The timing of *Drosophila* salivary gland apoptosis displays an *l(2)gl*-dose response

R Farkaš[1,2] and BM Mechler*,[1]

[1] Department of Developmental Genetics, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

[2] Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 83306 Bratislava, Slovakia

* Corresponding author: BM Mechler, Department of Developmental Genetics, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany. Tel: (49) 6221 424502; Fax: (49) 6221 424552; E-mail: dev.genetics@dkfz-heidelberg.de

Abstract

During *Drosophila* metamorphosis, larval tissues, such as the salivary glands, are histolysed whereas imaginal tissues differentiate into adult structures forming at eclosion a fly-shaped adult. Inactivation of the *lethal(2)giant larvae* (*l(2)gl*) gene encoding the cytoskeletal associated p127 protein, causes malignant transformation of brain neuroblasts and imaginal disc cells with developmental arrest at the larval-pupal transition phase. At this stage, p127 is expressed in wild-type salivary glands which become fully histolysed 12–13 h after pupariation. By contrast to wild-type, administration of 20-hydroxyecdsone to *l(2)gl*-deficient salivary glands is unable to induce histolysis, although it releases stored glue granules and gives rise to a nearly normal pupariation chromosome puffing, indicating that p127 is required for salivary gland apoptosis. To unravel the *l(2)gl* function in this tissue we used transgenic lines expressing reduced (~0.1) or increased levels of p127 (3.0). Here we show that the timing of salivary gland histolysis displays an *l(2)gl*-dose response. Reduced p127 expression delays histolysis whereas over-expression accelerates this process without affecting the duration of third larval instar, prepupal and pupal development. Similar *l(2)gl*-dependence is noticed in the timing of expression of the cell death genes *reaper, head involution defective* and *grim,* supporting the idea that p127 plays a critical role in the implementation of ecdysone-triggered apoptosis. These experiments show also that the timing of salivary gland apoptosis can be manipulated without affecting normal development and provide ways to investigate the nature of the components specifically involved in the apoptotic pathway of the salivary glands. *Cell Death and Differentiation* (2000) **7,** 89–101.

Keywords: salivary glands; *Drosophila*; apoptosis; *lethal(2)giant larvae* gene; ecdysone

Abbreviations: caspase, cysteine aspartic acid-specific protease; *GMR* system, glass multimer reporter system; *hid, head involution defective*; *l(2)gl, lethal(2)giant larvae*; *rpr, reaper*; RT–PCR, reverse transcriptase primed polymerase chain reaction; EcR, ecdysone receptor; USP, ultraspiracle protein; *br, broad*; *rbp, reduced bristles on palpus*; AP, after pupariation

Introduction

Inactivation of the *l(2)gl* gene which encodes the p127 protein,[1,2] a component of the cytoskeletal matrix underlying the plasma membrane,[3] leads to malignant transformation of brain neuroblasts and imaginal disc cells.[4,5] The p127 protein forms large homomeric complexes[6,7] and is associated with a series of proteins, among which were identified the heavy chain of nonmuscle myosin-II,[6] designated thereafter as myosin-II, and a yet unknown serine-kinase.[8] Activation of this kinase leads to specific phosphorylation of p127 and results in the dissociation of myosin-II from p127 without affecting its homomerisation.[8] The interaction between p127 and myosin-II is not only limited to *Drosophila* but also occurs in human,[9] further supporting the notion that mechanisms similar to those uncovered in *Drosophila* may also govern the intracellular organisation of human cells.

Drosophila larval salivary gland histolysis is dependent upon ecdysone[10] and cell death of the larval cells is nearly synchroneous proceeding in a distal-to-proximal gradient.[11,12] Based on the sequence of polytene chromosome puffings occurring during late larval and prepupal development,[13,14] a two-step regulatory hierarchy of early and late gene expression was proposed to account for the events that precede metamorphosis and the death of the salivary glands.[15,15] The expression of early genes, which is directly induced by the ecdysone-receptor complex, gives rise to the synthesis of a variety of proteins, including transcription factors, which trigger late gene expression. In turn the products of these genes are thought to induce expression of death genes, such as *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, which act as mediators between different signalling pathways and apoptosis implemented by caspases.[17–19] Along this line, the recent findings that *rpr* and *hid* are expressed in salivary glands prior to their histolysis[20] and that *rpr* expression can induce apoptosis indicate that death genes indeed contribute to salivary gland disintegration.

Similar to salivary gland histolysis, degeneration of different organs and tissues of higher vertebrates can be also elicited by steroid hormones.[21,22] In particular, thymic involution in rodents can be caused by glucocorticoid treatment and apoptosis of thymic lymphocytes is in part mediated by the glucocorticoid receptor.[23–27] Moreover, regression of many tissues, such as the prostate, uterus, oviduct and mammary glands, is negatively regulated by

sex steroids.[28–31] When the steroid hormones are withdrawn or when antagonists are used, these endocrine-dependent tissues undertake regression involving apoptosis. Moreover, a variety of human tumours arising from these tissues have been treated by using specific steroid hormones.[32–36]

Due to its particular location at the left end of the second chromosome, most tested *l(2)gl* alleles consist essentially in terminal deficiencies uncovering partially or completely *l(2)gl*,[1] and all so far examined homozygous loss-of-function mutations in *l(2)gl* cause tumorous overgrowth of the imaginal discs and excessive proliferation of the neuroblasts and ganglion mother cells in the larval brain.[1,37] In addition to neoplastic transformation, *l(2)gl* mutations affect other tissues (for review, see Hadorn,[38]), causing complete aplasia of the germ cells in the larval testes,[39,40] albeit not in the ovaries, and underdevelopment of the salivary glands,[41,42] the ring gland[43] and more particularly the prothoracic cells of the ring gland[44,45] which is the source of the molting hormone ecdysone. As a consequence, the larval life is prolonged from the normal 4 days to several weeks and the resulting bloated larvae then die without entering metamorphosis. The atrophy of the prothoracic cells appears to result from the invasive growth of malignant neuroblasts within the brain hemispheres that disrupt essential neuronal connections and functions required for the maturation of the ring gland.[46] This is supported by the observations that transplantation of a normal ring gland[47] or injection of ecdysone[48] into *l(2)gl*-deficient larvae can induce pupariation but cannot fully rescue the development of the mutant animals. Administration of ecdysone to *l(2)gl* late third instar larvae triggers formation of pseudopupae and induces a nearly normal pupariation puffing of the polytene chromosomes indicating that the ecdysone receptor is functional.[49] Moreover, salivary gland underdevelopment occurs before the outgrowth of the tumours and, thus, can be directly attributed to the absence of *l(2)gl* gene activity in these tissues, further supporting the idea that ecdysone deficiency alone is not sufficient to cause neoplastic growth.[50,51] Although p127 is expressed at a relatively high level in wild-type salivary glands,[3] no major dramatic abnormality could be observed in the salivary glands with the exception of a smaller than normal size[41] and a reduced degree of chromosomal polytenization,[42] suggesting that p127 functions differently in this tissue than in brain and imaginal discs. Investigations on the role played by p127 in the salivary glands may thus be highly informative to understand the pleiotropy of its cellular functions and will help to unravel new and unsuspected activities of *l(2)gl* during *Drosophila* development.

To determine the role played by p127 in salivary glands we used null *l(2)gl* alleles and transgenic lines expressing either reduced or increased levels of p127, and found that the timing of salivary gland histolysis is directly dependent upon the level of *l(2)gl* gene expression. In particular, histolysis of the salivary glands is considerably accelerated in presence of additional *l(2)gl* gene copies. To investigate the hierarchical step mediated by the p127 protein in the apoptotic pathway, we analyzed the expression of the death genes *rpr*, *hid* and *grim*, and investigated the pattern of salivary gland puffing. We found that the expression of the three death genes is prematurely induced in the presence of a high number of *l(2)gl* gene copies, but were unable to detect puffing in the chromosomal bands which normally give rise to early and late prepupal puffs. These results indicate that p127 acts at an early stage of the apoptotic pathway triggered by the steroid hormone ecdysone and can implement this mechanism without requiring the normal sequential induction and regression of puffs which normally occur in salivary glands during prepupal development.

Results

l(2)gl is required for salivary gland apoptosis

To examine the requirement for *l(2)gl* during salivary gland histolysis, we first investigated the ecdysone response following injection of 50 ng 20-hydroxyecdysone in Ringer's saline solution into 8-days old *l(2)gl*-deficient larvae. In response to ecdysone, we observed that, over a survival period of at least 3 days, all injected *l(2)gl* larvae formed pseudopupae, albeit without salivary gland histolysis (data not shown). We repeated this analysis on salivary glands isolated from *l(2)gl*-deficient larvae and maintained in Schneider culture medium for 24 h. By comparison to ready-to-pupariate wild-type salivary glands, 8 days-old *l(2)gl* salivary glands are considerably reduced in size and exhibit a shape in 'bunch of grapes'. The cells of these glands display a nearly spherical form with a reduced surface of cell-to-cell contact. Administration of 10^{-5} M 20-hydroxyecdysone failed to induce the histolysis of *l(2)gl* salivary glands whereas similar treatment produced marked alterations in the organisation of wild-type salivary glands as shown previously by Farkas and Sutáková[52] (data not shown). No sign of vacuolisation or abnormal pattern of organelles distribution could be detected in *l(2)gl*-deficient salivary gland cells treated with ecdysone (Figure 1c) and the overall organisation of the glands and the structure of the cells are similar to those of untreated *l(2)gl* glands (Figure 1b). Small amounts of glue proteins could be noticed in the lumen of the treated glands (data not shown) indicating that ecdysone can stimulate the release of the stored glue granules synthesised in *l(2)gl*-deficient glands.[49,53] Similarly, ecdysone dependent puffs characteristic of pupariation were observed (data not shown). These findings argue that p127 is required in the mechanisms of ecdysone-triggered apoptosis of salivary glands but not for pupariation chromosomal puffing, which can be nearly normally induced in salivary glands of *l(2)gl*-deficient larvae[49] (data not shown).

l(2)gl dose-dependent timing of salivary gland histolysis

A logical hypothesis drawn from the p127 requirement would be that salivary gland histolysis is dependent upon the level of *l(2)gl* gene expression. To substantiate this hypothesis we determined the duration of larval, prepupal and pupal development and the time needed for salivary gland
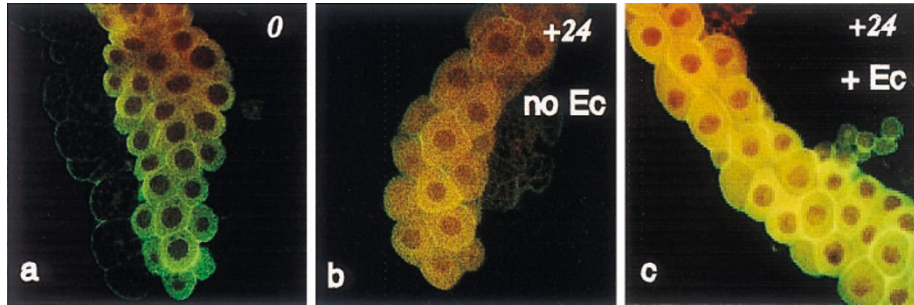
Figure 1 Treatment of *l(2)gl* salivary glands with 10^{-5} M 20-hydroxyecdysone fails to induce histolysis. Confocal micrographs of salivary glands from 8 days-old *l(2)gl* third instar larvae which were (**a**) freshly dissected, maintained for 24 h in Schneider culture medium (**b**) without or (**c**) with 20-hydroxyecdysone. The glands were stained with anti-myosin II monoclonal antibodies and anti-β-tubulin rabbit antibodies and secondary Cy5-conjugated anti-mouse of Cy3-conjugated anti-rabbit antibodies, respectively. Shown are projections of optical sections displaying the distribution of myosin-II (red) and β-tubulin (green). Superimposed signals for both proteins are shown in yellow

histolysis in wild-type and transgenic lines expressing either reduced or enhanced levels of p127. For a low expression of p127 we used the transgene *l(2)gl^{+t24}*, encoding a C-terminally truncation of p127 characterised by the loss of 141 amino acids and their replacement by 55 residues provided by the read-through into the flanking transposon sequence and producing a $\sim$120 kDa protein.[2,54] As a result, the truncated p127^{t24} protein is much less stable and consequently much less abundant than the normal protein (Figure 2), albeit fully functional as judged by the normal development of the *l(2)gl^{+t24}* animals.[2] For a high expression of p127 we used a transgenic line containing, in addition to the two wild-type *l(2)gl* genes, four *l(2)gl^{+ps}* transgenes,[55] or *P-4x[l(2)gl^{+ps}]*. The *l(2)gl^{+ps}* transgene encodes a fully functional *D pseudoobscura* p127 protein, or p127^{ps} exhibiting a slightly larger apparent size than the p127 protein from *D melanogaster* (Figure 2).

As shown in Figure 3a, the timing of salivary gland histolysis displayed an *l(2)gl*-dose response with acceleration of this process in *P-4x[l(2)gl^{+ps}]* prepupae and, conversely, with retardation in *l(2)gl^{+t24}* animals. Similarly, the process of cellular vacuolisation, which precedes histolysis and can be visualised by acridine orange staining on fixed salivary glands, exhibited identical *l(2)gl*-dependence (Figure 3b). However, the duration of larval (Figure 3c), prepupal (Figure 3d) and pupal development (Figure 3e) was identical in the three *l(2)gl* variants, as was the time required for ecdysone-induced release of glue granules in the lumen of the salivary glands (Figure 3f). We found also that head eversion occurring in wild-type prepupae at 12.5–13 h after pupariation (AP) and coinciding with salivary gland disintegration, takes place at the same time in both transgenic *P-4x[l(2)gl^{+ps}]* and *l(2)gl^{+t24}* pupae. These findings indicate that, within our experimental limits, variations in the level of *l(2)gl*-gene expression exert no visible outcome on the overall development of the transgenic animals but affect specifically the process of salivary gland apoptosis. Interestingly, the ability of the salivary glands to synthesize and release the glue granules, which is under the control of ecdysone, displays no *l(2)gl* gene dosage indicating that, in
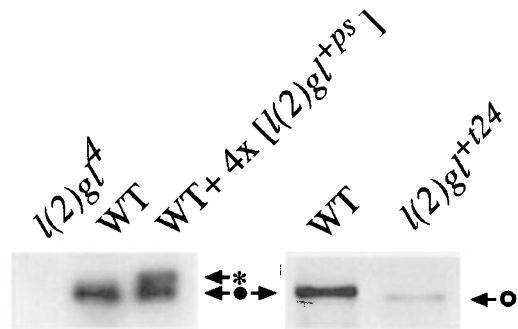

Figure 2 Levels of p127 expression in salivary glands of wild-type and transgenic *Drosophila* late third instar larvae. Immunoblots for p127 extracted from salivary glands of late third instar larvae expressing no p127: *l(2)gl^4*, normal p127 level in wild-type Oregon R (WT), low level in *l(2)gl^{+t24}*, and high level in *P-4x[l(2)gl^{+ps}]*. The p127^{ps} (*) protein displays an apparent higher molecular mass than the wild-type p127 protein (●), whereas the p127^{t24} (○) protein corresponds to a truncated form of p127, albeit fully functional as judged by its ability to restore development of *l(2)gl*-deficient animals

conjunction with ecdysone, p127 exerts no apparent function during glue secretion but is required during the apoptotic pathway.

Sequential changes in the structure of the salivary glands during prepupal development

To determine the pattern of morphological changes occurring in salivary gland cells, from very late larval stage up to the disintegration of the glands, we analyzed the distribution of p127 and myosin-II by immunofluorescence staining and confocal microscopy. This analysis revealed that, in a wild-type mature salivary gland with an empty lumen ($\sim$12 h before pupariation), p127 and myosin-II are distributed in the cytoplasm constituting a reticulated network around the secretory granules, forming a thin layer under the plasma membrane, and enveloping the nuclei (Figure 4a). When the glue proteins are released into the lumen of the glands from $\sim$6–4 h before pupariation, as indicated by the widening of the lumen, the intracellular distribution of p127 and myosin-II
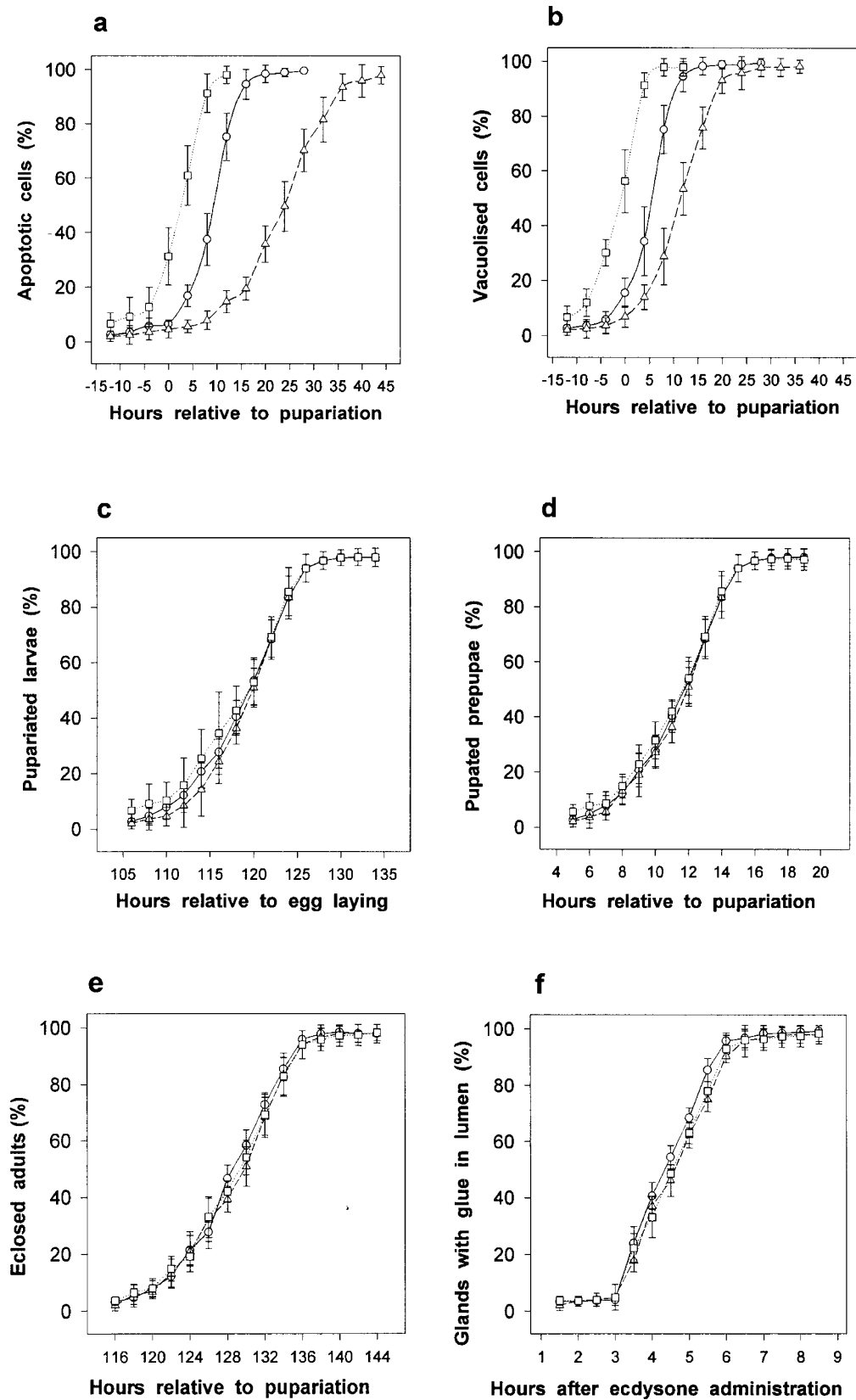
Figure 3 (a) Timing of salivary gland histolysis, (b) timing of vacuolisation of the cytoplasm, (c) duration of larval development, (d) duration of prepupal development, and (e) duration of pupal development in (○) wild-type, (□) $P-4x[l(2)gl^{+ps}]$ transgenic and (△) $l(2)gl^{+t24}$ animals. (f) Since the staging of third instar larvae is difficult to precisely estimate, the timing of glue secretion into the lumen of the salivary glands was measured *in vitro* on salivary glands dissected from

remained unchanged (Figure 4b). When the glue proteins fill the lumen of the salivary glands, the epithelial cells are much thinner and essentially devoid of secretory granules (Figure 4c). In these cells both p127 and myosin-II become diffusely distributed in the entire cytoplasm and much less abundant in the cytoskeletal matrix. Following expectoration of the glue from the glands (Figure 4d), the first vacuoles become visible ~2–4 h AP and p127 and myosin-II remain diffusely distributed between the vacuoles. The vacuoles increase gradually in size and fuse ultimately into a few large vacuoles which at ~8 h AP occupy a large portion of the cytoplasm (Figure 4e). As revealed by the strong staining of p127 and myosin-II in the lumen of the glands, cytoplasmic components are released into the lumen without giving rise to cells lysis. These cells retain their nucleus and an apparently normal size. Ultimately, at 12.5–13 h AP all the salivary gland cells become fully lysed, leaving a bag filled with nuclear and cellular debris (Figure 4f).

In *P-4x[l(2)gl*$^{+ps}$*]* salivary glands, the entire process of vacuolisation and histolysis is considerably moved forward (Figure 4g–i) resulting in the complete lysis of the salivary glands at ~3.5–4 h AP, similar to the histolysis observed in wild-type salivary glands (data not shown). By contrast in *l(2)gl*$^{+t24}$ salivary glands (Figure 4j–p), histolysis is retarded and takes place ~24–30 h AP. In these glands the appearance of cytoplasmic vacuoles is also delayed by comparison to wild-type. However, they persist over a longer period without giving rise to cell lysis. Similarly, the occurrence of p127 and myosin-II in the lumen of the glands (Figure 4n,o) could extend over several hours, suggesting that both processes can occur without resulting in immediate cell lysis.

p127 affects death gene expression

Analysis of the *Drosophila* genome for genes required for cell death during embryonic development led to the identification of three genes, *rpr*, *hid* and *grim*, whose expression presages apoptosis during embryogenesis.[17–19] Each of these genes can activate a death program mediated by caspases. Since the timing of salivary gland histolysis can be manipulated by varying the level of p127 expression, we wanted to determine whether the expression of the three cell death regulators is similarly affected. By using RT–PCR we analyzed the expression of *rpr*, *hid* and *grim* in the salivary glands of wild-type prepupae as well as in those of transgenic *P-4x[l(2)gl*$^{+ps}$*]* and *l(2)gl*$^{+t24}$ prepupae and pupae (Figure 5). In all three genetic backgrounds we found that *hid* expression precedes the induction of *grim* and *rpr*. By comparison to wild-type we observed that all three genes are prematurely expressed in *P-4x[l(2)gl*$^{+ps}$*]* prepupae with *hid* induced the first at the time of

puparation. By contrast, in *l(2)gl*$^{+t24}$, the expression of these genes was considerably delayed and spread over a longer period of time with *hid* induced at ~10–12 h AP, *grim* at ~12–14 h AP, and *rpr* at ~16–18 h AP. These results showed that the level of p127 expression can influence the timing of death gene expression.

Furthermore, we found that the expression of *rp49* is down regulated when the expression of cell death genes becomes up regulated,[20] and that the shift is similarly advanced or delayed according to the level of p127 expression. This finding suggests the selective repression of house-keeping genes at the time of the induction of cell death gene expression is also dependent upon the *l(2)gl* gene dosage.

Use of inhibitors or activators of programmed cell death reveals that cytoplasmic vacuolisation can be dissociated from cell lysis

The response to the activation of the *Drosophila* cell death regulators *rpr*, *hid* and *grim* can be inhibited by the baculovirus p35 protein blocking caspase activity or by specific inhibitors of caspases. We investigated first whether p35[56–58] can inhibit vacuolisation and/or histolysis of the salivary glands. For this purpose we expressed p35 by using the *glass/GMR* system. Late third instar larvae in the wandering stage or fresh prepupae carrying either the *hs-glass* transgene, or both *hs-glass* and *GMR-p35*, were heat shocked at 37°C for 1 h, allowed to recover for 3 h at 25°C and subjected to a new heat shock period of 1 h. Following treatment, we found that all the larvae had formed a puparium, indicating that the endogenous ecdysone release has taken place. These prepupae were allowed to develop for different periods of time (up to 36 h) at 25°C and their salivary glands were dissected and examined. At all time points, the salivary glands of prepupae expressing only *hs-glass* morphologically resembled to those seen in wild-type prepupae of similar age. By contrast, the salivary glands expressing p35 were blocked in their histolysis, confirming the results of Jiang *et al*,[20] but not in the vacuolisation of the cytoplasm. As shown in Figure 6a, 14 h after pupariation, the structure of the salivary glands expressing p35 was similar to that of ~4–6 h AP wild-type glands from which the glue has just been expectorated. Further examination revealed that 24 h AP 90% of the pupae expressing p35 contained viable salivary glands, as judged by trypan blue exclusion. This proportion decreased to ~40% in pupae 36 AP. Similar results could be re-constituted *in vitro* by treating cultured salivary glands with 10^{-5} M 20-hydro-xyecdysone in the presence of caspase inhibitors, including YVAD-CHO, Z-VAD-FMK and DEVD-CHO at a concentra-

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early wandering third instar larvae at puff stage (PS) 1[13,14] and incubated in presence of 10^{-5} M 20-hydroxyecdysone. By comparison to wild-type, salivary gland histolysis is accelerated in *P-4x[l(2)gl*$^{+ps}$*]* transgenic animals with six *l(2)gl*$^+$ copies and, conversely, retarded in *l(2)gl*$^{+t24}$ animals expressing a reduced level of p127. The process of cytoplasmic vacuolisation which precedes histolysis exhibits identical *l(2)gl*-dependence. However, the duration of larval, prepupal and pupal development as well as ecdysone induction of glue secretion are not affected in the different *l(2)gl* variants. Calculation of the number of apoptotic cells was determined by measuring the proportion of trypan blue stained cells whereas the number of vacuolized cells was measured by counting acridine orange fluorescent cells fixed with formalin/glutaraldehyde (see Materials and Methods). For each point, the number of positively stained cells were determined in ten independent glands. Standard deviations are represented by bars
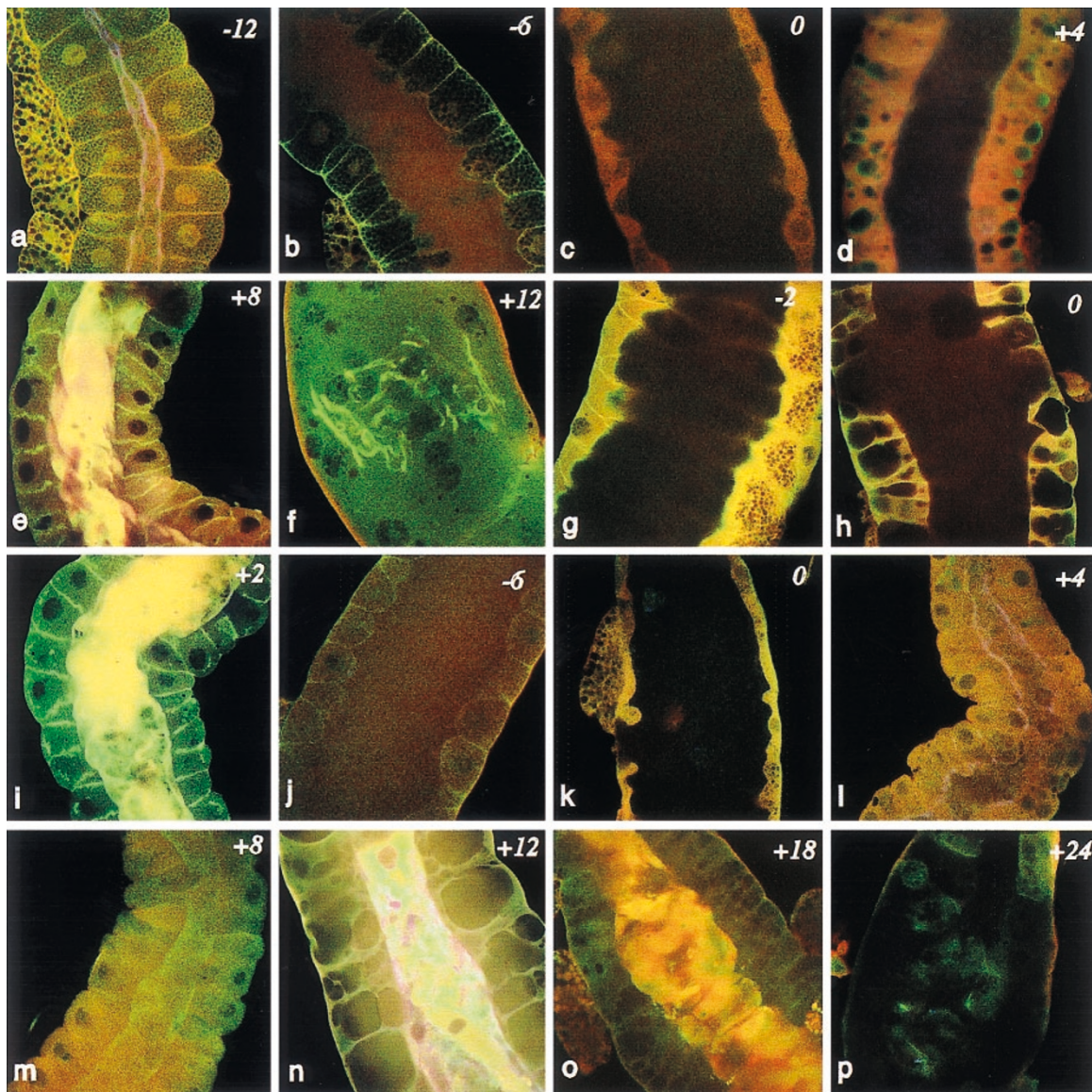
Figure 4 Histolysis of the salivary glands. Confocal micrographs of salivary glands dissected from (**a–f**) wild-type, (**g–i**) *P-4x[l(2)gl^{+ps}]* and (**j–p**) *l(2)gl^{+t24}* transgenic staged third instar larvae, prepupae and pupae. The glands were stained with anti-p127, anti-myosin-II antibodies, and fluorescently conjugated secondary antibodies. Shown are projections of optical sections displaying the distribution of p127 (green) and myosin-II (red). Superimposed signals for p127 and myosin-II are shown in yellow. Time relative to puparium formation is indicated at the upper right corner

tion of 10 nM. These inhibitors prevented histolysis of the salivary glands without affecting cytoplasmic vacuolisation (data not shown).

In a reciprocal experiment, we tested whether histolysis of the salivary glands could occur without prior cytoplasmic vacuolisation. For this purpose, we induced expression of the death gene *rpr* for 1 h in early wandering stage instar larvae (12–18 h before pupariation as indicated by the dark bromophenol blue staining of the gut), prior to ecdysone release, and found that *rpr* induction was sufficient to induce a rapid histolysis of the salivary glands in immobile late third instar larvae or white prepupae (Figure 6b). Examination of disintegrating salivary glands

revealed no sign of cellular vacuolisation prior to the complete lysis of the glands (data not shown). Altogether these findings indicate that the process of vacuolisation of the cytoplasm could be dissociated from the process of cellular lysis.

The temporal pattern of puff appearance and regression is altered in polytene chromosomes of *P-4x[l(2)gl^{+ps}]* salivary glands

The finding that cell death genes can be prematurely induced at the onset of puparation in *P-4x[l(2)gl^{+ps}]* animals prompted us to examine the puffing pattern of polytene chromosomes in
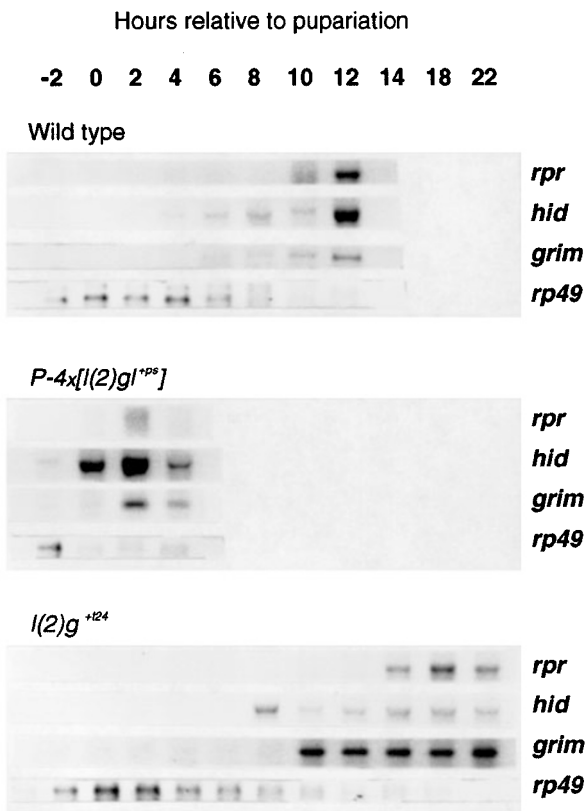
Hours relative to pupariation

-2 0 2 4 6 8 10 12 14 18 22

Wild type

rpr
hid
grim
rp49

P-4x[l(2)gl⁺ᵖˢ]

rpr
hid
grim
rp49

l(2)g ⁺ᵗ²⁴

rpr
hid
grim
rp49

Figure 5 Expression of cell death genes precedes salivary gland histolysis. Poly(A)⁺ RNA was isolated from salivary glands of (**a**) wild-type, (**b**) *P-4x[l(2)gl⁺ᵖˢ]*, and (**c**) *l(2)gl⁺ᵗ²⁴* staged animals and analyzed by RT–PCR to detect the patterns of *rpr*, *hid* and *grim* transcription. RT–PCR to detect *rp49* mRNA was used as a control
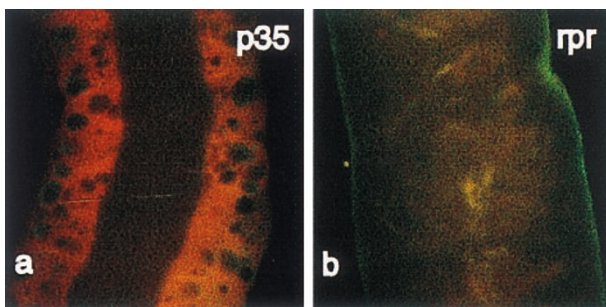

Figure 6 (**a**) The baculovirus p35 protein inhibits apoptosis of salivary glands but not vacuolisation,whereas (**b**) expression of the *reaper* (*rpr*) cell death induces apoptosis without prior vacuolisation. Wild-type third instar larvae in late wandering stage or fresh prepupae carrying *hs-glass* and *GMR-p35* transgenes were heat-shocked at 37°C for 1 h, allowed to recover for 3 h at 25°C and subjected to a new heat shock period of 1 h. Salivary glands were dissected from these animals at ∼14 h AP, stained with anti-p127 and anti-myosin-II antibodies and examined as indicated in Figure 4. Wild-type third instar larvae in early wandering stage carrying a *hs-rpr* transgene were heat-shocked at 37°C for 1 h and incubated at 25°C for 11 h. Immobile late third instar larvae or white prepupae were dissected and the salivary glands were processed and examined as indicated in Figure 4

1–2 h-old prepupae. In particular, we were interested to determine whether any late prepupal puff would occur earlier when salivary gland histolysis takes place prematurely. Two hours after puparation, the chromosomes of the salivary glands of *P-4x[l(2)gl⁺ᵖˢ]* prepupae exhibited none of the major puffs seen in wild-type prepupae of the same age. However, as shown in Figure 7, we observed consistently a puff in the region 52A, which usually occurs in very late prepupae. Moreover, we detected no puff in the region 75CD, encompassing the locus containing the cell death genes *rpr*, *hid* and *grim*, and the βFTZ-F1 ecdysone regulated gene.[59,60] In wild-type salivary glands this puff appears during prepupal development (7–9 h after puparium formation, see Becker[13]). These findings indicate that expression of the suicide genes can occur without puff formation. The absence of a normal pattern of chromosomal puffing in the salivary glands of *P-4x[l(2)gl⁺ᵖˢ]* prepupae shows that, with the exception of the 52A puff, the implementation of the death programme appears to be independent from the induction of prepupal chromosomal puffs and further indicates that ecdysone triggers different signalling pathways in salivary glands.

Discussion

In the reported work, we focused our investigations on the events that during prepupal and pupal development lead to the histolysis of the salivary glands and on the requirement for p127 in this process. In wild-type prepupae the disintegration of the salivary glands occurs relatively suddenly, 12–13 h after the onset of puparia-tion[11,12,20,61,87] and cell death takes place in a nearly synchronous manner in all larval cells. Our investigations show that this process can be studied *in vivo* with the analytical achievement of a tissue culture system while maintaining the physiological relevance of an intact organism.

p127 is required for salivary gland histolysis

Our studies confirm that salivary gland histolysis is a steroid-triggered programmed cell death response requiring the presence of the p127 protein. In the absence of p127, *in vitro* exposure of salivary glands from aged *l(2)gl* larvae to ecdysone results in a puffing pattern characteristic of pupariation,[49] and induces the secretion of the small amount of glue granules present in the glands, indicating that ecdysone receptors made of heterodimers between the ecdysone receptor EcR and the Ultraspiracle protein USP,[62–64] are present in *l(2)gl*-deficient salivary gland cells. However, these receptor are unable to induce any of the morphological changes that usually precede cell death, such as vacuolisa-tion of the cytoplasm and discharge of cytoplasmic compo-nents in the lumen of the glands. After 24 h of culture, ecdysone-treated *l(2)gl* salivary glands display the same morphology as untreated glands and the cells remain impermeable to vital dyes, such as trypan blue, demonstrating that apoptosis of *l(2)gl*-deficient glands cannot be triggered by ecdysone. These results indicate that p127 is specifically required for implementing ecdysone-triggered apoptosis of the salivary glands, including the early events leading to
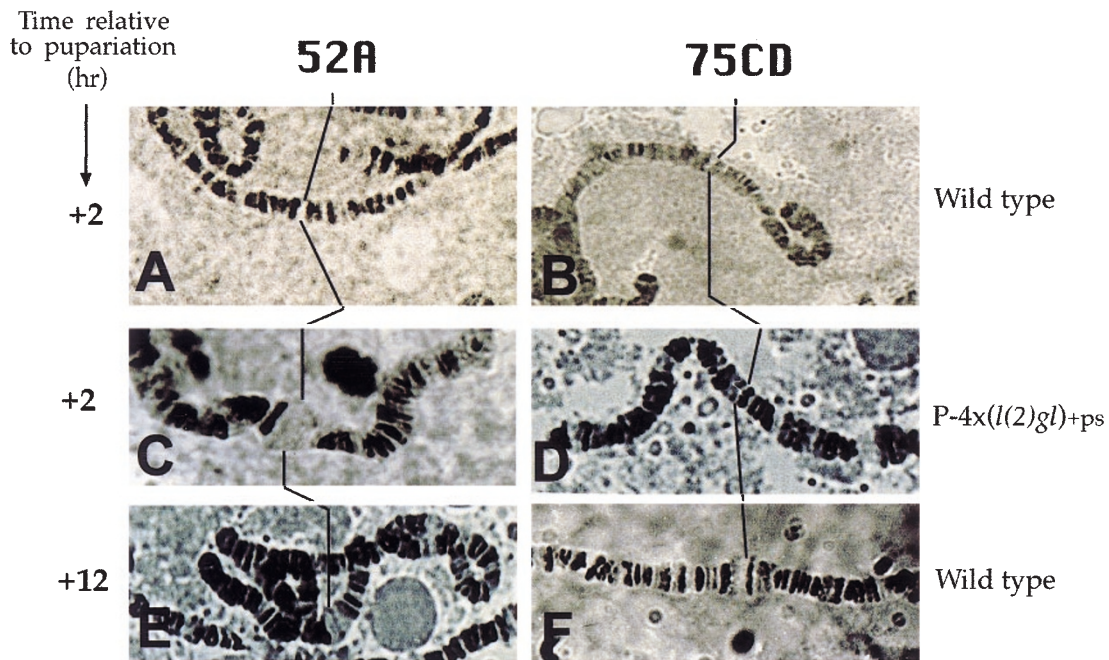
Time relative
to pupariation
(hr)

52A   75CD

Figure 7 Puffing pattern of salivary gland chromosomes around the 52A and 75CD loci in wild-type and *P-4x[l(2)gl⁺ᵖˢ]* prepupae. The late prepupal 52A puff which becomes only active in late wild-type prepupae is induced in early *P-4x[l(2)gl⁺ᵖˢ]* prepupae. By comparison, the 75CD puff which encompasses the locus containing the three death genes, *rpr*, *hid* and *grim* as well as the *βFTZ-F1* gene, and is induced in wild-type mid-prepupae, remains inactive in early *P-4x[l(2)gl⁺ᵖˢ]* prepupae. Time relative to puparium formation is indicated on the left

vacuolisation and down-regulation of the expression of house-keeping genes, such as the *rp49* ribosomal protein gene.

p127 gene dosage affects specifically the timing of salivary gland histolysis

Our experiments show that overexpression of p127 moves forwards the timing of salivary gland apoptosis and that a lower expression extends the survival of the glands. The differences in the timing of cell death can also be detected in other ecdysone-dependent apoptotic tissues, as revealed by the higher proportion of dying cells which can be observed in the midgut and fat bodies of *P-4x[l(2)gl⁺ᵖˢ]* prepupae (dat not shown). However, these tissues are more difficult to analyse due to asynchrony in cell death and deserve further studies. Remarkably, both an advanced apoptosis induced by supernumary copies of *l(2)gl*, or a delayed apoptosis resulting from a lower expression of *l(2)gl* exerts no apparent effect on the larval, prepupal and pupal development, demonstrating further that ecdysone-triggered apoptosis of the salivary gland cells is specifically controlled by p127. Moreover, these experiments show that, once ecdysone has induced the programme of cell death, disintegration of the salivary glands will ensue uniquely depending upon the level of p127 expression without affecting the development of the imaginal tissues. The findings that the induction of the *rpr*, *hid* and *grim* death gene expression preceding the destruction of the larval salivary glands, and the repression of *rp49* ribosomal protein gene displays also a *l(2)gl* time dependence, further sustain the idea that p127 mediates the steroid-triggered apoptotic process at a relatively early phase of the ecdysone response.

Salivary gland histolysis fails to occur in pupae mutated in the *reduced bristles on palpus* (*rbp*) allele[65] of the *Broad Complex* (*BRC*) locus.[66] Mutations in *BRC* functions disrupt numerous aspects of cell development autonomously during larval, prepupal and pupal development. In particular, the *BRC* function is required for the induction of intermolt puffs, early and late puffs as well as for the development of the imaginal tissues.[67,68] Molecular analysis show that the *BRC* products form a family of related proteins sharing a common N-terminal core domain and different C-terminal domains characterised by the presence of zinc-finger DNA-binding motifs,[69] and that the *rbp* function is provided by the BRC-Z1 protein isoform.[70–72] Interestingly, preliminary studies of BRC-Z1 in *l(2)gl*-deficient salivary glands showed that this protein is present in the cytoplasm (data not shown) instead of the nucleus.[73] This observation raises the possibility that p127 may facilitate the transport of ecdysone-responsive transcription factors into the nucleus of salivary gland cells which are required for the implementation of the apoptotic programme. In this respect, the finding that *Ebr*, an EMS-induced enhancer of *broad¹* isolated by Gotwals and Fristom,[74] is a mutation in the myosin II heavy chain locus[75] further supports the idea that cytoskeletal components may directly affect the *BRC* function. The survival of the salivary glands in both *rbp* pupae and aged *l(2)gl* larvae suggests that both BRC-Z1 and p127

function at an early stage of the ecdysone-triggered apoptosis of this tissue. A knowledge of the relative hierarchical position of BRC-Z1, myosin-II and p127 should provide a better understanding of the mechanism of salivary gland apoptosis.

Release of cytoskeletal components into the lumen of the salivary glands occurs without immediate cell lysis

The major function of the salivary glands is to synthesise and secrete glue proteins which affixes the pupal case to its substrates,[76] and earlier works have noticed that, following glue secretion at the time of puparium formation, the larval salivary glands display signs of histolysis and completely disappear after pupation.[11,12,77] However, electron microscopic examination of salivary glands following glue secretion reveals continued secretory function through the prepupal period, which were thought by Mitchell *et al*[61] to reflect the release of products resulting from the puffing activities.

During early third instar larval development, the gland is constituted by a monolayer of epithelial cells which gradually increase in size and accumulate granules from the most distal cells until all but the last proximal 20–25 cells.[78] Just before secretion takes place, the salivary gland cells become packed with glue granules relatively homogenous in size, leaving little space for the cytoplasm which forms a reticulated network around the granules, as indicated by the distribution of the p127 and myosin-II cytoskeletal proteins. Following secretion of the glue granules, the cytoskeletal proteins become first uniformly distributed in the cytoplasm of the salivary glands cells and then are gradually released in the lumen of the glands through a process which apparently leaves the cells intact, albeit strongly vacuolated. The release of these proteins appears to correspond to the second secretory cycle noticed by Mitchell *et al*.[61] Surprisingly, after the release of the cytoskeletal components, the *l(2)gl*[+t24] salivary gland cells which are essentially depleted of p127 and myosin-II retain however an apparent normal size and shape for several hours, albeit without regaining a normal cytoplasmic structure. In these glands, final histolysis occurs at least 12 h after the formation of cytoplasmic vacuoles. Our data indicate that the discharge of a large proportion of p127 and myosin-II into the lumen of the glands constitutes an event distinct from the final cell lysis.

Furthermore, in *l(2)gl*[+t24] the transcription of the death genes, which act upstream of putative executive caspases,[79–81] occurs essentially after the secretion of the cytoskeletal components, indicating that this process can be under a distinct control. This is further supported by the finding that *rpr* death gene expression can induce histolysis of salivary gland cells without formation of large vacuoles and release of cytoskeletal components into the gland lumen. However, expression of the baculovirus p35 caspase inhibitor blocks disintegration of wild-type salivary glands at the time when the cells become vacuolated but before any cytoskeletal component is released into the gland lumen, indicating that additional genes and mechanisms may contribute to salivary gland histolysis.

Absence of chromosomal puffing in *P-4x[l(2)gl*[+ps]*]* salivary glands suggests that prepupal puffing is not required for salivary gland apoptosis

Considerable attention has been dedicated to study variations in the pattern of chromosomal puffing during *Drosophila* late third instar larval and prepupal development of the salivary glands[13–16,82] and has led to the characterization of 21 puff stages. Some of the puff stages are correlated with particular morphological events such as glue synthesis at PS1, glue secretion at PS5, puparium formation at PS10/11 and head eversion at PS21. Molecular analysis of the genes located within puffs revealed that intermolt puffs contain genes for the glue proteins including the *Sgs4* at 3C, *Sgs1* at 25B, *Sgs3*, *Sgs7* and *Sgs8* clustered at 68C and *Sgs5* at 90BC, whereas early puffs contain essentially genes which may act as regulators of gene expression, such as transcriptional regulators at 2B5 and 74EF, or genes encoding the ecdysone receptor at 42A as well as members of the steroid hormone receptor superfamily at 2C, 75B and 78C (for review, see Russell and Asburner[16]). Interestingly, with the exception of the calcium-binding protein related to calmodulin encoded in the 63F early puff,[83] none of the so far identified puff sequences encode proteins regulating either the secretion and expectoration of the glue proteins, or the structural changes affecting the glands after completion of its major secretory function. These findings suggest that early puffs represent transcriptional activities of genes whose products will be responsible for inducing salivary gland histolysis. These regulatory proteins may activate or repress the function of constitutively present enzymes, or induce the synthesis of new proteins acting specifically in the apoptotic pathway. However, since the disintegration of the salivary glands occurs relatively rapidly after the ecdysone stimulus, particularly in the *P-4x[l(2)gl*[+ps]*]* prepupae, it would be advantageous that some of the components of signal transduction pathway leading to apoptosis are already present rather than waiting for protein synthesis. This is the case of p127 which is constitutively expressed in the salivary glands before the ecdysone trigger, indicating that disintegration of this tissue requires a pre-existing protein.

When we analyzed the puffing pattern of cells of the salivary glands of *P-4x[l(2)gl*[+ps]*]* prepupae, we were expecting to see the normal prepupal chromosomal puffs corresponding to this stage with additional late prepupal puffs which would represent puffs specifically required for apoptosis. To our surprise, we detected none of the major early or late prepupal puffs, with the exception of a puff at 52A whose products are not yet known. Furthermore, no puff at 75CD, which presumably contains the *rpr*, *hid* and *grim* cell death genes beside the *βFTZ-F1* gene, could be found in these animals although the transcription level of these genes is similar if not higher than in wild-type prepupae.

An interesting implication of our results is that normal ecdysone response is channelled in the salivary glands through at least two different pathways, with an *l(2)gl*-independent pathway leading to chromosomal puffing, which may mirror gene activities required for imaginal development, and an *l(2)gl*-dependent pathway resulting in

apoptosis. The occurrence of an apoptotic pathway distinct from the puffing pathway is further substantiated by the absence of chromosomal puffing in prepupae overexpressing p127, and the occurrence of a subset of prepupal puffs in *l(2)gl*-deficient salivary glands incubated with ecdysone.[49] Since p127 is constitutively expressed in the salivary glands,[3,84] the absence of prepupal chromosome puffing in *P-4x[l(2)gl^{+ps}]* salivary glands suggests that p127 acts at a relatively early step of the ecdysone response to elicit an apoptotic pathway. When p127 is overexpressed, the component inducing normally chromosome puffs could be removed from the puffing pathway and shifted to the apoptotic pathway. As a consequence the timing of histolysis is considerably accelerated, and no puffing occurs with the exception of a puff in the chromosomal band 52A. Conversely, when p127 becomes rate-limiting, the apoptotic pathway is considerably delayed and a normal pattern of chromosome puffing takes place. Alternatively, it is also possible to envisage that the accelerated pace of apoptosis occurring after glue secretion in *P-4x[l(2)gl^{+ps}]* prepupae results in transcriptional activation without chromosomal puffing. Further analyses on gene expression will help clarify the mechanism that controls the unusual puffing pattern in *P-4x[l(2)gl^{+ps}]* salivary glands and the timing of apoptosis.

Our data show the importance played by a cytoskeletal protein in the implementation of an apoptotic process, and offer new ways to investigate the mechanism by which steroid-triggered stimuli activate cell death. Explanation on how a cytoskeletal protein could be involved in the transduction of an apoptotic signal mediated by ecdysone would be found in the identification of interacting partners. In these respects, the findings that, on the one hand, p127 interacts with myosin-II[6] and acts as a negative regulator of myosin-II contractility (G Merdes, D Strand, WH Zu, D Kiehart and BM Mechler, personal communication), and, on the other hand, a mutation in myosin-II is an enhancer of *broad*[1,74] are consistent with the idea that cytoskeletal proteins can indeed regulate the function of ecdysone-responsive transcription factors. Detailed genetic and molecular analyses on the interaction between these three genes and their products are currently being conducted and may help to resolve the role played by cytoskeletal proteins played in apoptosis mediated by ecdysone. As the death programme is conserved during evolution, these investigations will provide new insights into understanding how steroid hormones, in conjunction with the cytoskeleton, may regulate apoptosis in higher organisms.

Materials and Methods

Staging larvae and pupae

Stocks were raised at 25°C on a standard cornmeal, molasses and agar food supplemented with 0.05% bromophenol blue.[85] Eggs were collected for 1 h and maintained at 25°C for the reported times. Time points prior to puparium formation were estimated on the degree of bromophenol blue coloration of the gut and the initiation of the wandering stage.[86] To avoid the effects of accumulated modifiers on

the second chromosome and to obtain large numbers of null mutant *l(2)gl* larvae, heterozygous *l(2)gl^4/SM5* and *l(2)gl^{U334}/SM1* flies[1] were crossed, and 8–10 days after oviposition, heteroallelic *l(2)gl* larvae (distinguished from their heterozygous sibs by their bloated appearance) were collected. In these larvae, the size of the glands is about one-third of the size of wild-type glands.

Detection of cell death and vacuolisation

Dissected salivary glands in Ringer's solution were stained either for 5 min with 0.2% Trypan Blue dissolved in Ringer's solution and directly examined under a light microscope or for 3 min with 5 μg/ml acridine orange in phosphate-buffered saline (PBS), washed in PBS and immediately viewed under epifluorescence illumination set for FITC channel. For detecting vacuolisation, we found that acridine orange preferentially accumulates in vacuoles of salivary glands treated with 4% paraformaldehyde and 1.5% glutaraldehyde, washed in PBS and examined under epifluorescence illumination using a fluresceine emission filter.

Evaluation of puffing activity

Salivary glands were dissected, immediately fixed in 45% acetic acid and stained with 2% orcein in 50% acetic acid and 30% lactic acid. The preparation was squashed and examined under a Leitz photomicroscope.

Immunohistochemistry

Salivary glands were fixed in 4% paraformaldehyde and 1.5% glutaraldehyde in PBS, permeabilized with 0.3% Triton X-100 in PBS, blocked with 2% BSA and 1% normal goat serum. P39 and P38 rabbit polyclonal antibodies (1 : 300 dilution) for detecting p127[3] in wild-type and *P-4x[l(2)gl^{+ps}]* or *l(2)gl^{+t24}* transgenic glands, respectively, and mouse monoclonal antibodies against myosin-II (1 : 20 dilution), kindly provided by D Kiehart (Duke University, Durham, NC, USA), were added and the salivary glands were incubated for 16 h at 4°C. The salivary glands were washed with 0.3% Triton X-100 in PBS and blocked. Then Cy3 goat anti-rabbit antibodies and Cy5 goat anti-mouse antibodies (Jackson Immunoresearch, Inc., West Groove, PA, USA) as well as fluoresceine conjugated phalloidin from Sigma, St Louis, MO, USA (1 : 200 dilution) were added and the glands were further incubated for 2 h at room temperature, then washed, mounted in Mowiol (Calbiochem, La Jolla, CA, USA). Optical sections, 1 μm thick, were collected using a Zeiss LSM-410 laser confocal microscope.

Protein analysis

For Western blot analysis, ten pairs of salivary glands were homogenised in 20 μl 0.125 M Tris [pH 6.8], 5% β-mercaptoethanol, 1% SDS, diluted in 1 : 1 in 2 × Laemmli sample buffer and boiled for 5 min. The proteins were separated on a 8% SDS-polyacrylamide gels. After electrophoresis, proteins were electrotransferred to Immobilon-P polyvinylidenfluoride membranes (Millipore Corp., Bedford, MA, USA) using semi-dry blotter. After blocking, the membranes were probed with anti-p127 N-terminal P38 or C-terminal P39 rabbit polyclonal antibodies, visualised with alkaline phosphatase-conjugated secondary antibodies and Nitroblock/CSPD as provided in the Tropix system (Serva Feinbiochemica GmbH, Heidelberg, Germany) and used as recommended by the manufacturer.

RT – PCR analysis of gene expression

For each time point, RNA was extracted from five pairs of salivary glands in 10 μl 4 M guanidium isothiocynate, 1% SDS, 10 mM Tris, pH 8.0, and the poly(A)$^+$ RNA was isolated using Oligotex suspension and mini-spun columns (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed in 10 μl from one-fifth of the extracted poly(A)$^+$ RNA using the Moloney murine leukemia virus reverse transcriptase (Cetus-Perkin Elmer, Branchburg, NJ, USA) with reverse primers, followed by amplification with the AmpliTaq DNA polymerase (Cetus-Perkin Elmer) in presence of forward and reverse primers. The following primers were used: *rpr*-reverse (5'-TCATTGCGATGGCTTGCGAT-3') and *rpr*-forward (5'-TGGCATTCTACATACCCGAT-3'); *hid*-reverse (5'-CGTAAAGTTGTCGTAGCG-AT-3') and *hid*-forward (5'-GCACGGC-CATCCGAATCCGA-3'); *grim*-reverse (5'-CTTGGAGGTGGCATCGG-TAA-3') and *grim*-forward (5'-GTTGGCCAGAAGCTATCAGC-3'); *rp49*-reverse (5'-GTGTATTCCGACCACGTTACA-3') and *rp49*-forward (5'-TCCTACCAGCTTCAAGATGAC-3'). The temperatures were as follows: denaturation 94°C for 1 min, primer annealing 55°C for 2 min, polymerase extension 72°C for 3 min and the MgCl$_2$ concentration was 1.5 mM. The number of cycles was 30. To exclude DNA contamination in the case of the intronless *rpr* and *grim* genes, extracted poly(A)$^+$ RNA was treated with DNase devoid of RNase (Boehringer GmbH, Mannheim, Germany). Reactions were analyzed on a 2% agarose gel and stained with ethidium bromide.

Acknowledgements

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