

AP-1, but not NF- κ B, is required for efficient steroid-triggered cell death in *Drosophila*

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Abstract

Extensive studies in vertebrate cells have assigned a central role to Rel/NF- κ B and AP-1 family members in the control of apoptosis. We ask here whether parallel pathways might function in *Drosophila* by determining if Rel/NF- κ B or AP-1 family members contribute to the steroid-triggered death of larval salivary glands during *Drosophila* metamorphosis. We show that two of the three *Drosophila* Rel/NF- κ B genes are expressed in doomed salivary glands and that one family member, *Dif*, is induced in a stage-specific manner immediately before the onset of programmed cell death. Similarly, *Djun* is expressed for many hours before salivary gland cell death while *Dfos* is induced in a stage-specific manner, immediately before this tissue is destroyed. We show that null mutations in the three *Drosophila* Rel/NF- κ B family members, either alone or in combination, have no apparent effect on this death response. In contrast, *Dfos* is required for the proper timing of larval salivary gland cell death as well as the proper induction of key death genes. This study demonstrates a role for AP-1 in the stage-specific steroid-triggered programmed cell death of larval tissues during *Drosophila* metamorphosis.

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Abbreviations: JNKs, Jun N-terminal kinases; MAPK, MAP kinase, hid, head involution defective; RPR, *Drosophila* grim reaper

Introduction

Programmed cell death is a critical aspect of metazoan development, providing a means of removing obsolete or

damaged cells and sculpting organs to assume their appropriate function. The regulation of programmed cell death is under precise spatial and temporal control in order to ensure that only obsolete or unnecessary cells are destroyed. Accordingly, multiple signal transduction systems act together to direct a programmed cell death response. Rel/NF- κ B transcription factors function as critical downstream effectors of these death pathways.^{1,2} Rel/NF- κ B factors form homo- and heterodimers and are defined by the Rel homology domain, a region important for DNA binding, protein dimerization, interaction with cytoplasmic inhibitors, and nuclear translocation.^{2,3} Prior to their activation, Rel/NF- κ B factors are restricted to the cytoplasm via interactions with I κ B inhibitor proteins. A wide range of agents activate an I κ B kinase complex that phosphorylates I κ B, leading to its degradation via a proteasome-mediated pathway.⁴ This, in turn, frees the Rel/NF- κ B factor, allowing it to translocate into the nucleus where it can activate target gene transcription. Rel/NF- κ B proteins can either inhibit or promote programmed cell death in a cell-type and signal-dependent manner.^{1,5,6} Interestingly, these responses have been linked directly to the transcription of downstream effector genes. Thus, NF- κ B directly regulates a pro-survival Bcl family member in immune tissues where NF- κ B prevents cell death, and NF- κ B directly regulates Fas ligand expression in T lymphocytes where this transcription factor drives activation-induced cell death.^{7–13}

A number of studies have also implicated the AP-1 transcription factor as either a death inhibitor or death trigger depending on cellular context.^{9,14–16} AP-1 is a heterodimer of c-Jun and c-Fos.¹⁷ AP-1 activity is regulated by Jun N-terminal kinases (JNKs) that phosphorylate specific serine residues in c-Jun.¹⁸ JNKs, in turn, integrate a wide range of upstream signaling events through MAP kinase (MAPK) signaling cascades. Growth factors can activate these pathways through interaction with G protein-coupled receptors, and proinflammatory cytokines can activate MAPK through cell surface receptors. Extracellular stress such as irradiation, toxic drugs, or oxidative stress can also trigger the activation of AP-1 through JNK.^{16,19,20} The mechanisms by which divergent signals coordinate cellular responses through appropriate regulation of AP-1 activity remains a focus of current research.

Hormones also function as critical regulators of programmed cell death.^{21,22} For example, glucocorticoids regulate cell death in the central nervous system, sex steroids control cell death in the ovary, and progesterone can inhibit cell death in breast cancer cells.^{23–25} Steroid hormones have also been used for treatment of prostate and breast cancers^{26,27} and extensive efforts are underway to alter apoptotic signaling pathways in order to control other diseases, including viral infections, autoimmune diseases, and neurodegenerative disorders.^{28–30}

We are studying the fruit fly, *Drosophila melanogaster*, as a model system for defining the hormonal regulation of programmed cell death. The *Drosophila* genome encodes homologs of all the major players defined in both *C. elegans* and mammalian cell death pathways, including TRAF, Bcl-2 family members, an Apaf/CED-4 homolog, seven caspase genes, and Inhibitor of Apoptosis (IAP) homologs.^{31–33} In addition, three key death inducer genes were defined in *Drosophila*, *grim*, *reaper* (*rpr*), and *head involution defective* (*hid*).^{34–36} These genes are necessary and sufficient for programmed cell death and appear to function in pairs to trigger the appropriate patterns of cell death during development.^{34–38} The expression of *rpr* foreshadows cell death throughout *Drosophila* development, while *hid* and *grim* display close, but not exact correlation. HID activity can be regulated through the Ras pathway.^{39,40} Recent studies have indicated that the mammalian Smac/DIABLO proteins exert a similar function to *rpr*, *hid*, and *grim*, acting as key triggers of apoptosis by suppressing the ability of IAP proteins to inhibit caspase activation.^{41,42} Thus, the basic components of the cell death machinery have been faithfully conserved through evolution, from worms and flies to humans.

Drosophila metamorphosis entails the simultaneous death of obsolete larval organs and the growth and differentiation of the adult fly, in response to sequential pulses of the steroid hormone 20-hydroxyecdysone (henceforth called ecdysone).⁴³ A high titer ecdysone pulse at the end of larval development signals the destruction of the larval midgut and anterior muscles as the animal undergoes puparium formation, initiating metamorphosis and the prepupal stage of development.⁴⁴ This is followed ~10 h later by another ecdysone pulse that triggers the prepupal-pupal transition and the massive destruction of the larval salivary glands. Most salivary glands are destroyed rapidly in wild-type animals, at ~15 h after puparium formation.⁴⁵ Destruction of the larval midgut and salivary glands occurs by steroid-triggered autophagy with hallmark features of cell death, including permeability to the vital dye acridine orange, DNA fragmentation, and caspase activation.^{45–47} In addition, *rpr* and *hid* are coordinately induced by ecdysone in a stage- and tissue-specific manner immediately before the onset of larval midgut and salivary gland cell death.⁴⁵ Genetic studies have identified ecdysone-inducible transcription factors that direct *rpr* and *hid* induction in the doomed salivary glands. The zinc finger transcription factors encoded by the *Broad-Complex* and the E74A ETS domain transcription factor are required for *rpr* and *hid* induction.⁴⁸ Similarly, the stage-specific ecdysone-inducible *E93* gene is required for *rpr* and *hid* expression, along with the induction of several other key death regulators.⁴⁹ Moreover, mutations in the *BR-C*, *E74A*, or *E93* result in defects in larval salivary gland cell death.^{48,49} Finally, the ecdysone receptor directly induces *rpr* transcription through a hormone response element in its promoter.⁴⁸

In this paper, we integrate *Drosophila* Rel/NF- κ B and AP-1 transcription factors into the ecdysone-triggered genetic hierarchy that directs larval salivary gland cell death. The *Drosophila* genome encodes three Rel/NF- κ B

transcription factors, Dorsal, Dif, and Relish. Most homodimeric and heterodimeric combinations of these factors can regulate gene expression.^{50–53} Like their vertebrate homologs, these proteins can be inhibited by *Drosophila* I κ Bs, Cactus and the C-terminus of Relish.^{52,54,55} *Dorsal* is required for patterning the dorsal–ventral axis of the early embryo.⁵² As in vertebrates, the Cactus–Dorsal complex is restricted to the cytoplasm until signaling through the Toll receptor (related to the vertebrate interleukin-1 and Toll-like receptors) results in Cactus phosphorylation and degradation.⁵² This allows Dorsal to translocate to the nucleus where it can directly activate target gene transcription. In a further parallel with vertebrate Rel/NF- κ B function, all three *Drosophila* family members are key regulators of *Drosophila* immunity to microbial infection.^{56–58} The striking conservation in the regulation and function of *Drosophila* Rel/NF- κ B family members raises the possibility that these proteins may also play a role in the control of programmed cell death.

Like its mammalian counterpart, *Drosophila* AP-1 is a heterodimer of *Djun* and *Dfos* transcription factors, and its activity is regulated by the *Drosophila* JNK.^{59–64} Unlike mammals, however, *Dfos* can function as a homodimer.⁵⁹ *Djun* and *Dfos* are critical regulators of epithelial morphogenetic responses during both mid-embryogenesis and early metamorphosis.^{60–63,65–68}

We show here that members of both of the Rel/NF- κ B and AP-1 families are induced in larval salivary glands in a stage-specific manner, immediately before the onset of cell death and in synchrony with *rpr* and *hid* induction. Although expression of a truncated Dif, with only the Rel homology domain, led to a significant delay in salivary gland cell death, extensive loss-of-function genetic studies did not uncover any direct role for the three Rel/NF- κ B proteins in this death pathway. In contrast, we found that *Dfos* is required for efficient destruction of larval salivary glands as well as maximal levels of *rpr* and *hid* transcription. This study demonstrates a role for *Dfos* in the steroid-triggered death of the *Drosophila* larval salivary glands during metamorphosis.

Results

Rel/NF- κ B and AP-1 transcripts are induced immediately before the onset of larval salivary gland cell death

As a first step toward identifying a possible function for *Drosophila* Rel/NF- κ B and AP-1 family members in programmed cell death, we set out to determine if these genes are expressed in larval salivary glands during the onset of metamorphosis. RNA was isolated from salivary glands dissected from staged late third instar larvae and prepupae and analyzed by Northern blot hybridization (Figure 1). Transcription of the *E74A* early ecdysone-inducible gene marks the two high titer pulses of ecdysone, in late third instar larvae and late prepupae.⁶⁹ The second ecdysone-induced peak in *E74A* expression coincides with the coordinate induction of *rpr* and *hid*, consistent with the essential role of *E74A* in *hid* induction and salivary gland cell death.⁴⁸ *Dorsal*

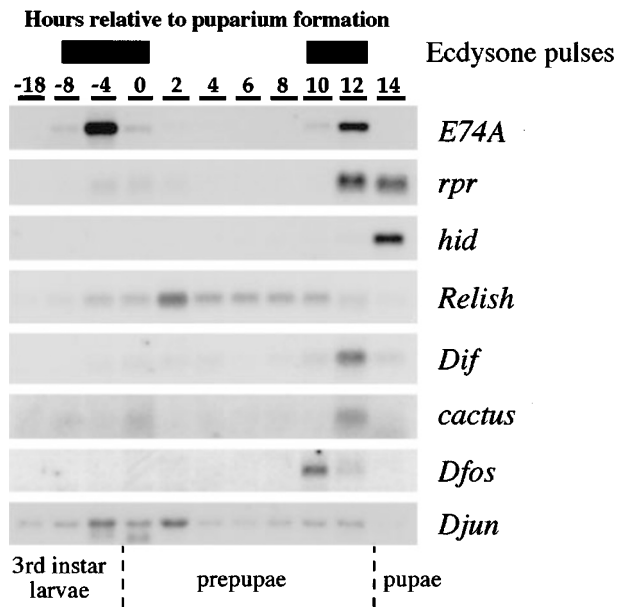


Figure 1 Temporal profiles of Rel/NF- κ B and AP-1 gene expression before the onset of salivary gland cell death. RNA was isolated from the salivary glands of staged late third instar larvae, at either ~18, 8, or 4 h before puparium formation, or from prepupae and pupae staged at 2 h intervals after puparium formation. Equal amounts of total RNA were fractionated by formaldehyde agarose gel electrophoresis and transferred to nylon. This blot was sequentially probed and stripped to detect *E74A*, *rpr*, *hid*, *Relish*, *Dif*, *cactus*, *Dfos* and *Djun* mRNA. The boxes at the top represent the peaks of the late larval and prepupal ecdysone pulses. Developmental stages are shown at the bottom

mRNA is not detectable at these stages in larval salivary glands (data not shown). In contrast, *Relish* mRNA is induced in parallel with the high titer late larval ecdysone pulse, peaks in 2 h prepupal salivary glands, and is then repressed in synchrony with the prepupal ecdysone pulse (Figure 1). Interestingly, *Dif* is induced as *Relish* is repressed, and expressed for a brief 2–4 h interval in late prepupal salivary glands, immediately before the onset of cell death (Figure 1). The *Drosophila* I κ B homolog *cactus* is expressed in parallel with *Dif*, indicating that other components of the Rel/NF- κ B signaling pathway are active at this time. *Djun* is expressed much like *Relish*. It is induced in late third instar larval salivary glands and then repressed in late prepupae (Figure 1). In contrast, *Dfos* is expressed in a stage-specific manner, in parallel with *Dif*, immediately before the onset of cell death (Figure 1). Thus, *Dif* and *Dfos* expression correlates well with the induction of *rpr* and *hid*, suggesting that these transcription factors might contribute to the ecdysone-triggered regulatory hierarchy that directs larval salivary gland cell death. Below we describe a series of experiments that test this hypothesis.

Expression of a truncated *Dif* protein inhibits salivary gland cell death and impairs *hid* induction

As an initial test of the role of Rel/NF- κ B family members in salivary gland cell death, we used a transformant line that expresses a truncated form of *Dif* under the control of a heat-

inducible promoter. This construct, referred to here as P[*hs-Dif(RD)*], directs expression of the Dif Rel homology domain but lacks the transactivation domain of this protein. P[*hs-Dif(RD)*] prepupae were selected at 10.5 h after puparium formation, maintained at either 25°C or given a brief heat treatment, and then allowed to recover at room temperature for 3 or 5 h. Salivary glands were dissected from these pupae and the levels of *rpr* and *hid* transcription were determined by Northern blot hybridization (Figure 2). Transcription of *rpr* is not significantly affected in salivary glands expressing *Dif(RD)*, but *hid* transcription is reduced to approximately one-fifth of the control level (Figure 2). Consistent with this effect, 75% of the animals that express *Dif(RD)* had persistent salivary glands at 18 h after puparium formation ($n=12$), 3 h after the destruction of salivary glands in control animals. These results suggest that Rel/NF- κ B family members may play a role in salivary gland cell death through the regulation of *hid* transcription.

Rel/NF- κ B family members are known to function as dimers, and the Rel domain is sufficient to mediate this interaction.^{50–53} Thus, the *Dif(RD)* construct could recruit an active Rel/NF- κ B family member to target promoters and thereby act in a manner similar to wild-type *Dif*, in spite of the absence of its activation domain. To test this possibility, we asked if ectopic overexpression of wild-type *Dif* would have a similar inhibitory effect on salivary gland cell death. Prepupae carrying both *hs-GAL4* and *UAS-Dif* constructs were subjected to a single 30 min heat treatment at 38°C either 9 or 10 h after puparium formation, and persistent salivary glands were scored 9 h later. Of 49 animals examined, however, only one showed a detectable delay in salivary gland cell death. We thus conclude that *Dif(RD)* has a novel function that is distinct from that of wild-type *Dif* protein.

An alternate possibility is that the *Dif(RD)* construct is functioning as a dominant negative by preventing Rel/NF- κ B family members from activating their normal targets in the genome. To test this possibility, we overexpressed a known dominant-negative regulator of Rel/NF- κ B signaling. Deletion of the N-terminus of the Cactus protein results in a stable protein that holds Rel/NF- κ B family members in the cytoplasm, rendering them unable to bind their genomic targets.⁵⁵ We thus used either the *hsp70* or a GAL4-dependent promoter to express a truncated form of Cactus that is missing the coding region for the N-terminal 125 amino acids.⁷⁰ Constitutive expression of this protein in larval salivary glands using an appropriate GAL4 driver resulted in normal larval salivary gland cell death, with 6% of the 18 h pupae examined having persistent salivary glands ($n=34$). This is similar to the 7% persistent salivary glands detected in 18 h pupae of the control genotype ($n=14$). Heat-induced overexpression of the dominant-negative Cactus protein using a 30 min 38°C heat treatment at 10 h after puparium formation resulted in 11% of the 18 h pupae having persistent salivary glands ($n=28$). Although this per cent is slightly higher than that seen with a constitutive GAL4 driver, it is within the range that is seen in heat-treated *w¹¹¹⁸* control animals. Moreover, no reproducible effects were detected on either *rpr* or *hid* transcription by Northern blot analysis of salivary gland

RNA (data not shown). We thus conclude that the *Dif(RD)* construct is not functioning in the same pathway as that regulated by the Cactus I κ B inhibitor.

Neither Dif nor Relish is required for *hid* induction in salivary glands

Definitive proof of a role for Rel/NF- κ B family members in larval salivary gland cell death requires an examination of null mutants in this pathway. *Dorsal* has no essential functions during zygotic development. It maps directly adjacent to *Dif* and, under certain conditions, can replace *Dif* function in the immune response.^{57,58} We thus analyzed the phenotypes associated with a deletion for both *dorsal* and *Dif*, *Df(2L)J4*.⁵⁷ As a control for this study we examined the *dl^{P01313}* mutant,

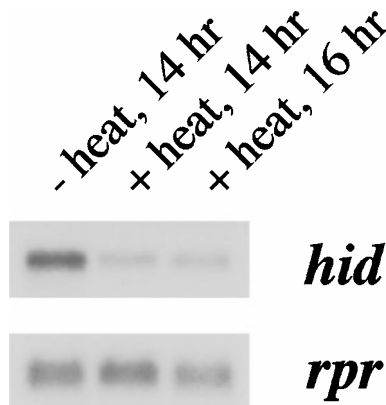


Figure 2 Ectopic expression of *Dif(RD)* results in reduced levels of *hid* transcription. *P[hs-Dif(RD)]* 10.5 h prepupae were maintained at either 25 or 37°C for 30 min and allowed to recover at 25°C for 3 or 5 h. Salivary glands were dissected from these 14 and 16 h pupae and the levels of *rpr* and *hid* mRNA were determined by Northern blot hybridization

which specifically inactivates *dorsal* function.⁵⁷ Salivary glands were dissected from staged homozygous *dl^{P01313}* and *Df(2L)J4* prepupae and expression of *rpr* and *hid* was determined by Northern blot hybridization (Figure 3A). Both death genes, however, are induced normally in *Df(2L)J4* mutant pupae, indicating that *Dif* is not required for their expression. In addition, the timing of salivary gland cell death is normal in *Df(2L)J4* mutant pupae (data not shown).

We also examined a possible contribution of *Relish* to programmed cell death because this Rel/NF- κ B family member is expressed in the larval salivary glands before they are destroyed (Figure 1). Salivary glands were dissected from staged *Df(3R)E20* *Relish* mutant and control prepupae,⁵⁶ and the expression of *rpr* and *hid* was determined by Northern blot hybridization (Figure 3B). Again, this study revealed normal patterns of *rpr* and *hid* induction, indicating that *Relish* is not required for death gene expression in doomed larval salivary glands. Moreover, cell death occurred normally in *Df(3R)E20* mutant salivary glands (data not shown).

There are no redundant functions between Drosophila Rel/NF- κ B family members in salivary gland cell death

One final possibility is that the three *Drosophila* Rel/NF- κ B family members could exert redundant functions in larval salivary gland cell death. Thus, for example, removing either *Dif* or *Relish* function alone might not reveal the role of a Rel/NF- κ B response in this pathway. Accordingly, we examined the phenotypes associated with a triple mutant that is missing all members of the *Drosophila* Rel/NF- κ B family. Animals carrying the *Df(2L)J4* deletion were crossed to *Df(3R)E20* animals and homozygotes were established for both deletions. Consistent with the expected weakness of this genetic background and, in particular, its susceptibility to infection,

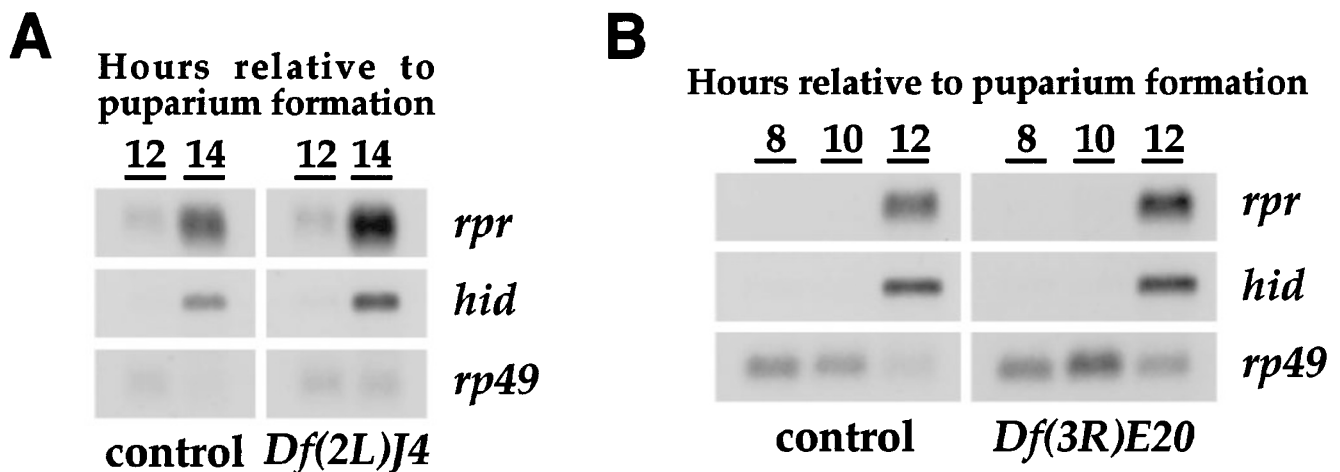


Figure 3 *Dif* and *Relish* mutations have no effect on *rpr* and *hid* transcription in doomed larval salivary glands. (A) Total RNA was isolated from salivary glands dissected from either control *dl^{P01313}* or *Df(2L)J4* 12 and 14 h pupae, and the levels of *rpr* and *hid* mRNA were determined by Northern blot hybridization. *Df(2L)J4* is a deletion mutation for both *dorsal* and *Dif*.⁵⁷ Hybridization to detect *rp49* mRNA was used as a control for loading and transfer. (B) Total RNA was isolated from salivary glands dissected from either control *E23* or *Df(3R)E20* 8, 10 and 12 h pupae, and the levels of *rpr* and *hid* mRNA were determined by Northern blot hybridization. *Df(3R)E20* is a null mutation for *Relish*.⁵⁶ Hybridization to detect *rp49* mRNA was used as a control for loading and transfer. The 2 h difference in *rpr* and *hid* induction in panels A and B is consistent with the known differences in the timing of the prepupal ecdysone pulse in different genetic backgrounds.⁸⁷

very few of the triple mutants survived until puparium formation. Eleven such animals were selected, staged until 14.5–16 h after puparium formation, and their salivary glands were examined for signs of impending cell death. Of these animals, 18% had intact salivary glands, 18% had no salivary glands, and 64% had salivary glands that were being degraded. These numbers are comparable to those seen in wild-type animals at this stage in development indicating that there is no contribution of Rel/NF- κ B family members to larval salivary gland cell death.

To confirm the genetic identity of the stock we had established, RNA was isolated from the triple mutants and analyzed for *Dif* and *Relish* expression by Northern blot hybridization (Figure 4). As expected, control flies show normal levels of *Dif* and *Relish* (Figure 4, lanes 1,5). In

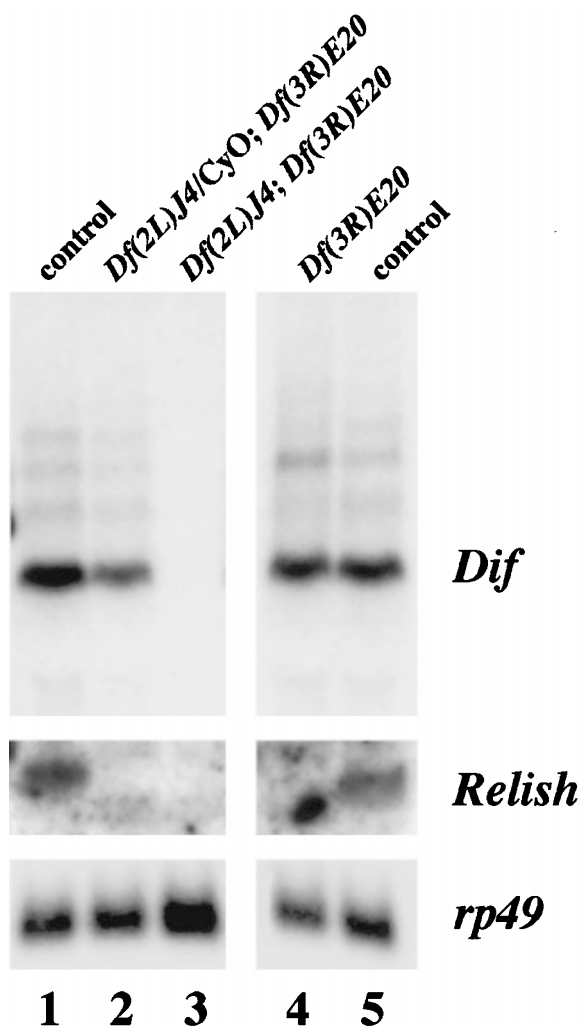


Figure 4 *Dif* and *Relish* are not expressed in *Df(2L)J4; Df(3R)E20* mutants. RNA was isolated from adult flies of the depicted genotypes and *Dif* and *Relish* mRNA were detected by Northern blot hybridization. The control samples that express all Rel/NF- κ B family members were derived from either *w¹¹¹⁸* or *E23* animals (lanes 1 and 5, respectively). *Df(3R)E20 Relish* mutants with either one copy (lane 2) or two copies (lane 4) of the *dorsal* and *Dif* genes were also tested, as was the *Df(2L)J4; Df(3R)E20* double mutant that is missing all Rel/NF- κ B family members (lane 3). Hybridization to detect *rp49* mRNA was used as a control for loading and transfer

addition, *Df(3R)E20* mutants and *Df(3R)E20* mutants that are missing one copy of the *Dif* locus lack *Relish* mRNA but continue to express *Dif* (Figure 4, lanes 2,4). Finally, flies carrying both homozygous deficiencies express neither *Dif* nor *Relish* (Figure 4, lane 3). These observations confirm the identity of the double deficiency stock and support the conclusion that Rel/NF- κ B family members are not required for salivary gland cell death.

***Dfos* mutants display defects in larval salivary gland cell death as well as *rpr* and *hid* induction**

Both halves of the *Drosophila* AP-1 transcription factor, *Dfos* and *Djun*, are expressed in the salivary glands of late prepupae, with a brief burst of *Dfos* expression immediately preceding the induction of *rpr* and *hid* (Figure 1). We therefore tested whether these factors might contribute to salivary gland cell death through regulating *rpr* and/or *hid* transcription. *Djun* mutants die during embryogenesis and thus could not be used for this study.^{60–62} Similarly, most *kay* alleles lead to embryonic lethality. However, *kay²* is a hypomorphic *Dfos* mutation that allows a few animals (~1%) to survive to adulthood,^{63,66} allowing us to analyze the role of *Dfos* in larval salivary gland cell death. Control *kay²/TM6B* pupae displayed no defects in salivary gland cell death, with glands either partially or completely destroyed by 16 h after puparium formation (Figure 5). However, 23% of 16 h *kay²* mutant pupae and 5% of 17–20 h *kay²* mutant pupae have persistent salivary glands (Figure 5). These *kay²* mutants underwent ecdysone-triggered adult head eversion at approximately the same time as the control pupae, indicating that there is no overall effect on hormone signaling in this genetic background, or a general developmental delay. We thus conclude that *Dfos* is required for the proper timing of this death response.

The observed delay in the destruction of *kay²* mutant larval salivary glands suggests that there might be a detectable effect on *rpr* and/or *hid* expression in this tissue. To test this possibility, salivary glands were isolated from staged *kay²/TM6B* control pupae at either 12 or 14 h after puparium formation and *kay²* mutant pupae at 14 and 16 h after puparium formation. RNA was extracted from these tissues and the patterns of *rpr* and *hid* transcription were analyzed by Northern blot hybridization (Figure 6A). Both *rpr* and *hid* are induced normally in control salivary glands, and normal levels of *rpr* and *hid* mRNA are detected in 14 h *kay²* mutant salivary glands (Figure 6A, lanes 1–3). In salivary glands isolated from 16 h *kay²* mutants, however, little or no *hid* mRNA is detectable and the levels of *rpr* mRNA appear to be reduced (Figure 6A, lane 4). The absence of any effects on *rpr* and *hid* mRNA levels in 14 h *kay²* mutant salivary glands is most likely due to the fact that many of these salivary glands would have died on time (Figure 5). In contrast, *kay²* mutant glands that have survived to the 16 h timepoint, when all salivary glands from the control genotype have been destroyed (Figure 5), are more likely to reveal a role for *Dfos* in death gene expression.

To confirm this result, we used a salivary gland GAL4 driver to express a dominant negative form of *Dfos*, Fbz, in

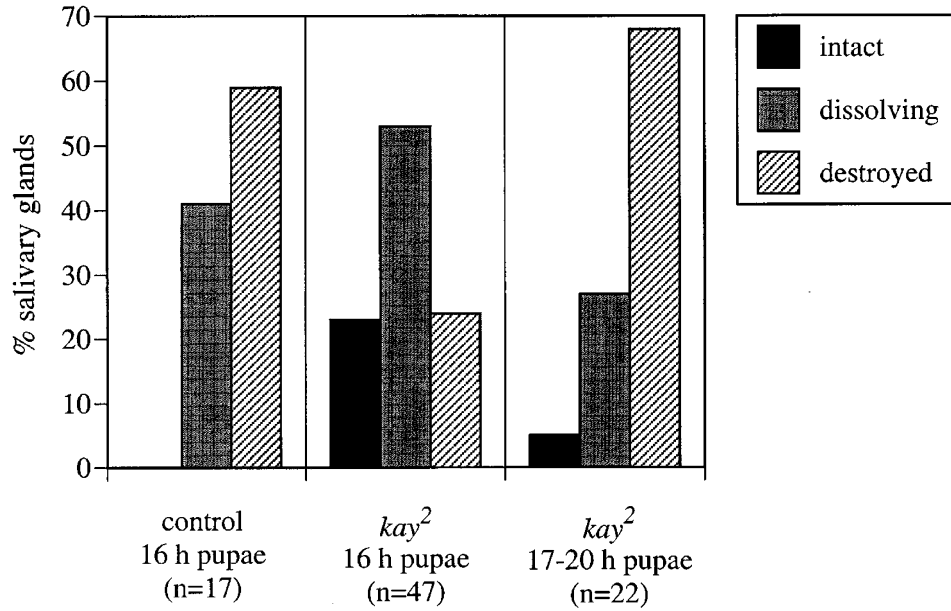


Figure 5 Salivary gland cell death is delayed in *kay*² mutants. Salivary glands were dissected from either control *kay*²/TM6B 16 h pupae, or 16 h and 17–20 h *kay*² mutant pupae. *kay*² mutant pupae that were delayed in their overall development were discarded. Fully intact, partially destroyed (dissolving), and completely destroyed salivary glands were scored for each genotype and the numbers are presented in graphic format

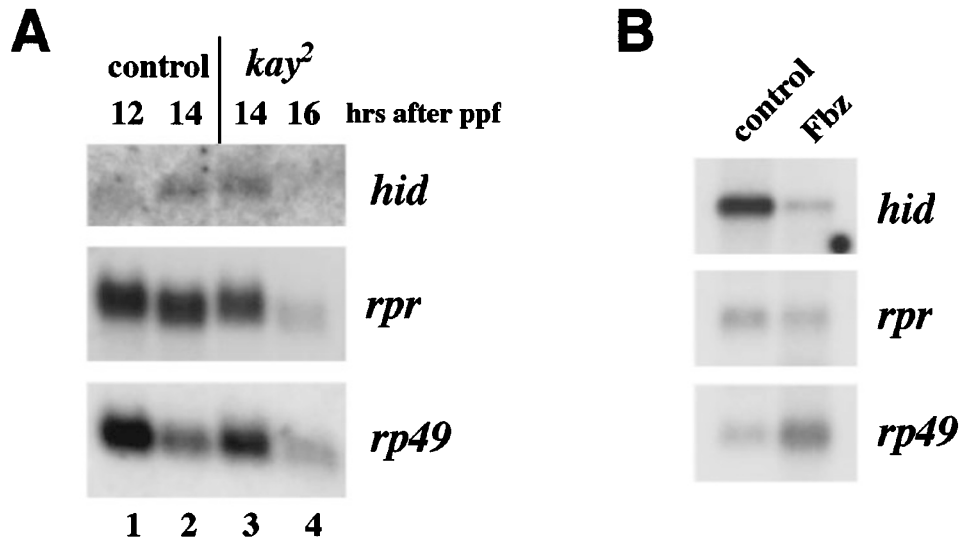


Figure 6 Reduced *Dfos* activity results in lower levels of *rpr* and *hid* transcription. (A) Total RNA was isolated from salivary glands of either *kay*²/TM6B control animals at 12 or 14 h after puparium formation (hours after ppf) or *kay*² homozygotes at 14 or 16 h after puparium formation, and the levels of *rpr* and *hid* mRNA were determined by Northern blot hybridization. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer. (B) Total RNA was isolated from 13 h pupae carrying either one copy of *UAS-Fbz* (control) or one copy of the D59 salivary gland GAL4 driver and one copy of the *UAS-Fbz* transgene (Fbz). The levels of *rpr* and *hid* mRNA were determined by Northern blot hybridization. Hybridization to detect *rp49* mRNA was used as a control for loading and transfer

the salivary glands of late prepupae.⁶⁵ RNA was isolated from salivary glands at 13 h after puparium formation and the levels of *rpr* and *hid* mRNA were analyzed by Northern blot hybridization (Figure 6B). In agreement with the *kay*² loss-of-function study, salivary glands expressing the dominant negative form of *Dfos* have reduced levels of *hid* mRNA. Moreover, correcting for the levels of the internal *rp49* control, the levels of *rpr* mRNA also appear to be reduced. Similar results were seen in an independent

duplicate experiment (data not shown). We thus conclude that *Dfos* is required for the proper timing of salivary gland cell death and that it exerts this effect by directing maximal levels of *rpr* and *hid* transcription.

Discussion

Several studies have shown that vertebrate steroid hormones can regulate programmed cell death through NF- κ B and AP-1

activity.^{71–75} In all of these cases, however, the downstream targets of NF- κ B and AP-1 are not known, and thus it remains unclear how hormonal regulation of these transcription factors initiates a death response. In this study, we ask whether Rel/NF- κ B and/or AP-1 family members contribute to the steroid-triggered destruction of the larval salivary glands during *Drosophila* metamorphosis. Although we find no direct role for Rel/NF- κ B family members in this pathway, we do show that AP-1 is required for efficient salivary gland cell death at the prepupal-pupal transition, and that it can mediate this effect through *rpr* and *hid* induction. This work provides a foundation for integrating AP-1 activity into the genetic cascade that controls larval salivary gland cell death during *Drosophila* metamorphosis.

NF- κ B and AP-1 family members are induced in a stage-specific manner before the onset of cell death

Drosophila Rel/NF- κ B and AP-1 family members all show some apparent regulation by ecdysone in doomed larval salivary glands. Both *Relish* and *Djun* are induced in late third instar larval glands, in synchrony with the *E74A* early ecdysone-inducible gene (Figure 1). Both peak in 2 h prepupae, are down-regulated in 4 h prepupae, and are repressed at 12–14 h after puparium formation, immediately following the prepupal ecdysone pulse. In contrast, *Dif*, *cactus*, and *Dfos* are all induced in a stage-specific manner, in synchrony with the prepupal ecdysone pulse and immediately preceding the induction of *rpr* and *hid* and the onset of larval salivary gland cell death (Figure 1). Whereas *Dif* and *cactus* are induced in parallel with *E74A*, *Dfos* is expressed somewhat earlier, just as *E74A* and *Dif* mRNA are first detected (Figure 1). This early induction could be due to increased sensitivity of the *Dfos* promoter to ecdysone, resulting in induction by a lower hormone concentration than that required for *E74A*.⁷⁶

The stage-specific induction of *Dif* and *Dfos* provides a model for explaining the stage-specificity of *rpr* and *hid* expression in larval salivary glands. The *rpr* and *hid* death genes show no response to the late larval pulse of ecdysone but rather are selectively induced by the prepupal ecdysone pulse, ~12 h after puparium formation (Figure 1).⁴⁵ The similar stage-specific expression patterns of *Dif* and *Dfos* raise the possibility that these factors could direct the timing of *rpr* and *hid* induction, providing a molecular mechanism whereby the repetitive ecdysone signal is refined into a stage-specific death response. We test this model and provide evidence of a role for *Dfos* in larval salivary gland cell death.

A potential role for Rel/NF- κ B binding sites in controlling *hid* expression and cell death

We used five different genetic approaches to determine whether Rel/NF- κ B family members might play a role in larval salivary gland cell death. These included ectopic expression of either wild-type *Dif*, a truncated form of *Dif* carrying only the DNA binding and dimerization domain, or a dominant-negative form of Cactus that should inactivate all NF- κ B

responses. In addition we examined larval salivary gland cell death in loss-of-function mutants for either *dorsal* and *Dif*, *Relish*, or all three NF- κ B family members. Of these, only ectopic expression of the truncated *Dif* protein resulted in a delay in larval salivary gland cell death and reduced levels of *hid* transcription (Figure 2). Unfortunately, the mechanism of action of this mutant form of *Dif* remains unclear. The absence of any clear effect on salivary gland cell death in the different mutant backgrounds, however, as well as in the presence of dominant-negative Cactus, strongly argues against a direct role for these transcriptional regulators in the salivary gland death response.

The observation that ectopic *Dif*(RD) expression can lead to death defects while no effect is seen in Rel/NF- κ B mutants argues that this truncated protein is functioning in a different pathway. One possibility is that *Dif*(RD) can occupy Rel/NF- κ B binding sites in *hid* regulatory sequences, thereby blocking the binding of other critical factors. These factors could either recognize the NF- κ B site directly or bind to an overlapping target sequence. The latter is more likely since we have tested all three genes predicted by the *Drosophila* genome sequence to have canonical Rel DNA binding domains – *dorsal*, *Dif*, and *Relish*. The presence of four optimal Rel/NF- κ B binding sites within a 28 kb region encompassing the ~20 kb *hid* gene supports this model. It would be interesting to determine whether these sites are required for maximal *hid* induction by ecdysone. Regardless of the mechanism of *Dif*(RD) action, however, the key conclusion from this study is that Rel/NF- κ B family members are not required for the steroid-triggered cell death of larval salivary glands during *Drosophila* metamorphosis.

Morphological and genetic studies have demonstrated that *Drosophila* larval salivary glands die by autophagy rather than apoptosis.^{46,47} Autophagy is characterized by the formation of multiple acidic autophagic vacuoles within the doomed cells followed by massive cellular degeneration. Studies in vertebrate cells have shown that Rel/NF- κ B and AP-1 family members play a critical role in controlling apoptosis.^{1,5,6,9,14–16} No studies, however, have addressed a possible role for these factors in autophagic cell death. The results of this work indicate that Rel/NF- κ B family members have no function in at least one autophagic death response, and suggest that the activity of this pathway may be specific to apoptotic programmed cell death. Further studies of Rel/NF- κ B function in other autophagic death responses should provide insight into its role in regulating programmed cell death during development.

Dfos is required for efficient larval salivary gland cell death

In contrast to our results with Rel/NF- κ B family members, we find that the *Drosophila* homolog of Fos is required for maximal *rpr* and *hid* transcription as well as the proper timing of larval salivary gland cell death. Intact larval salivary glands can be detected in 16–20 h *kay*² mutant pupae, a time when glands in the control genotype have all entered cell death (Figure 5). In addition, the levels of both *rpr* and *hid* are reduced in persistent *kay*² mutant salivary glands (Figure 6A, lane 4) as well as

salivary glands that express a dominant negative form of *Dfos* (Figure 6B). It is important to note, however, that this is a partially penetrant phenotype. In 16 h *kay*² pupae, 77% of the larval salivary glands are either completely or partially destroyed, and only 5% of the salivary glands persist until 17–20 h after puparium formation (Figure 5). This is similar to the partial salivary gland death defects observed in *E74A* mutants and supports the proposal that multiple independent pathways are required to direct efficient larval salivary gland cell death.⁴⁸ We thus conclude that *Dfos* is required for directing efficient larval salivary gland cell death in response to the prepupal pulse of ecdysone. It is interesting to note that there are three AP-1 binding sites in the 7 kb region of the *rpr* promoter that confers maximal expression, suggesting that *Dfos* may directly regulate death gene expression.

Ecdysone triggers two apparently opposite biological responses at the onset of metamorphosis – the death of larval tissues and the growth and differentiation of adult tissues.⁷⁷ It is interesting to note that AP-1 functions in both of these pathways. The late larval ecdysone pulse triggers morphogenesis of the leg and wing imaginal discs resulting in the formation of rudimentary adult appendages.⁷⁸ The thoracic portions of the wing discs then fuse during prepupal development to form a smooth intact thorax that is evident on the dorsal surface of the adult fly. This fusion is dependent on AP-1 signaling.^{67,68} Thus, it appears that AP-1 exerts distinct stage- and tissue-specific functions during the onset of metamorphosis. Initially required for fusion of the thoracic imaginal discs, AP-1 is later required for efficient destruction of the larval salivary glands in response to ecdysone.

It is interesting to note that the results reported here for *Dfos* in larval salivary gland cell death are similar to those seen in mice. The expression of c-Fos precedes programmed cell death during normal mouse development.⁷⁹ In spite of this correlation, however, mutant mice lacking c-Jun alone or c-Jun and c-Fos show only minor effects on programmed cell death responses.^{80,81} But, unlike *Drosophila*, the presence of multiple AP-1 family members encoded by the mouse genome complicates the interpretation of these results. Further functional studies of AP-1 should provide a better understanding of its roles in regulating programmed cell death during fly and mouse development.

Materials and Methods

Drosophila stocks

Canton S was used as a wild-type stock. The *dl*^{P01313} *dorsal* mutant and *Df(2L)J4 dl, Dif* double mutant stocks⁵⁷ were maintained as *yw, dl*^{P01313}/*Cyo, y+* and *yw, Df(2L)J4/Cyo, y+* or *Df(2L)J4/Cyo GFP*. Homozygous *dl*^{P01313} and *Df(2L)J4* mutant third instar larvae were identified by their yellow mouth hooks. The *Relish* mutant *Df(3R)E20* and its control line *E23* have been described and were kindly provided by Dan Hultmark. *E23* is a precise excision of a P element inserted in the 5' end of the *Relish* gene.⁵⁶ *Df(3R)E20* is an imprecise excision of this P element that removes all four *Relish* transcription start sites and some 5' untranslated sequences.⁵⁶ Both *E23* and *Df(3R)E20* are homozygous viable. The GAL4/UAS system was used to direct ectopic

expression in larval salivary glands.⁸² *hs-GAL4* transformants were obtained from the Bloomington stock center. D59 was used to drive GAL4-dependent expression in larval salivary glands.⁸³ The dominant negative *cactus* BP4- Δ N125*cact* Δ PEST cDNA was kindly provided by D Stein,⁷⁰ the *kay*² mutant stock was provided by D Bohmann⁶³ and *UAS-Fbz* transformants were provided by M Bienz.⁶⁵

Developmental staging

Late third instar larvae were staged by maintaining them on food containing 0.1% bromophenol blue, as described.⁸⁴ Blue gut mid-third instar larvae are referred as –18 h relative to puparium formation, partially clear gut late third instar larvae are referred to as –8 h, and clear gut late third instar larvae are referred to as –4 h. Prepupae and pupae were staged relative to puparium formation (0 h) as described.⁸⁴

Ectopic expression studies

The P[*hs-Dif(RD)*] construct was generated by cutting the *Dif* cDNA with *NdeI* (artificially generated at codon 16)⁵³ and *EcoRV* (at codon 432). The 1.25 kb fragment was inserted into the *StuI* site of the pCaSpeR-*hs* vector by blunt end ligation. This construct removes the C-terminal 235 amino acids of *Dif* which should function as the transcriptional activation domain. This construct was introduced into *yw*⁶⁷ flies by P element-mediated germline transformation.⁸⁵ To ectopically express *Dif(RD)*, P[*hs-Dif(RD)*] transformants were synchronized at puparium formation and allowed to develop at 25°C for 10.5 h. They were incubated in a 37°C water bath for 30 min, and allowed to recover at 25°C for 3 or 5 h, after which their salivary glands were dissected. As a control, transformants were selected at puparium formation and allowed to develop at 25°C for 14 h before salivary glands were dissected. Total RNA was then extracted from the salivary glands and analyzed by Northern blot hybridization. The *cactus* Δ N125*cact* Δ PEST cDNA⁷⁰ was inserted into both pCaSpeR-*hs*-act and pUAST. The vectors were cut with *EcoRI*, filled-in, and then cut with *NotI*, and the Δ N125*cact* Δ PEST cDNA was inserted as a 1.5 kb *HindIII* (filled-in)-*NotI* fragment. Transgenic lines were established for these constructs by P element-mediated germline transformation of the recipient strain *w*¹¹¹⁸.

Northern blot hybridization

Total RNA isolated from 12 pairs of dissected salivary glands was fractionated by formaldehyde gel electrophoresis and transferred to nylon membranes as described.⁶⁹ For analysis of the *dl, Dif*, and *Relish* triple mutant (Figure 4), 20 μ g total RNA from adult flies was loaded per lane. Each blot was sequentially hybridized with one or more radioactive probes and stripped for rehybridization.⁶⁹ The *E74A* and *rpr49* probes were prepared as described,⁸⁶ *rpr* and *hid* probes were prepared as described.⁴⁵ The following DNA fragments were used to detect *Djun, Dfos, Relish, Dif* and *cactus* – *Djun*: a 300 bp fragment corresponding to the coding region for the first 100 amino acids of the *Djun* protein; *Dfos*: a 1195 bp *Bam*HI fragment; *Relish*: an 804 bp *Bam*HI–*Hind*III fragment; *Dif*: an 855 bp *Bam*HI–*Eco*RI fragment; *cactus*: an 1162 bp *Bgl*II fragment.

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*kay*² mutant, M Bienz for the *UAS-Fbz* stock, and the Bloomington Stock Center for providing GAL4 driver stocks. M Lehmann is the recipient of a research fellowship (Le870/4-1) from the Deutsche Forschungsgemeinschaft. This work was supported by National Institutes of Health grant RO1 GM53269 to YT Ip and RO1 GM60954 to CS Thummel.

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