Activation of the cAMP/PKA signaling pathway is required for post-ecdysial cell death in wing epidermal cells of *Drosophila melanogaster*

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Summary

At the last step of metamorphosis in *Drosophila*, the wing epidermal cells are removed by programmed cell death during the wing spreading behavior after eclosion. The cell death was accompanied by DNA fragmentation demonstrated by the TUNEL assay. Transmission electron microscopy revealed that this cell death exhibited extensive vacuoles, indicative of autophagy. Ectopic expression of an anti-apoptotic gene, *p35*, inhibited the cell death, indicating the involvement of caspases. Neck ligation and hemolymph injection experiments demonstrated that the cell death is triggered by a hormonal factor secreted just after eclosion. The timing of the hormonal release implies that the hormone to trigger the death might be the insect tanning hormone, bursicon. This was supported by evidence that wing cell death was inhibited by a mutation of *rickets*, which encodes a G-protein coupled receptor in the glycoprotein hormone family that is a putative bursicon receptor. Furthermore, stimulation of components downstream of bursicon, such as a membrane permeant analog of cAMP, or ectopic expression of constitutively active forms of G proteins or PKA, induced precocious death. Conversely, cell death was inhibited in wing clones lacking G protein or PKA function. Thus, activation of the cAMP/PKA signaling pathway is required for transduction of the hormonal signal that induces wing epidermal cell death after eclosion.

Movies available online

Key words: Programmed cell death, *Drosophila*, Metamorphosis, cAMP, PKA, *rickets*, Bursicon, Wing

Introduction

Programmed cell death is crucial for the development and maintenance of multicellular organisms, to control cell number, to remove infected, mutated or damaged cells, and to eliminate cells that are no longer required at a given stage of development (Jacobson et al., 1997; Vaux and Korsmeyer, 1999). During development, apoptotic, autophagic and non-lysosomal cell death have been widely reported (Schweichel and Merker, 1973; Clarke, 1990). Apoptosis is characterized by initial DNA condensation, followed by nuclear and cytoplasmic fragmentation. Further removal of those fragments occurs through phagocytosis. Autophagy is accompanied by the formation of autophagic vacuoles that encapsulate cytosolic components for degradation. It does not appear to require phagocytes for the majority of cell removal, although the remnants of autophagic cell death are still cleared by phagocytes (Clarke, 1990).

The components and mechanisms for programmed cell death are conserved in a wide variety of organisms, from worms and flies to humans. These mechanisms involve the activation of cysteine proteases, known as caspases, which act as executioners (Kumar and Doumanis, 2000). Caspase activity is inhibited by negative regulators, such as the Inhibitor of Apoptosis Proteins (IAPs), that prevent cell death (Hay, 2000). In *Drosophila*, the products of the pro-apoptotic genes *reaper* (*rpr*) (White et al., 1994), *head involution defective* (*hid*; *Wrinkled*, *W* – FlyBase) (Grether et al., 1995), *grim* (Chen et al., 1996) and *sickle* (*skl*) (Christich et al., 2002; Srinivasula et al., 2002; Wing et al., 2002a) promote cell death by the inhibition of IAP function (Wang et al., 1999; Holley et al., 2002; Ryoo et al., 2002; Wing et al., 2002b; Yoo et al., 2002). Various death and survival signals should converge onto the pro-apoptotic genes during development. The nature of these extracellular signals and the signal transduction pathways that activate or suppress the death program are largely unknown.

During metamorphosis in *Drosophila*, most of the larval tissues die and are removed. The timing of these events is orchestrated by changes in the levels of the steroid hormone 20-hydroxyecdysone (ecdysone) in the larval salivary glands and midgut (Jiang et al., 1997). Ecdysone regulates expression of a number of early and late genes (Lee et al., 2002a; Lee et al., 2002b) that act in part by inducing expression of the cell death activator genes *rpr* and *hid*, thereby repressing the *Drosophila* IAP gene *Diap2* (*Iap2* – FlyBase) (Jiang et al., 1997). Thus, changes in hormone titre, in combination with the fine-scale expression of the ecdysone receptor, control timing and spacing of cell death during metamorphosis. Though ecdysone-dependent regulation of cell death is the best

understood pathway, it is likely that peptide hormones also regulate programmed cell death in *Drosophila* metamorphosis.

At the last step of metamorphosis, newly emerged adults undergo extensive cell death. Many of the abdominal muscles and associated neurons die within a day of adult emergence (Finlayson, 1975; Truman, 1983; Kimura and Truman, 1990). In addition, the wing epidermis is also removed by cell death at the time of wing spreading in the large fly *Lucilia cuprina* (Seligman et al., 1975) and in *Drosophila melanogaster* (Johnson and Milner, 1987). In the present study, we focused on this epidermal cell death during maturation of the wings of *Drosophila*. Marking of wing epidermal cells by GFP using the GAL4/UAS system (Brand and Perrimon, 1993) enabled us to follow the fate of the epidermal cells and facilitated further analysis of the regulation of the cell death. We report that the cell death is triggered by a hormone, probably bursicon, which is released just after eclosion, and that the hormonal signal is received by RICKETS (DLGR2), one of glycoprotein hormone receptors identified as a member of the G-protein-coupled receptor family (Eriksen et al., 2000; Hewes and Taghart, 2001; Baker and Truman, 2002). We also present evidence that this signal is transmitted through the cAMP/PKA signaling pathway. These results reveal a novel mechanism to regulate programmed cell death by a hormone other than ecdysone.

Materials and methods

Drosophila strains

Flies were reared on cornmeal-yeast medium at 25°C under constant illumination. To follow the fate of the epidermal cells after eclosion, we marked wing epidermal cells with nuclear GFP in most experiments. We made a stock with *engrailed (en)-Gal4* (Dormand and Brand, 1998) and *UAS-GFPN* (Shiga et al., 1996) on the same second chromosome and used it as wild-type. A stock of His2AvDGFP (His-GFP) (Clarkson and Saint, 1999) was also used to mark the wing epidermal cells.

The same *en-Gal4* line was used to express the following constructs: UAS-GsαWT and UAS-Gsα* (*Drosophila* wild-type Gsα subunit and constitutively active Gsα subunit, respectively) (Wolfgang et al., 1996); UAS-mC* (constitutively active form of mouse PKA catalytic subunit mC*) (Li et al., 1995); UAS-R* (dominant-negative form of the regulatory subunit of PKA) (Li et al., 1995); or UAS-p35 (anti-apoptotic protein) (Zhou et al., 1997), using the Gal4/UAS expression system (Brand and Perrimon, 1993).

For clonal analysis of the Gsα or PKA mutations, somatic clones were produced using the FLP/FRT recombination system (Xu and Rubin, 1993). First or second instar larvae, generated by crossing *y hs-flp1 / y^{1} w^{1118}; FRT42D y^{+} /+; +/+* females to *y w/Y; FRT42D sha bw dgs^{R60C} /+; His-GFP/+* males, were heat shocked for 1 hour at 37°C to induce mitotic recombination, and adult flies were examined for clones of *sha*$^{-}$ wing tissue, where hairs are missing or replaced by smaller hairs. Similar treatment of first or second instar larvae, derived from the cross of *y hs-flp1; hs-CD2 y+ hs-myc FRT39E/CyO; MKRS/TM2* females to *y w/Y; DC0^{E95} stc FRT39E/+; His-GFP/+* males was carried out and adult flies were examined for clones of *stc*$^{-}$ wing tissue, which replaces hairs by smaller hairs or tufts of hairs. The stocks for generation of *dgs* or *DC0* mutant clones were generously provided by Wolfgang et al. (Wolfgang et al., 2001) and Jiang and Struhl (Jiang and Struhl, 1995), respectively.

The mutant stock of *rickets* (*rk*) used was *rk^{1} cn^{1} bw^{1}; His-GFP/+*. An allele of *rk^{1}* carries a mutation in the transmembrane domain that results in a premature termination codon. This mutation should prevent the production of a functional membrane receptor (Baker and Truman, 2002).

Observation of wing epidermal cells and detection of cell death

Wings were dissected in PBS and mounted in PBS on a slide glass. Expression of GFP in the central part of the wing blade was observed under an Olympus AX70 fluorescence microscope equipped with an Olympus DP50 camera and images obtained were processed with Photoshop software. The extent of cell death was graded into three classes based on the number of cells undergoing death at the time of observation: 'occurrence of cell death', 80% or more of cells were dead; 'partial cell death', 20-80% of cells were dead; 'no cell death', less than 20% of cells were dead.

Movie images of dying cells in the wing of an intact fly, *en-Gal4 UAS-GFPN*, were captured, after fixing a ventral portion of the fly on a slide glass with melted myristyl alcohol. The wing just after spreading was observed under a fluorescence microscope equipped with a Sony video camera DXC-930, and images were recorded on videotape.

Ligations and injections

Newly eclosed adults were collected at 3-minute intervals. Staged flies after eclosion were anesthetized on an ice-chilled Petri plate, ligated at the neck with a thin silk thread, and the head was cut away. Pharate adults were collected on a strip of double-sided tape attached to a slide glass and the opercula of the puparium was removed. They were staged under a dissection microscope according to features of the head (Kimura and Truman, 1990).

Briefly, at about 9 hours before eclosion, the pupal cuticle of the head has a smooth appearance [smooth (S) stage]. At 6 hours before eclosion, wrinkles appear in the pupal cuticle over the head [smooth/grainy (S/G) stage], because of the initiation of molting fluid resorption. By about 3 hours before eclosion, molting fluid resorption is well advanced and the pupal cuticle has a granular appearance [grainy (G) stage]. At about 50 minutes before eclosion, the head acquires a whitish sheen [white (W) stage], because air fills the space between the pupal and adult cuticle. At about 40 minutes before eclosion, the ptilinum protrudes from the front of the head [extended ptilinum (EP) stage].

After removal of the anterior half of the puparium, staged pharate adults were ligated at the neck. For injections, neck-ligated or intact flies were anesthetized on an ice-chilled Petri dish and were injected with solutions using a glass capillary injection needle connected to a glass syringe. The volume of solution injected was approximately 10-30 nl. Solutions used for injections were saline (PBS), 8-bromo-cAMP (Sigma) solution at various concentrations and DAPI solution at the concentration of 0.1 mg/ml in PBS. Flies ligated or/and injected were kept in a moist Petri dish till the desired time.

For collection of hemolymph, we cut legs of a staged fly and compressed the thorax of the fly using forceps. A drop of hemolymph was collected in a glass capillary needle and was injected into a host fly.

Histology

TUNEL (TdT-mediated dUTP nick-end labeling) assays were carried out, using the In Situ Cell Death Detection Kit, POD (Roche), as described by the manufacturer. Staged flies of Canton-Special strain (wild type) were fixed in 3.7% formaldehyde for 2 hours and paraffin-sectioned preparations of wings were prepared by standard methods. The sections were incubated in TUNEL reaction mixture containing TdT and fluorescein-conjugated dUTP to label the ends of DNA fragments. After washing, the labeled preparations were stained with DAPI solution.

Wings were fixed for electron microscopy (EM) in 2.5% glutaraldehyde in 0.1 M phosphate buffer, and embedded into Epon 812 by standard procedures. EM sections were stained with uranyl

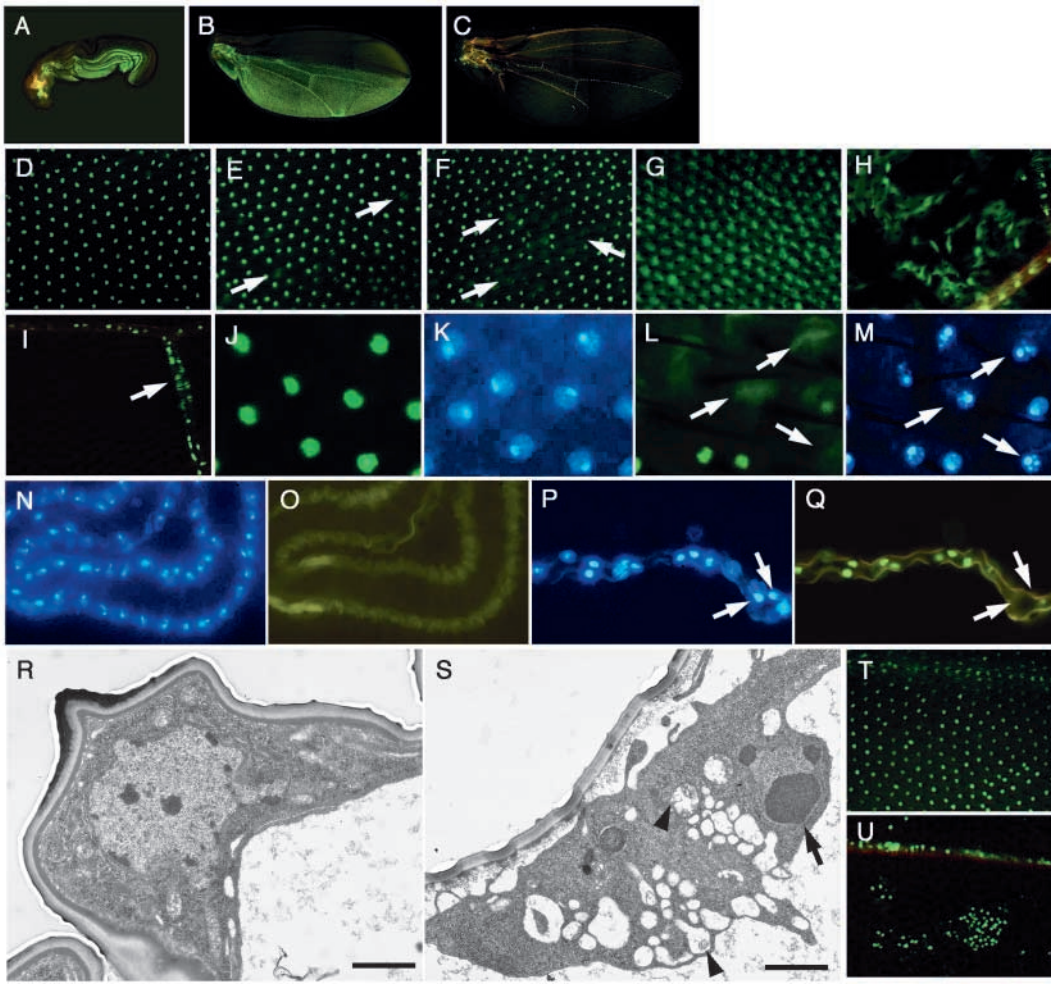

Fig. 1. (A-M) Programmed cell death of wing epidermal cells in *en-Gal4 UAS-GFPN* flies. Wing epidermal cells were visualized by nuclear-localized GFP. GFP was apparent in wing epidermal cells of the posterior compartment at 0 hours (A) and 0.5 hours (B) after eclosion, but disappeared by 2 hours after eclosion (C). Higher magnification images of cell death at 0 minutes (D), 10 minutes (E), 20 minutes (F), 30 minutes (G), 40 minutes (H) and 60 minutes (I) after wing spreading. At wing spreading, wing epidermal nuclei are labeled by GFP (D). The GFP signal was then dispersed into the cytoplasm from the nucleus following breakdown of the nuclear membrane (E,F; arrows). GFP was undetectable in the nuclei of all cells after 30 minutes (G), and the cells were detached from the wing cuticle and floated in the cavity formed by the dorsal and ventral wing surfaces (H). After 60 minutes, the wing epidermal cells had disappeared and GFP was detectable only in the nuclei of vein cells (I, arrow). At wing spreading, GFP in the nuclei of wing epidermal cells (J) corresponded to nuclei stained with DAPI (K). At 20 minutes after wing spreading, GFP was dispersed in the cytoplasm (L, arrows). DAPI staining showed that the chromatin of these cells was fragmented (M, arrows). (N-Q) Transverse sections of adult wings in wild-type flies. At eclosion, two layers of wing epidermal cells were stained with DAPI (N) but were not labeled with TUNEL (O). At 20 minutes after wing spreading, the wing epidermal cells were stained with DAPI (P) and were labeled with TUNEL (Q), except for vein cells (arrows; P,Q). Transmission electron microscopy in a wing epidermal cell at 0 hours after eclosion (R) and at 20 minutes after wing spreading (S). The arrow (S) indicates representative condensed chromatin structures. Arrowheads (S) denote cytoplasmic vacuoles that contain cellular structure including mitochondria. Scale bars: 1 μm in R,S. Ectopic expression of *p35* inhibited the death of wing epidermal cells in *en-Gal4 UAS-GFPN /UAS-p35* flies at 2 hours (T) or even at 48 hours after eclosion (U).

acetate and lead citrate, and examined on a JEOL 1010 electron microscope.

Results

Wing epidermal cells die after eclosion

The wings of newly eclosed flies are initially folded but most flies expand them within 30 minutes by executing a stereotyped behavior pattern (Baker and Truman, 2002). Wing epidermal cells in *en-Gal4 UAS-GFPN* flies expressed nuclear-localized GFP in the posterior compartment of wings (Fig. 1A,B). At 2 hours after eclosion, GFP expression in wing epidermal cells disappeared, except for in cells associated with veins (Fig. 1C).

Close observation showed that the disappearance of GFP is due to programmed cell death in the wing epidermal cells. At wing spreading, a sheet of wing epidermal nuclei labeled by GFP were seen in dorsal and ventral layers (Fig. 1D). Within 30 minutes after wing spreading, the GFP in the nuclei became obscure and dispersed in the cytoplasm due to the breakdown of nuclear membrane (Fig. 1E,F,G). Double staining with DAPI showed that the breakdown of the nuclear membrane was followed by the fragmentation of chromatin (Fig. 1J-M). During this procedure, TUNEL-positive cells appeared in wing epidermal cells, except for the vein cells (Fig. 1N-Q), indicating that DNA fragmentation is also accompanied by death. Subsequently, the cells detached from the wing cuticle and dispersed in the cavity between the dorsal and ventral wing cuticular sheets (Fig. 1H). Within 1 hour after wing spreading, these dying cells were absorbed into thoracic cavity through the veins (Fig. 1I; see movie at http://dev.biologists.org/supplemental/). For subsequent studies, we took the dispersion of GFP from the nucleus into cytoplasm to be an indication of cell death.

Transmission electron microscopy (TEM) analysis revealed the association of dynamic changes in vacuole structure with this cell death process. A wing epidermal cell at eclosion

possessed a spherical nucleus with some electron dense materials (Fig. 1R). At 20 minutes after wing spreading, the cell possessed condensed chromatin and many vacuoles that contain cellular components including mitochondria (Fig. 1S). Thus, TEM indicated that this cell death exhibited features indicative of autophagy, but this required further detailed analysis. Further studies in the detailed processes of the wing epidermal cell death will be described later.

We examined the effect of the forced expression of an anti-apoptotic gene, *p35*, the products of which inhibit the activity of caspases. We followed the fate of wing epidermal cells in *en-Gal4 UAS-GFPN /UAS-p35* adults. Ectopic expression of *p35* inhibited the death of wing epidermal cells at 2 hours after eclosion (Fig. 1T). Then, the cells detached from the wing cuticle without breakdown of the nuclear membrane, and many persisting cells remained between the cuticular sheets at least until 48 hours after eclosion (Fig. 1U), indicating that the caspases are involved in the cell death process.

Inhibition of the cell death also prevented the subsequent adhesion of the ventral and dorsal cuticular sheets (data not shown). As a result, wings remained filled with hemolymph and some persisting cells or debris, and, in some cases, ectopic expression of *p35* in the epidermal cells produced blistered wings (data not shown).

Neck ligation or wing isolation prevents the death of wing epidermal cells

To determine whether a humoral signal coming from the head triggers the cell death, we ligated the necks of flies at various times after eclosion and examined them for cell death at 2 hours after the ligation. Ligation just after eclosion suppressed cell death and GFP was still detectable in the nuclei of wing epidermal cells after 2 hours (Fig. 2A). By contrast, when flies were ligated at 20 minutes after eclosion, the normal pattern of cell death was observed (Fig. 2B). Ligation at later stages correlated with an increased percentage of flies with wing epidermal cell death (Fig. 2C). Thus wing epidermal cell death is triggered by a signal emanating from the head shortly after eclosion.

Neck ligation at eclosion inhibited cell death, but some changes in the wing epidermal cells were seen even after ligation (data not shown). Just after eclosion, GFP was expressed uniformly in the nucleus of wing epidermal cells, but the GFP expression was distributed at the circumference of the nucleus at 2 hours after ligation, which might represent a region of chromatin condensation. Sometimes, distorted features of the nucleus were seen, although breakdown of the nuclear membrane did not occur. The features were not changed 24 hours after ligation.

We isolated wings from wild-type flies at various times after eclosion and examined cell death 2 hours after the isolation. Cell death was blocked in wings isolated at eclosion, whereas cell death was unaffected by isolation 20 minutes after eclosion in most cases (Fig. 2D,E). Even in folded wings, cell death proceeded normally, indicating that the spreading of the wing itself is not necessary to induce cell death. As in the case of neck ligation, the increment in time of wing isolation after eclosion correlated with an increase in the percentage of flies showing wing epidermal cell death (Fig. 2F).

Experiments on hemolymph injection confirmed that a hormonal factor is a direct signal to induce the cell death. We

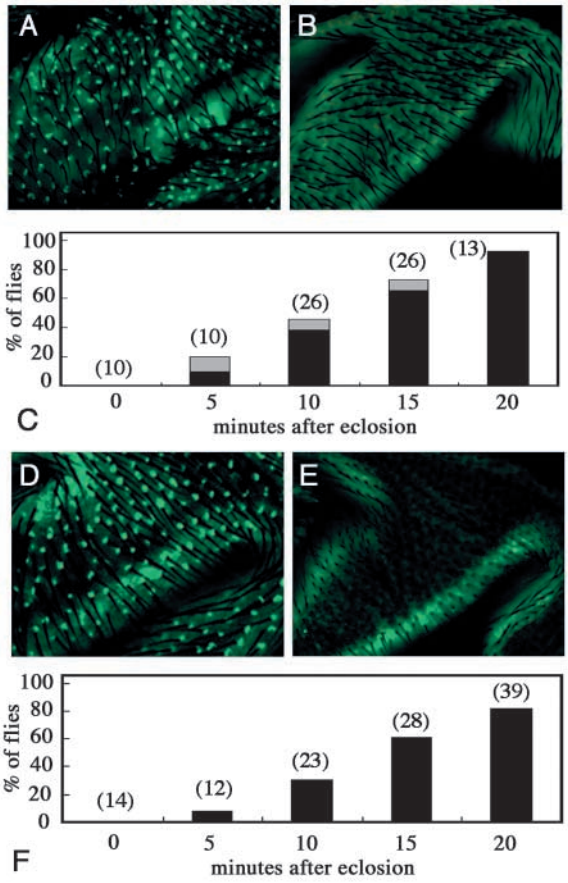

Fig. 2. Effects of neck ligation or wing isolation on the death of wing epidermal cells in *en-Gal4 UAS-GFPN* flies. Induction of cell death was examined at 2 hours after neck ligation (A-C) or wing isolation (D-F). Neck ligation or wing isolation in flies just after eclosion suppressed cell death (A,D, respectively), but treatment at 20 minutes after eclosion did not prevent the death (B,E). The percentage of flies showing wing epidermal cell death increased with time of treatment (C,F). Black and gray bars indicate the percentage of flies showing cell death extensively or in restricted domains of the wing, respectively (see Materials and methods). The number of flies examined is shown in parentheses.

injected hemolymph from wild-type flies at various times after eclosion into flies neck-ligated at eclosion and examined the induction of cell death at 2 hours after injection. Injection of hemolymph from flies at eclosion did not induce the cell death (Fig. 3A). However, injection of hemolymph from flies at 30 minutes and 60 minutes after eclosion induced the cell death (Fig. 3B,C). Hemolymph collected from flies at 120 minutes after eclosion was less able to induce the cell death. Control flies injected with PBS after neck ligature showed no induction of cell death (Fig. 4B). These results indicate that the death inducing hormonal factor is secreted just after eclosion but disappears at 2 hours and later after eclosion.

To assay the time-dependent responsiveness of wing epidermal cells to the death-inducing hormonal factor, hemolymph from wild-type flies at 30 minutes after eclosion was injected into pharate adult flies neck-ligated at various stages. Wings from these flies were then assayed for incidence of cell death. Flies injected at the S or S/G stage did not initiate

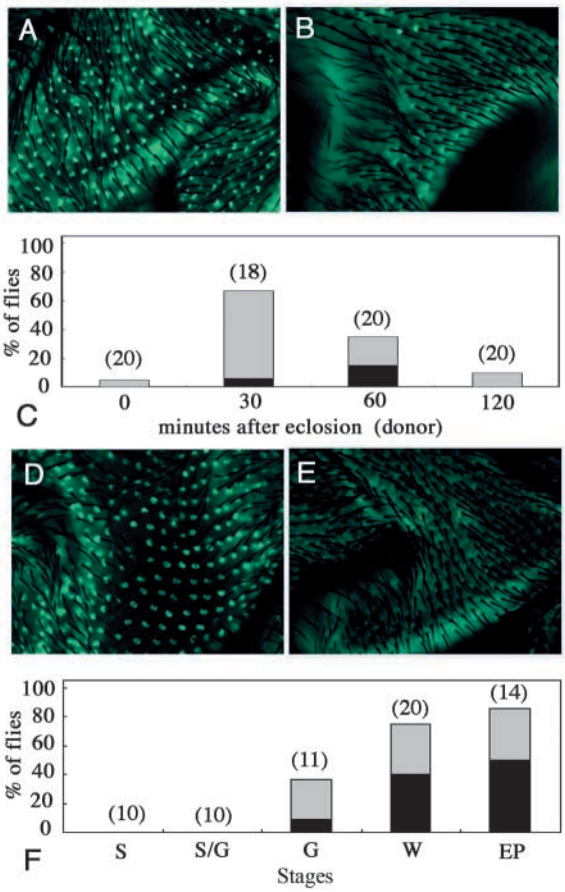

Fig. 3. Effects of hemolymph injection on the death of wing epidermal cells in *en-Gal4 UAS-GFPN* flies. Hemolymph collected from wild-type flies at various times after eclosion was injected into flies neck-ligated at eclosion and wings were examined for induction of the cell death 2 hours later (A-C). Cell death was not induced by injection of hemolymph from flies at eclosion (A), but was induced by it at 30 minutes after eclosion (B). Hemolymph from flies at 30 minutes after eclosion was injected into pharate adults neck-ligated at various stages and induction of the cell death was examined 2 hours after injection (D-F). Injection of hemolymph into the flies at S stage did not induce cell death (D), but it did at W stage (E). (C,F) Black and gray bars indicate the percentage of flies showing cell death extensively or in restricted domains of the wing, respectively. The number of flies examined is shown in parentheses.

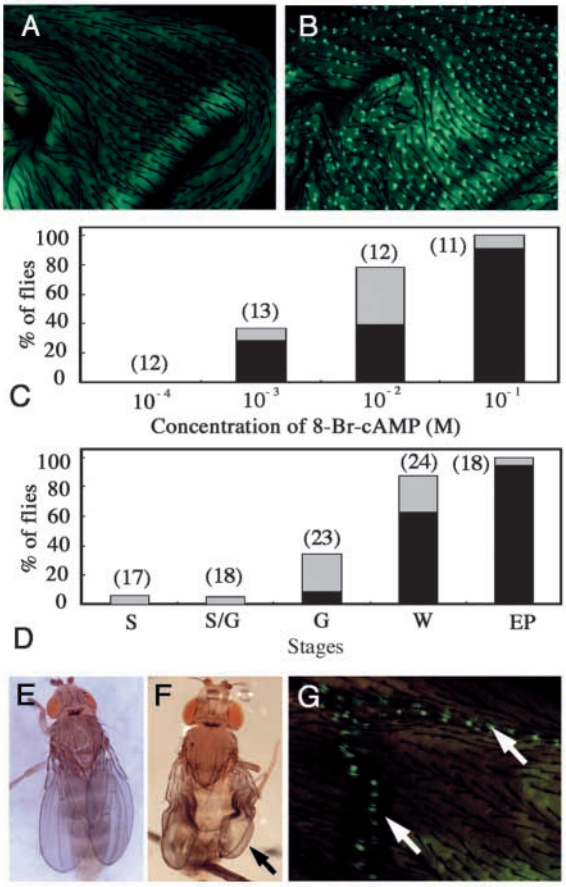

Fig. 4. Effects of 8-Br-cAMP injection on the death of wing epidermal cells in *en-Gal4 UAS-GFPN* flies neck-ligated at eclosion. Cell death was induced by injection of 8-Br-cAMP at a concentration of 10^{-1} mol/l (A) but not by injection of PBS (B). Injection of 8-Br-cAMP produced a dose-dependent effect on the induction of cell death (C). 8-Br-cAMP (10^{-1} mol/l) was injected into pharate adult flies neck-ligated at various stages and wings were examined for cell death 2 hours after injection (D). (C,D) Black and gray bars indicate the percentage of flies showing cell death extensively or in restricted domains of the wing, respectively. The number of flies examined is shown in parentheses. 8-Br-cAMP (10^{-1} mol/l) was injected into intact flies at EP stage (just before eclosion). A control fly injected with PBS spread its wings normally (E). A fly injected with 8-Br-cAMP had blistered wings (F, arrow), in which the epidermal cells had died prior to spreading (G). Arrows (G) indicate the persistence of GFP in vein cells.

cell death in the wings (Fig. 3D). However at G and later stages (W and EP), cell death was induced (Fig. 3E,F). Thus, the responsiveness to a triggering hormone appears at the G stage, about 3 hours before eclosion.

cAMP causes the death of wing epidermal cells

To examine the effect of cAMP on the induction of cell death in *Drosophila*, a membrane-permeant 8-bromo analog, 8-Br-cAMP, was injected at various concentrations into flies neck-ligated at eclosion and cell death induction was examined 2 hours after injection. Injection of 8-Br-cAMP produced a dose-dependent induction of cell death (Fig. 4A,C). As a control, injection of PBS showed no induction of cell death (Fig. 4B). cAMP did not inhibit GFP expression by affecting the *en* promoter, because GFP-marked wing veins were present in folded wings that had undergone the death (data no shown). In subsequent experiments, we used 8-Br-cAMP at a concentration of 10^{-1} mol/l. We followed the time course of cell death after the injection of 8-Br-cAMP. In all cases (32/32), cell death proceeded within 1 hour of injection.

The timing of responsiveness of cells to cAMP was investigated. 8-Br-cAMP was injected into pharate adult flies neck-ligated at various stages and induction of the cell death was examined at 2 hours after injection (Fig. 4D). As in the case of the death inducing hormone, when cAMP was injected into flies at S or S/G stage, cell death was not induced. However, when cAMP was injected at G or later (W and EP) stages, the induction of the cell death was observed.

We examined the effect of injection of PBS or 8-Br-cAMP prior to eclosion in intact flies. After injection into pharate adults at EP stage (about 0-40 minutes before eclosion), the flies were released from the pupal cases. They then walked around and spread their wings. Flies injected with PBS showed normal wing-spreading behavior, and the wing epidermal cells were still alive at wing spreading in all cases (8/8) (Fig. 4E). Flies injected with 8-Br-cAMP showed blistered wings in all case (7/7) (Fig. 4F), due to the precocious death of the epidermal cells at wing spreading (Fig. 4G).

Activated Gsα* mimics the effects of cAMP

These results demonstrate that death in wing epidermal cells can be induced by elevation of cAMP. To test this in intact flies, we manipulated adenylyl cyclase activity by overexpressing a constitutively active Gsα subunit (Gsα*), generated by substitution of leucine 215 by glutamine 215 (Quan et al., 1991; Wolfgang et al., 1996). Expression of Gsα* results in receptor-independent activation of the α subunit, and thus activates an endogenous adenylyl cyclase (Quan et al., 1991; Chyb et al., 1999). Targeted expression of wild-type Gsα in wing epidermal cells using *en-Gal4* driver had no effect on cell death (Fig. 5A). By contrast, targeted expression of Gsα* caused wing blisters (Fig. 5B). Observation of the GFP expression pattern in cells at wing spreading demonstrated that precocious cell death occurred in the blistered wings (Fig. 5C).

As the *en-Gal4* line expresses GAL4 in the posterior compartment throughout development, the ectopic expression of Gsα* should be continuous in the posterior compartment of the wing. The cuticular pattern of adult wings was normal, indicating that the wings were formed normally during adult development. Hence, the precocious cell death must occur late in development, at least after cuticular deposition. We examined when the cell death was first induced by expression of Gsα* (Fig. 5D). At the S and S/G stages of pharate adults, no sign of cell death was seen. At G and later stages, precocious cell death had been induced.

We examined the effect of elimination of Gsα activity by generating clones of *dgs* mutant cells within the developing wings. A dgs^{R60C} mutation is likely to be a null allele that is generated by the change of nucleotide 723 from a T to an A, resulting in the change of residue 241 in the protein from a Tyr to a stop codon (Wolfgang et al., 2001). In this mosaic analysis, we marked wing epidermal cells using Histone-GFP. The cells of the clones remained at 2 hours after wing spreading, although the cells around clones had already disappeared as a result of cell death (Fig. 5E,F). Thus, elimination of Gsα activity prevents the death of wing epidermal cells.

cAMP effects are mediated by protein kinase A

The cellular effects of cAMP are usually mediated by PKA (for reviews, see Gottesman, 1980; Francis and Corbin, 1994). To determine whether this is also the case for wing epidermal cell death, we examined the effect of reduction or elimination of PKA activity on cell death.

We initially used a dominant-negative form of the regulatory subunit of PKA (R*), whose ectopic expression is known to reduce the activity of endogenous PKA (Li et al., 1995). When R* was ectopically expressed using the *en-Gal4* driver, many cells of the wings remained at 2 hours, or even at 8 hours, after wing spreading (Fig. 6A,B), resulting in separation between the ventral and dorsal cuticular sheets in posterior compartment. Targeted expression of R* caused wavy or curly wings (data not shown), probably due to the distortion between normal adhesion of dorsoventral cuticles in the anterior compartment and detachment of the cuticle in the posterior compartment.

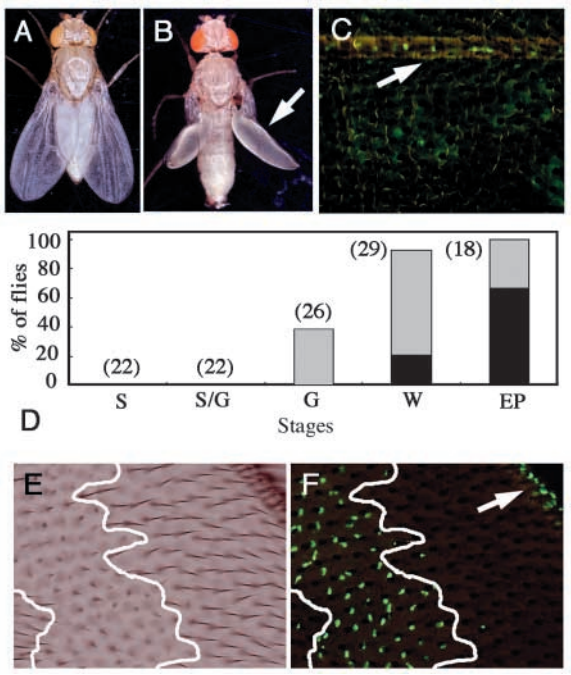

Fig. 5. Effects of ectopic expression of the wild-type Gsα subunit in *en-Gal4 UAS-GFPN / +;UAS- Gsα/+* flies (A), or a constitutively active form of Gsα (Gsα*) in *en-Gal4 UAS-GFPN / +;UAS- Gsα*/+* flies (B,C). Ectopic expression of wild-type Gsα produced no visible phenotype (A), whereas Gsα* caused wing blisters (B, arrow). Precocious cell death had occurred in blistered wings at the time of wing spreading (C). The arrow (C) indicates GFP remaining in the vein cells. The onset of precocious cell death induced by ectopic expression of Gsα* was examined at various stages in pharate adults (D). Black and gray bars indicate the percentage of flies showing cell death extensively or in restricted domains of the wing, respectively. The number of flies examined is shown in parentheses. Effects of elimination of Gsα activity on the cell death of wing epidermal cells (E,F). Mutant clones of *dgs* were marked by the sha^{-} phenotype of missing or smaller hairs (E, enclosed by white lines). The cells of the clones remained at 2 hours after wing spreading, although neighboring cells had died (F). The arrow (F) indicates the persistence of GFP in anterior wing margin cells.

Next we eliminated DC0-dependent PKA activity by generating clones of *DC0* mutant cells within the developing wings. The *DC0* gene in *Drosophila* encodes a catalytic subunit, one of the components of PKA (Kalderon and Rubin, 1988). As clones of *DC0* mutant cells in the anterior compartment produce anterior duplication of the normal wing pattern (Jiang and Struhl, 1995; Li et al., 1995), we examined the clones in the posterior compartment to investigate whether the death of wing epidermal cells marked with Histone-GFP is suppressed or not. The cells of the clones remained at 2 hours after wing spreading, although the surrounding cells had already been eliminated by cell death (Fig. 6C,D). Thus, reduction or elimination of PKA activity prevents the death of wing epidermal cells.

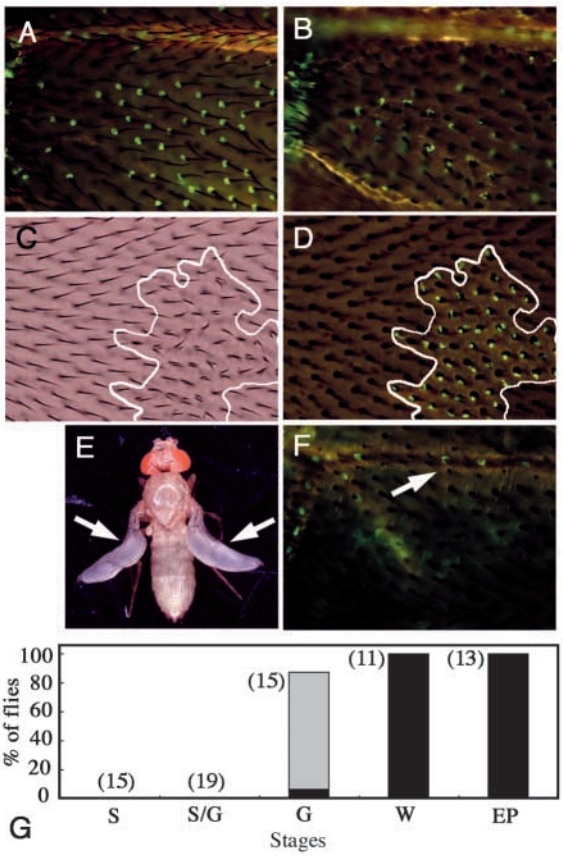

Fig. 6. Effects of reduction or elimination of PKA activity on the death of wing epidermal cells. Ectopic expression of a dominant-negative form of the regulatory subunit of PKA (R*) inhibited cell death at 2 hours (A) or even at 8 hours (B) after wing spreading in *en-Gal4 UAS-GFPN /+;UAS-R*/+* flies. Mutant clones of *DC0* were marked by the *stc*$^{-}$ phenotype of smaller hairs or of tufts of hairs (C, enclosed by white lines). Cells of the clones remained 2 hours after wing spreading, although neighboring cells had died (D). Effects of ectopic expression of a constitutively active form of the PKA catalytic subunit (mC*) in *en-Gal4 UAS-GFPN /UAS-mC** flies (E-G). Ectopic expression of mC* caused wing blisters (E, arrows). Precocious cell death had occurred in blistered wings by the time of wing spreading (F). The arrow (F) indicates GFP in vein cells. Precocious cell death induced by ectopic expression of mC* was examined at various stages of pharate adult (G). Black and gray bars indicate the percentage of flies showing cell death extensively or in restricted domains of the wing, respectively. The number of flies examined is shown in parentheses.

We examined the effects of constitutive activation of PKA on cell death. We used a mutationally altered mouse catalytic subunit (mC*) that is resistant to inhibition by the regulatory subunit (Orellana and McKnight, 1992). The mutant catalytic subunit is constitutively active, irrespective of cAMP concentration, and can function in *Drosophila* cells (Jiang and Struhl, 1995; Li et al., 1995). Using the *en-Gal4* driver, we expressed the constitutively active catalytic subunit of mC* in wing epidermal cells. All eclosing flies had blistered wings (Fig. 6E). The wing epidermal cells died prior to wing spreading (Fig. 6F). Thus, constitutive activation of PKA causes the precocious death of wing epidermal cells.

We examined the induction of cell death at various stages of pharate adults. As seen in the cases of cAMP injection and of ectopic expression of Gsα*, precocious cell death was induced at G stage and later (Fig. 6G). This indicates that wing cells acquire competence to respond to PKA activity by G stage, about 3 hours before eclosion.

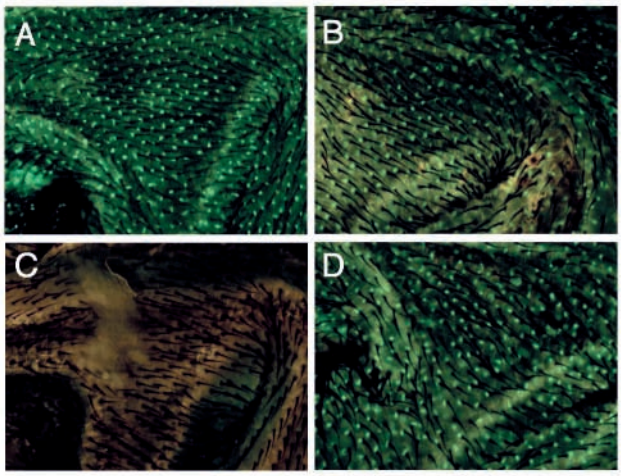

Fig. 7. Effects of a *rickets* mutation on the death of wing epidermal cells in *rk*^{1} *cn*^{1} *bw*^{1}*; His-GFP/+* flies. In *rk*^{1} mutants, cell death was inhibited at 2 hours (A) or even at 8 hours (B) after eclosion. In *rk*^{1} mutants neck-ligated at eclosion, cell death was induced by injection of 8-Br-cAMP (10^{-1} mol/l) (C), but not by injection of hemolymph from wild-type flies at 30 minutes after eclosion (D).

A mutation in the G-protein coupled receptor gene *rickets* inhibits wing epidermal cell death

In *Drosophila*, the *rickets* gene is a member of the glycoprotein hormone receptor family of the G-protein-coupled receptors (Ashburner et al., 1999; Eriksen et al., 2000) and has been suggested to encode a bursicon receptor (Baker and Truman, 2002). We examined wing epidermal cell death, marked by Histone-GFP, in *rk* mutants. In the mutants, wing epidermal cells remained at 2 hours, or even at 8 hours, after eclosion (Fig. 7A,B).

To determine whether the inhibition of cell death is caused by a failure in the reception of a hormonal signal inducing death, we examined the effects of 8-Br-cAMP and hemolymph injection into *rk* mutants neck-ligated at eclosion. In wild-type flies, injection of hemolymph from wild-type flies at 30 minutes after eclosion and injection of 8-Br-cAMP induced cell death (Fig. 3B, Fig. 4A, respectively). However, in *rk* mutants, cell death was induced by injection of 8-Br-cAMP but not by injection of hemolymph (Fig. 7C,D). This indicated that the mutant cells could not receive the hormonal signal in the hemolymph, although the activation of cell death by cAMP/PKA signaling was normal in the mutant cells.

Discussion

Role of programmed cell death during wing expansion

We have used the GAL4/UAS system and nucleus-localized GFP, to follow the fate of wing epidermal cells after eclosion. Within 1 hour after wing expansion, epidermal cells initiate

breakdown of the nuclear membrane, followed by fragmentation of the DNA and nucleus. TEM indicates that this cell death exhibits features indicative of autophagy with extensive cytoplasmic vacuoles and chromatin condensation. Although DNA fragmentation and chromatin condensation are known as typical features of apoptosis, autophagic cell death can also be accompanied by them in some cases (Clarke, 1990; Jiang et al., 1997; Lee and Baehrecke, 2001; Bursch, 2001). The dying cells are detached from the wing cuticle and are absorbed into the thoracic cavity through wing veins. This process is followed by tanning and hardening of the wing cuticle and by the adhesion of two layers of dorsal and ventral cuticle.

Programmed cell death in wing epidermal cells plays an important role in the maturation of wings. We showed that induction of precocious cell death induced by injection of 8-Br-cAMP, or by ectopic expression of a constitutively activated form of Gsα or PKA, caused a blistered wing phenotype. During wing development, wing epidermal cells connect the two surfaces of the wing together by a highly specialized system of cytoskeletal supports, the trans-alar array, which is a mechanically continuous structure consisting of microtubules and microfilaments (Tucker et al., 1986). The trans-alar array is anchored by integrin-mediated basal adhesion (Fristrom et al., 1993). Disruption of these connections in integrin mutants also results in blistered wings (Brower and Jaffe, 1989; Brabant and Brower, 1993; Brower et al., 1995; Brabant et al., 1996). Thus, the wing epidermal cells are necessary to connect the two layers of the wing until wing expansion.

Inhibition or delay of the cell death also disturbs the subsequent maturation of the wing. In the flies ectopically expressing p35 or a dominant-negative form of PKA, inhibition of the cell death prevented adhesion of the dorsal and ventral cuticle, and sometimes caused blistered wings. Thus, the precise regulation of cell death is essential for the formation of functional wings.

A peptide hormone trigger for wing epidermal cell death

The peptide hormone, bursicon, is known to play a role in the post-ecdysial phase of development (Cottrell, 1962; Fraenkel and Hisao, 1962). Bursicon has been shown to be released before wing expansion and to hasten the tanning reaction, serving to harden the newly expanded cuticle. Our results suggest that the hormone that induces cell death of the wing epidermis could be bursicon.

First, neck ligation and hemolymph injection experiments demonstrated that the triggering signal to induce death is a humoral factor released after eclosion. This temporal pattern of death-inducing activity in the hemolymph corresponds to that of bursicon. Second, injection of cAMP induced cell death, implicating cAMP as the second messenger in the cell death pathway. Studies in blowflies have shown that bursicon also acts through cAMP (Seligman and Doy, 1972; Seligman and Doy, 1973). Recently, in *Drosophila*, cAMP was shown to induce cuticular melanization in a fashion similar to bursicon (Baker and Truman, 2002). Third, reception of the hormonal signal inducing cell death is mediated by a probable bursicon receptor, RICKETS (DLGR2), which also act through cAMP (Eriksen et al., 2000; Hewes and Taghart, 2002; Baker and Truman, 2002). Finally, in *Lucilia cuprina*, it was proposed that bursicon is the same as fragment disaggregating hormone (FDH), which increases the circulating filamentous cellular fragments derived from post-ecdysial death of the wing epidermal cells (Seligman and Doy, 1973; Seligman et al., 1975). Taken together, it is likely that bursicon coordinates events such as the cell death of wing epidermis and the subsequent tanning and hardening of the cuticle. However, we cannot rule out another possibility, namely that several humoral factors could signal through the pathway. Identification of a bursicon gene in *Drosophila* (Riehle et al., 2002) will facilitate genetic approaches to understand the role of bursicon in wing epidermal cell death.

cAMP/PKA signaling is required for the wing epidermal cell death

The fact that cell death can be induced by the injection of cAMP implicates cAMP in the humoral signal transduction pathway. The binding of ligand to G-protein-coupled receptors is known to stimulate Gsα, resulting in adenylyl cyclase activation and the production of cAMP (for reviews, see Tang and Gilman, 1992; Neer, 1995). Our gain-of-function analyses, using constitutively activated Gsα, and loss-of-function analyses, using a *dgs* mutation, indicate that the activity of Gsα is sufficient and necessary for the death of wing epidermal cells. Consistent with this, endogenous Gsα is expressed in the basal and trans-alar membranes of the wing epithelium of pharate adults (Wolfgang et al., 1996).

Cellular effects of cAMP are usually mediated by PKA (for reviews, see Gottesman, 1980; Francis and Corbin, 1994), and this holds true in the case of cell death in the wing epidermis. Ectopic expression of a constitutively active form of PKA in wing epidermal cells induced cell death. Contrastingly, ectopic expression of a dominant-negative form of PKA or a mutation of the *Pka-C1* gene prevented the cell death. Thus, the death of wing epidermal cells is stimulated by and requires PKA activity.

Activated PKA phosphorylates substrates that control diverse cellular phenomena. The signaling mechanisms used by cAMP/PKA to control programmed cell death are likely to be complex and cell-type specific. For example, cAMP-mediated activation of PKA stimulates apoptosis in thymocytes (McConkey et al., 1990) and leukemic cell lines (Lanotte et al., 1991). By contrast, it protects neutrophils (Parvathenani et al., 1998), thyroid follicular cells (Saavedra et al., 2002) and spinal ganglion neurons (Bok et al., 2003) from apoptosis. With respect to the suppression of cell death, one molecular mechanism has been elucidated: PKA can phosphorylate the pro-apoptotic regulator Bad and inhibit its function (Harada et al., 1999; Bok et al., 2003). However, little is known about the molecular mechanisms through which cAMP/PKA promotes cell death.

How could PKA regulate the death of wing epidermal cells? In *Drosophila*, pro-apoptotic genes, such as *rpr*, *hid* and *grim*, induce cell death (Chen et al., 1996; Grether et al., 1995; White, at al., 1994). In the case of *rpr* and *grim*, death induction is controlled at the level of transcription – the pattern of *rpr*/*grim* expression mimics the pattern of apoptosis. Intriguingly, the gene *hid* is more broadly expressed in both cells fated to die and those fated to live (Grether et al., 1995). Furthermore, HID-induced apoptosis in midline glia cells or in eyes is suppressed through posttranslational regulation of

HID by the RAS-MAP Kinase pathway (Bergmann et al., 1998; Bergmann et al., 2002). The death of wing epidermal cells proceeds promptly, within 1 hour after the hormonal signal triggers it. This prompt induction of the cell death should be controlled by posttranslational rather than transcriptional regulation. Our preliminary studies show that the *hid* gene is involved in the cell death of wing epidermal cells (K.-i.K., unpublished). The HID protein sequence (Grether et al., 1995) contains three consensus PKA phosphorylation sites, suggesting that HID could be a target of PKA.

Possible mechanism to acquire the competence

Flies acquire competence to respond to the death-triggering hormone before eclosion. At about 3 hours before eclosion (G stage) (Kimura and Truman, 1990), wing epidermal cell death can be induced either by hemolymph injection, cAMP injection, ectopic expression of Gsα* or ectopic expression of mC*. Thus, the PKA-dependent cell death machinery is assembled at G stage, which coincides with the time of eclosion hormone (EH) release (Baker et al., 1999). In our preliminary experiments, EH-cell knockout flies did not show cell death of the wing epidermis after eclosion (K.-i.K. and Y.H., unpublished). It is possible that EH triggers expression of the components of the cell death pathway. It is also possible that EH release simply induces the post-eclosion release of bursicon, as in *Lepidoptera* (Truman, 1973), and that other developmental mechanisms regulate assembly of the cell death machinery in the wings.

In conclusion, these studies illustrate that regulation of programmed cell death plays an essential role in the maturation of the wings in *Drosophila*. The post-ecdysial cell death is regulated temporally and spatially by the hormone-receptor RICKETS through the cAMP/PKA signaling pathway. In *Drosophila*, the molecular components involved in cell death are well studied. Further analysis of the targets of PKA will link the signaling pathway with the components that directly regulate cell death.

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