degradation of cell death regulatory molecules has been reported (Yang et al., 2000), and this mechanism is thought to be involved in insect cell autophagy (Schwartz et al., 1993). Clearly, rapid cell degradation is complicated, and invoking simple singular mechanisms to explain autophagy and apoptosis may prevent us from seeing the complexity of death regulatory mechanisms during development.

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.

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