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The *imd* gene is required for local *Cecropin* expression in *Drosophila* barrier epithelia

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Surfaces of higher eukaryotes are normally covered with microorganisms but are usually not infected by them. Innate immunity and the expression of gene-encoded antimicrobial peptides play important roles in the first line of defence in higher animals. The immune response in *Drosophila* promotes systemic expression of antimicrobial peptides in response to microbial infection. We now demonstrate that the epidermal cells underlying the cuticle of larvae respond to infected wounds by local expression of the genes for the antimicrobial peptide cecropin A. Thus, the *Drosophila* epidermis plays an active role in the innate defence against microorganisms. The *immune deficiency* (*imd*) gene was found to be a crucial component of the signal-induced epidermal expression in both embryos and larvae. In contrast, melanization, which is part of the wound healing process, is not dependent on the *imd* gene, indicating that the signalling pathways promoting melanization and antimicrobial peptide gene expression can be uncoupled.

INTRODUCTION

Surfaces of higher eukaryotes are naturally covered with microorganisms but are usually not infected by them. In mammals, recent discoveries have highlighted the importance of antimicrobial peptides produced by barrier epithelia (Schröder, 1999). Innate immunity and the expression of gene-encoded antimicrobial peptides have shown to play an important role as a first line of defence in higher animals, including mice and humans. Insects have been proven to be very useful models for the study of innate immunity as they are notably resistant to microorganisms and can be subjected to biochemical and genetic analyses (Boman *et al.*, 1972; Hultmark, 1993). Recently, the molecular basis of pathogen recognition, signal transduction and effector mechanisms have been found to share considerable similarities between insects and mammals, suggesting the existence of an evolutionary relationship in the immune defence (Medzhitov and Janeway, 1998; Engström, 1999; Hoffmann *et al.*, 1999).

Insects have successfully inhabited the terrestrial part of the biosphere and often lead their lives in habitats infested with microorganisms. When infected, insects mount a potent humoral immune response by the activation of proteolytic cascades resulting in blood clotting, melanin formation and production of an array of antimicrobial peptides with broad and overlapping specificity (reviewed in Bulet *et al.*, 1999). Experimental infection with bacteria, or injections of microbial substances such as lipopolysaccharide (LPS), lead to induction of antimicrobial peptide genes in the fat body of third instar larvae, pupae and adults of *Drosophila* and other insects (reviewed in Engström, 1998). In the *Drosophila* embryo, we recently demonstrated the presence of an inducible immune response in the embryonic yolk and epidermis, but surprisingly not in the embryonic fat body (Önfelt Tingvall *et al.*, 2001). It has thus been an open question as to when the fat body becomes immune-competent. Here we show that an immune response in the fat body can be induced during the first larval instar, and that this response persists through all larval stages.

In insects, and other arthropods, the integument is composed of an epicuticle and a procuticle that are secreted by a single cell layer of epidermis. Immune responses in the epithelia underlying the larval cuticle of insects have been reported in *Bombyx mori* and *Hyalophora cecropia* (Brey *et al.*, 1993; Ashida and Brey, 1995), and *Ceratitis capitata* (Marmaras *et al.*, 1993). Recently, it was described that a drosomycin promoter–green fluorescent protein (GFP) fusion was expressed in epithelial tissues covering the trachea of *Drosophila* in response to fungi. This local expression of drosomycin was not dependent on the *Toll* pathway, suggesting that local and systemic expression of antimicrobial peptides might be regulated by different signalling pathways (Ferrandon *et al.*, 1998).

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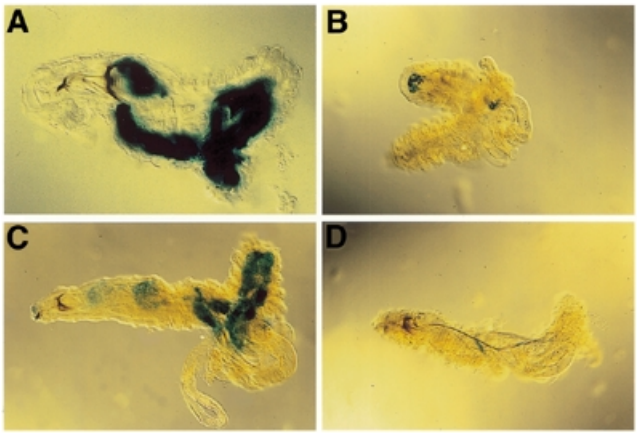

Fig. 1. Injection of LPS induces a systemic immune response in the fat body of first and second instar larvae. (**A–D**) Distribution of β-gal staining in opened A10 *CecA1–lacZ* transgenic larvae of first (A, B) and second (C, D) larval instar. Larvae were injected with LPS into the haemocoel (A, C), or were not injected (B, D), kept at 25°C for 3–4 h, opened up with forceps, fixed and stained. Anterior is to the left.

The present study demonstrates that cuticular abrasion of *Drosophila* larvae in the presence of bacteria induced expression of the *Cecropin A1* (*CecA1*) gene in the cuticular epithelium. Analysis in mutant background revealed that the immune deficiency (*imd*) gene product (Lemaitre *et al.*, 1995; Corbo and Levine, 1996) is necessary to mount an immune response in embryonic and larval epidermis in response to microbial challenge. Interestingly, the formation of melanin in the wound area was not dependent on the *imd* gene, suggesting that different signalling pathways are involved in activating expression of antimicrobial peptides and the pro-phenoloxidase cascade.

RESULTS

CecA1 gene expression is inducible in fat body of first and second instar larvae

Although the fat body is the major site of *CecA1* expression in flies and third instar larvae (Samakovlis *et al.*, 1990; Roos *et al.*, 1998), no expression could be induced in the embryonic fat body (Önfelt Tingvall *et al.*, 2001). To determine the onset of inducible *CecA1* expression in the fat body we used transgenic fly strains carrying the A10 construct, which contains 760 bp of the *CecA1* upstream region fused to the *Escherichia coli lacZ* gene. These constructs were previously shown to mimic the induction of the *CecA1* gene in terms of tissue-specificity and inducibility by infectious agents in embryos, third instar larvae and adults (Engström *et al.*, 1993; Roos *et al.*, 1998; Petersen *et al.*, 1999; Önfelt Tingvall *et al.*, 2001). All LPS-injected first and second instar A10 larvae displayed strong reporter gene expression in the fat body (Figure 1A and C). The uninjected control larvae did not promote any reporter gene expression (Figure 1B and D), while injection of sterile phosphate-buffered saline (PBS) promoted faint fat body staining in 20–25% of the larvae (data not shown), suggesting that wounding may trigger a weak response. In conclusion, a maturation of the fat body had occurred during the first larval instar, rendering it immune-competent. Analysis of deletion constructs in the *CecA1* upstream region showed that inducible expression in the fat body of first and second instar larvae required the presence of a conserved region including a κB-like motif, a GATA motif and another putative regulatory element called R1 (data not shown). These results are the same as those found for *CecA1* regulation in third instar larvae (Roos *et al.*, 1998; Petersen *et al.*, 1999) indicating that LPS-inducible *CecA1* expression in the fat body is controlled via the same regulatory promoter elements in all three larval instars.

The *CecA1* gene is induced in the fat body of larvae which have been infected as embryos

We decided to infect late stage embryos with bacteria and study the effects on *CecA1* expression in the hatched larvae. Embryos, which were injected with Gram-negative bacteria and aged to 2–6 h after hatching, displayed similarly patched staining patterns in the larval epidermis, as was observed in late stage embryos (Figure 2A) (Önfelt Tingvall *et al.*, 2001). Most interestingly, however, when the larvae were aged to a later stage of development (10–14 h after hatching), activation of *CecA1* expression occurred in the fat body (Figure 2B), while the expression in the epidermis had declined. These results suggest that the *CecA1* gene can be induced in the larval fat body by the persistent presence of bacteria, or that cells that were activated in the embryo have a 'memory' and provide a secondary signal in the larval stage, which activates the response in the fat body. To differentiate between these two models we repeated the experiment by injecting LPS instead of live bacteria, since LPS is a short-lived molecule *in vivo*, and not likely to be transferred from the embryo into the hatched larva. LPS-injection of late stage embryos, followed by aging into larvae, mounted a similar pattern of staining in the larval epidermis as seen in late stage embryos (Figure 2C), but could not elicit any response in the larval fat body even after longer aging (data not shown). LPS-induction leads to a peak of *CecA1* expression that reaches a maximum after 1–3 h (Samakovlis *et al.*, 1990). Therefore, the epidermal expression of *CecA1* in LPS-injected embryos is probably transient and the staining observed in the larval epidermis (Figure 2C), mainly the result of high stability of the β-gal protein. Our conclusion is therefore that live bacteria, but not LPS, transmitted the inducing signal from embryos to larvae, which promoted activation of the *CecA1* reporter gene in the larval fat body, but not in the larval epidermis. Thus, a maturation in the immune-competence of the fat body had occurred during a time window ranging from 6 to 14 h of larval development.

Wounding induces local *CecA1* expression in the larval epidermis

How do *Drosophila* larvae deal with skin infections, and what are the effects of non-sterile abrasion on epidermal and fat body *CecA1* expression? We tested this by direct challenge of the larval epidermis using sandpaper or the ragged edge of a sharp preparation needle, and inoculation with a solution of *Enterobacter cloacae*. All three larval instars reacted by turning on the *CecA1* promoter in cells in the epidermis while in the fat body

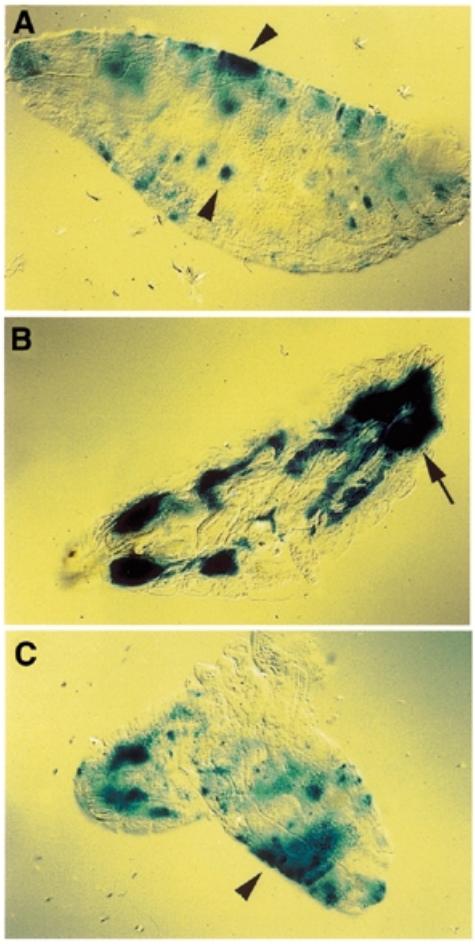

Fig. 2. The fate of embryonally induced *CecA1* expression was analysed in hatched first instar larvae after injections of bacteria or LPS in late stage embryos. (A–B) Injection with *E. cloacae*. (**A**) 8 h post-injection (2–6 h after hatching) the pattern of induced cells in the larval epidermis (arrowheads) remained the same as in embryos (Önfelt Tingvall *et al.*, 2001), (**B**) while staining of the fat body (arrow) was predominant in larvae aged to 16 h post-injection (10–14 h after hatching). (**C**) Injection with LPS promoted expression in the epidermis (arrowheads) but not in the fat body.

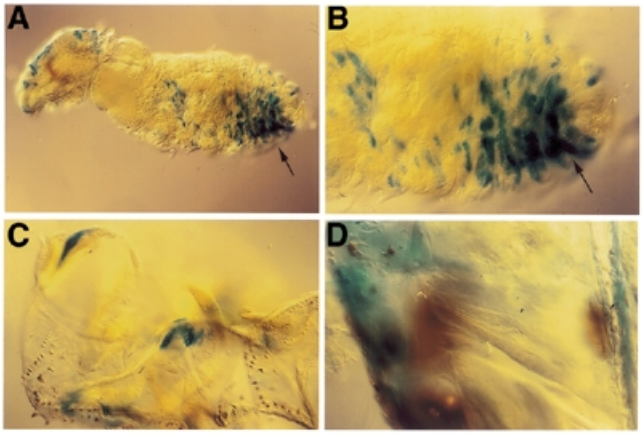

Fig. 3. Cuticular wounding and exposure to bacteria induces a local immune response in the epidermis of larvae. (A–D) The epidermis layer of larvae carrying the A10 *CecA1–lacZ* construct was abraded and incubated with *E. cloacae*, and analysed for reporter gene expression using X-gal. (**A**) First instar larva, and (**B**) a close up of the same larva demonstrating the local distribution of β-gal staining in the same areas as had been scratched (arrows). Dissected cuticle of abraded second (**C**) and third (**D**) instar larvae showing melanization of the wound (brown) and β-gal staining mimicking *CecA1* expression (blue) in adjacent and partly overlapping regions of the cuticle.

the *CecA1* promoter remained inactive (Figure 3). The response was local and on the same side as the wound had been inflicted (Figure 3A and B). The expression pattern closely resembled the one found previously in embryos, with a network of stained cells elongated in the dorsovetral axis (Önfelt Tingvall *et al.*, 2001). However, a major difference is that the expression in the larval epidermis was restricted to the wounded region, while in the embryo, it occurred throughout the embryo. Occasionally, a more general β-gal staining occurred, which included the underlying fat body. This was probably due to wounding through the cuticle, allowing bacteria to enter the haemocoel, giving rise to a systemic induction of the immune response. The β-gal expression in the epidermis was in most cases accompanied by a local melanization reaction. To study this in detail, the larval cuticle was dissected and the rest of the larval tissue, including the fat body, was removed carefully. In Figure 3C and D, dissected cuticle from second and third stage larvae show melanized tissues adjacent to the β-gal positive cells.

Wounding and incubation with LPS instead of live bacteria promoted a weaker response, suggesting that bacteria may trigger several signal transduction pathways, leading to stronger upregulation of *CecA1* expression. Abrasion during aseptic conditions did not promote any *CecA1* expression in the epidermis, indicating that the presence of bacterial substances is required. In conclusion, we have demonstrated that the larval epidermis of *Drosophila* is not only a passive barrier against foreign invaders, but has the capacity to induce the synthesis of antimicrobial peptides in response to infected wounds.

The *imd* signalling pathway is involved in local *CecA1* expression in the epidermis of embryos and larvae, but not in the melanization of the wounded cuticle

Genetic analysis of the regulation of *Drosophila* antimicrobial peptide genes implies that several signalling pathways for activation exist (reviewed in Engström, 1999). In the fly mutant *imd*, systemic expression of the genes for the antimicrobial peptides diptericin, drosocin and cecropin was severely impaired upon bacterial challenge, while inducible expression of the gene for the antifungal peptide drosomycin was unaffected (Lemaitre *et al.*, 1995; Corbo and Levine, 1996). We tested whether the *imd* pathway was involved in the activation of *CecA1* in epidermis by analysing the expression of A10 *CecA1–lacZ* in homozygous *imd* embryos and larvae. The β-gal expression in the epidermis of immuno-challenged *imd* embryos was reduced strikingly. To reveal the differences in β-gal staining between *imd* and wild-type embryos the incubation time with X-gal was reduced to 1 h. Under these conditions, no β-gal staining was observed in the

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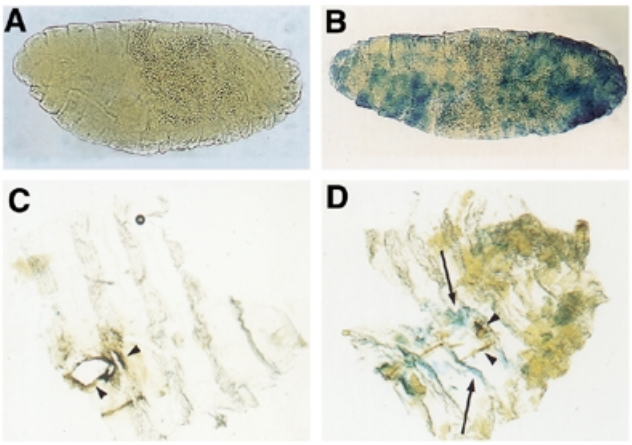

Fig. 4. The *imd* gene is required for epidermal *CecA1* expression, but not for melanization. (A–B) β-gal staining of A10 *CecA1–lacZ* in (**A**) homozygous *imd* and in (**B**) wild-type embryos shown in lateral views, anterior to the left, dorsal up. (C–D) Local expression of A10 *CecA1–lacZ* in the cuticle of wild-type and *imd* mutant larvae. (**C**) Dissected cuticle from *imd* larvae did not reveal any reporter gene staining but clear melanization (brown, arrowheads) in the wounded area. Cuticle from control A10 larvae (**D**) displayed both reporter gene staining (blue, arrows) and melanization (arrowheads).

epidermis or in any other tissues in the *imd*⁻ mutant background (Figure 4A), while 80% of the A10 embryos revealed substantial staining (Figure 4B). Longer incubation time shows a low level of β-gal staining in 44% of the *imd*⁻ embryos. This is in correspondence with previous data showing that *CecA* gene expression is reduced but not abolished in *imd* mutant larvae and adults (Lemaitre *et al.*, 1995; Corbo and Levine, 1996; Lemaitre *et al.*, 1996).

We investigated further the role of the *imd* pathway for the local expression of *CecA1* in the larval cuticle. Wounding of the larval cuticle and incubation with *E. cloacae* did not promote any β-gal staining in the epidermis of the *imd*⁻ mutant larvae (Figure 4C), in contrast to wild-type larvae (Figure 4D), demonstrating that the *imd*⁻ gene is crucial for the activation of a local immune response in the epidermis of larvae. Most interestingly, the formation of melanin in the wounded area of the abraded cuticle occurs in both the *imd* mutant and in the wild type (Figure 4C and D). This indicates that the signalling pathway(s) leading to activation of the pro-phenoloxidase cascade, resulting in melanin formation, is independent of the *imd* pathway. Taken together, our data show that the *imd* gene product is an important component of signal-induced, local *CecA1* expression in the epidermis of both embryos and larvae.

DISCUSSION

We propose the following model to explain the transition of *CecA1* expression in epidermal tissues of the embryo to the larval fat body. During embryogenesis, *CecA1* is induced in the epidermis as a result of direct contact between microbial substances in the privitelline fluid and epidermal cells. (Önfelt Tingvall *et al.*, 2001). When the cuticle is formed during late embryogenesis, this direct contact is broken, and the presence of bacteria or LPS in the larval haemocoel does not promote *CecA1* expression in the larval epidermis. During the larval stages, *CecA1* expression is restricted to wounded areas of the epidermis, suggesting that either the cuticle needs to be removed to allow direct contact between microbial substances and the epithelial cell layer, or that the wounding is an important signal *per se*. Our conclusion is therefore, that the epidermal cells are immuno-competent from the embryonic stage onwards. The fat body, on the other hand, seems to undergo maturation during the first larval instar. Expression of *CecA1* could be induced in the fat body of all three larval instars, but not in embryos. Injecting live bacteria into embryos and aging them into larvae showed that a maturation in the immuno-competence of the fat body occurs during first larval instar.

We were able to restore *CecA1*-driven reporter gene expression in the larval epidermis by inflicting infected wounds in all three larval instars. In addition to the cuticle being a physical barrier it is possible that the larval epidermis at the stage of hatching normally contains cecropins, since a low level of constitutive *CecA1* expression is observed in the epidermis of late stage embryos (Önfelt Tingvall *et al.*, 2001). It was shown recently that some of the antimicrobial peptides have a half-life of 2–3 weeks in *Drosophila* adults (Uttenweiler-Joseph *et al.*, 1998), suggesting that a low level of expression may also suffice to load the epidermis layer with substantial amounts of antimicrobial peptides. In addition, it was demonstrated recently that *Drosophila* cecropins are not only active against Gram-negative and Gram-positive bacteria (Samakovlis *et al.*, 1990), but also possess strong antifungal activity (Ekengren and Hultmark, 1999) making them powerful weapons against most classes of microorganisms.

Genetic evidence has provided a model in which at least two different pathways are involved in the activation of antimicrobial peptides, the *Toll* pathway and the *imd* pathway, both suggested to activate members of the *Drosophila* Rel family (Lemaitre *et al.*, 1997; Engström, 1999). Although the systemic expression of the peptide drosomycin was dependent on the *Toll* pathway and the Rel protein Dif, the local expression of drosomycin in tracheal epithelium was independent of the same pathway (Ferrandon *et al.*, 1998). The present study demonstrates that the *imd* gene seems to play an important role in the local expression of cecropin. In *imd* mutants, the expression of *CecA1* was impaired dramatically in the epidermis of embryos, and was undetectable in the epidermis underlying the cuticle of wounded larvae. Interestingly, the pro-phenoloxidase cascade was activated by injury in both wild-type and *imd* larvae, showing that the processes of antimicrobial defence and melanization can be uncoupled. In addition, the inducible, local expression of *CecA1* in infected wounds indicates that the insect epidermis is not only a passive barrier against infection, but plays an active role in the innate defence against microorganisms.

In this study, we demonstrate that antimicrobial cecropins are expressed locally in infected wounds in the epidermis of *Drosophila* larvae, and that this response is amenable to genetic dissection in mutants of the fly's immune response. This has a strong potential to become valuable for the understanding of innate and epithelial immunity in higher organisms, including mammals.

METHODS

Flies. Transgenic fly strains, with A10 (A10–1, A10–3 and A10–4) reporter constructs have been described previously (Roos *et al.*, 1998). Flies carrying a recombination of A10 to the X-chromosome, the *b pr imd* stock and the A10; *imd* stock were generously provided by Bruno Lemaitre. Flies were reared on standard cornmeal agar medium in vials and flasks in humid culture rooms at 25 or 18°C.

Injections and staining of embryos. Embryos were collected on apple juice agar plates for 3 h at 25°C and aged to the desired stage at 18°C. Dechorionated embryos were mounted on a coverslip with double-stick tape and covered with 10 S voltalef oil (Elf Autochem). Injections were with LPS (10 µg/ml) or with a 1:10 dilution of log phase *E. cloacae*, β*12* or with sterile PBS. After LPS injection the embryos were kept at 25°C for 3–4 h, fixed in glutaraldehyde-saturated heptane, devitellinized by hand, and stained using 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) for 15–18 h or as indicated.

Injection and wounding of larvae. Larvae were injected with LPS (10 µg/ml) on the dorsal side using fine glass capillaries. Injected larvae were kept on humid filter paper at 25°C for 3–4 h before fixation and staining. Abrasion of larvae was done by holding a larva with forceps and scratching the cuticle against sandpaper or a ragged, sharp preparation needle. Larvae with such wounds in the epidermis were inoculated with *E. cloacae*, 1:10 dilution of an overnight culture in Ringer solution. The abraded larvae were incubated for 18 or 24 h at 18°C, fixed in 1% glutaraldehyde in PBS pH 7.3 for 20 min and stained as described (Roos *et al.*, 1998).

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