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The *Drosophila* Toll-9 activates a constitutive antimicrobial defense

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The Toll family of transmembrane proteins participates in signaling infection during the innate immune response. We analyzed the nine *Drosophila* Toll proteins and found that wild-type Toll-9 behaves similar to gain-of-function Toll-1. Toll-9 activates strongly the expression of *drosomycin*, and utilizes similar signaling components to Toll-1 in activating the antifungal gene. The predicted protein sequence of Toll-9 contains a tyrosine residue in place of a conserved cysteine, and this residue switch is critical for the high activity of Toll-9. The *Toll-9* gene is expressed in adult and larval stages prior to microbial challenge, and the expression correlates with the high constitutive level of *drosomycin* mRNA in the animals. The results suggest that Toll-9 is a constitutively active protein, and implies its novel function in protecting the host by maintaining a substantial level of antimicrobial gene products to ward off the continuous challenge of microorganisms.

INTRODUCTION

Insects possess a self-defense system equivalent to the innate immune system of humans (Imler and Hoffmann, 2001; Kimbrell and Beutler, 2001). Similar to the acquired immune response, an important aspect of the innate immune system is the recognition of pathogens and the stimulation of downstream events. A central component for recognition is the so-called pattern recognition receptors (Medzhitov and Janeway, 2000; Takeuchi and Akira, 2001). These receptors function to distinguish molecules that are common within a group of pathogens, rather than to recognize specific antigens present in individual microorganisms.

Several lines of evidence suggest that the Toll family of transmembrane proteins may serve as parts of pattern recognition receptors (Medzhitov and Janeway, 2000; Imler and Hoffmann, 2001; Kimbrell and Beutler, 2001; Takeuchi and Akira, 2001). The first Toll protein was identified in *Drosophila*, where Toll functions to transmit extracellular signals into the cytoplasm to control dorsal–ventral patterning in the early embryo. Toll and many components in this signaling pathway also mediate antifungal response at post-embryonic stages. The identification of Toll-like receptors (TLRs) in mammals further demonstrates that the Toll family play important roles in innate immunity (Medzhitov and Janeway, 2000; Takeuchi and Akira, 2001). TLR proteins including TLR2, TLR4, TLR5, TLR6 and TLR9 are essential in mediating the effects of Gram-positive and -negative bacterial, mycobacterial and fungal products, as well as bacterial DNA (Medzhitov and Janeway, 2000; Takeuchi and Akira, 2001).

The *Drosophila* genome has nine genes that encode Toll-related proteins (Tauszig *et al.*, 2000), while 10 TLRs have been identified in humans (Medzhitov and Janeway, 2000; Takeuchi and Akira, 2001). However, it is not clear whether the detailed pathogen recognition mechanism is conserved in *Drosophila* and humans (Imler and Hoffmann, 2001). Antimicrobial response in *Drosophila* utilizes two largely separable pathways. The antifungal response depends on the Toll/Cactus signaling pathway (Imler and Hoffmann, 2001; Kimbrell and Beutler, 2001), and the antibacterial response pathway is composed of Imd, TAK1, Dredd, IKK and Relish, with the receptor yet to be identified (Hedengren *et al.*, 1999; Elrod-Erickson *et al.*, 2000; Leulier *et al.*, 2000; Rutschmann *et al.*, 2000b; Silverman *et al.*, 2000; Vidal *et al.*, 2001). To better understand the functions of *Drosophila* Toll receptors, we tested whether full-length Toll proteins could regulate the expression of immunity genes. We found that Toll-9 can activate constitutively the expression of the antifungal gene *drosomycin*, similar to those of gain-of-function mutants of Toll-1.

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RESULTS

Toll proteins in the activation of antimicrobial peptide genes

The prototypic Toll and 18Wheeler, called here Toll-1 and Toll-2, respectively, have been well characterized (Imler and Hoffmann, 2001; Kimbrell and Beutler, 2001). *Toll-3* to *Toll-9* are predicted genes based on the *Drosophila* genome sequence (Tauszig *et al.*, 2000; www.fruitfly.org). In the experiments presented in this report, we used Toll constructs that contain both extracellular and cytoplasmic domains to ask whether any of them could augment the lipopolysaccharide (LPS) response or activate antimicrobial peptide gene expression in S2 culture cells. In transient transfection assays, we found that Toll-3 and Toll-5 caused a 3- to 7-fold increase in *cecropin* and *defensin* promoter-reporter gene activities (Figure 1A and B). Toll-9 activated both *cecropin* and *defensin* reporters 10- to 20-fold higher than the vector. However, none of these proteins could enhance the LPS response significantly above that of the vector alone. We also tested whether transfection of combinations of Toll proteins, particularly Toll-3, -5 and -9, would have a synergistic effect. We observed no augmentation of the LPS response (data not shown).

When assayed for the *drosomycin* reporter gene, we found that Toll-5 and Toll-9 could highly activate the expression (Figure 1C). Toll-5 activated the reporter by ~30-fold, which is similar to that reported using a chimeric Toll-5 construct (Tauszig *et al.*, 2000). Toll-9 showed even better stimulation, and increased the luciferase activity by >150-fold. The activating capability of Toll-9 is similar to that of a gain-of-function allele of Toll-1, Toll^{10b} (Figure 1C). It has been clearly demonstrated in whole animals that Toll^{10b} activates constitutively the antifungal response, mostly through the dorsal–ventral signaling components and the NF-κB factor Dif (Lemaitre *et al.*, 1996; Meng *et al.*, 1999; Rutschmann *et al.*, 2000a).

Stably transfected cell lines harboring individual *Toll* genes were also established to examine their ability to activate endogenous antimicrobial peptide genes. While some Toll proteins (e.g. Toll-3) caused slightly increased expression of the antibacterial peptide genes, none of the stably transfected Toll cell lines had enhanced LPS response when compared to that of parental cells (Figure 2). On the other hand, we observed significant *drosomycin* gene activation from the expression of Toll-9. Quantification of the signals showed a 5-fold increase in the expression of the antifungal gene in the cells. Therefore, both transient and stable transfection demonstrated that Toll-9 is a modest activator of antibacterial peptide genes and a strong activator of an antifungal peptide gene.

Toll-9 is a constitutively active allele

Based on the observation that Toll-9 has an activity similar to Toll^{10b}, we surmized that the naturally existing Toll-9 could be a constitutively active protein. Previous genetic screens have isolated a class of three strong gain-of-function mutants of Toll-1. Each of these three mutants has one of the cysteine residues, located just outside the transmembrane domain, changed to tyrosine (Schneider *et al.*, 1991). We aligned the *Drosophila* Toll protein sequences in this region and found that all except Toll-9

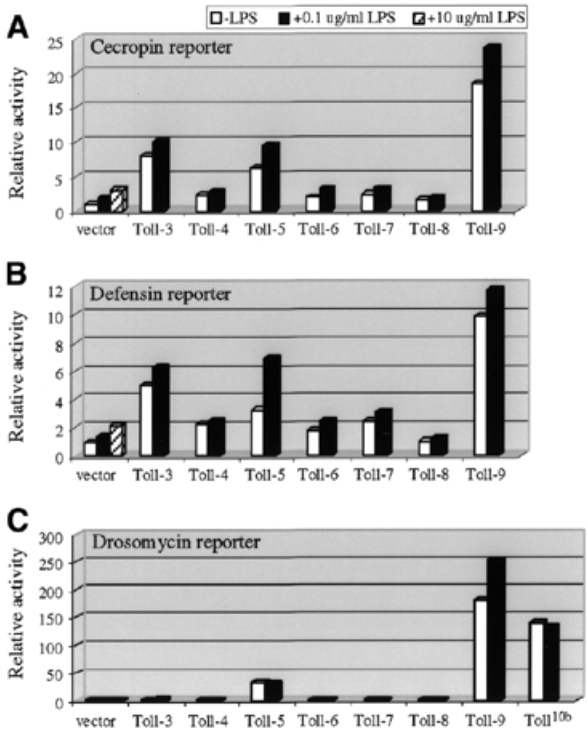

Fig. 1. Activation of antimicrobial peptide reporter genes by Toll proteins. Transient transfections of the Actin5C promoter-driven Toll and the luciferase reporters under the control of *cecropin* (**A**), *defensin* (**B**) or *drosomycin* (**C**) promoter were performed. The *copia–lacZ* plasmid was included as a transfection control. Three hours prior to harvest, LPS (L2637; Sigma) was added to the samples as indicated. The luciferase activity was normalized with the β-galactosidase activity. Each normalized luciferase activity was divided by that of the vector alone without LPS treatment. The results obtained were plotted as relative activity. The results are the average of three independent transfection experiments. Expression of Toll-3 and Toll-5 have modest effects on *cecropin* and *defensin-luciferase* expression, whereas Toll-9 shows higher activity. LPS treatment does not have a synergistic effect on the reporter activity when compared to the vectors alone. Toll-5 and Toll-9 strongly activate *drosomycin* reporter expression.

have strict conservation of these cysteine residues (Figure 3A). In particular, the N-terminal CXC motif is clearly absent from the predicted Toll-9 protein. Due to the sequence divergence of Toll-9, there are different possible alignments in this cysteine-rich region. One of the possible alignments, as shown in Figure 3A, illustrates a tyrosine residue (656) in place of a conserved cysteine. The Toll^{1} gain-of-function mutant allele of Toll-1 has an equivalent change of cysteine to tyrosine (Schneider *et al.*, 1991). Therefore, it is possible that the high activity of Toll-9 is due to the loss of the cysteine or the presence of the tyrosine residue.

We first confirmed Toll-9 clones from different strains by sequencing, and the tyrosine codon was identical to that of the Berkeley *Drosophila* Genome Project published sequence. Therefore, it is unlikely to be a sequencing error or a polymorphism only present in the strain used for genome sequencing. We then introduced a nucleotide substitution to change the amino acid to a cysteine (the construct is named

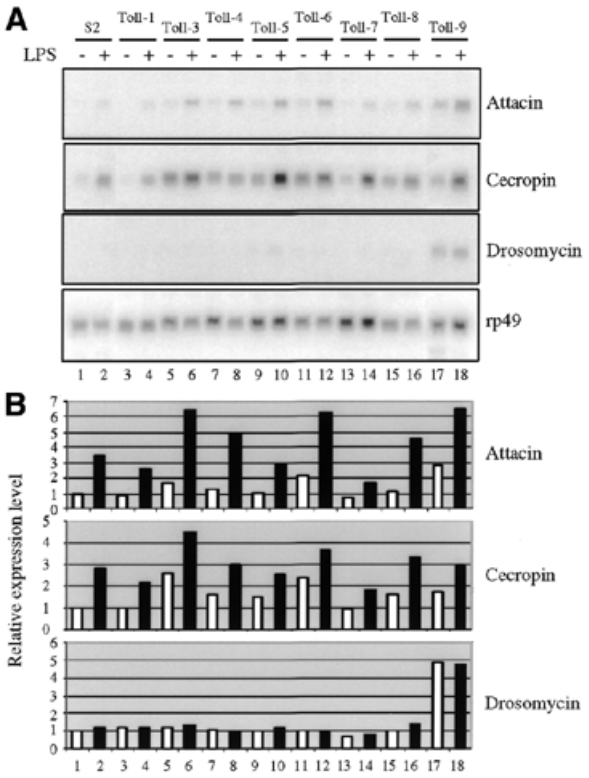

Fig. 2. Stable transfection of Toll-9 activates endogenous *drosomycin* expression. The Actin5C–Toll constructs were transfected to obtain stable cell lines. RNA expression of endogenous immunity genes was analyzed and the results are shown in (**A**). They are representative blots of two independent northern experiments. The hybridized blots were quantified using a phosphoimager. The expression of the immunity genes was normalized with that of the ribosomal protein 49 gene. The average result of two independent experiments was plotted and is shown in (**B**). Expression of some Toll proteins, such as Toll-3, can lead to a slight increase in *attacin* and *cecropin* gene expression. The addition of LPS to the stably transfected cell lines has little effect when compared with the parental S2 cells. The stable expression of Toll-9, in contrast, clearly increases the endogenous *drosomycin* gene expression.

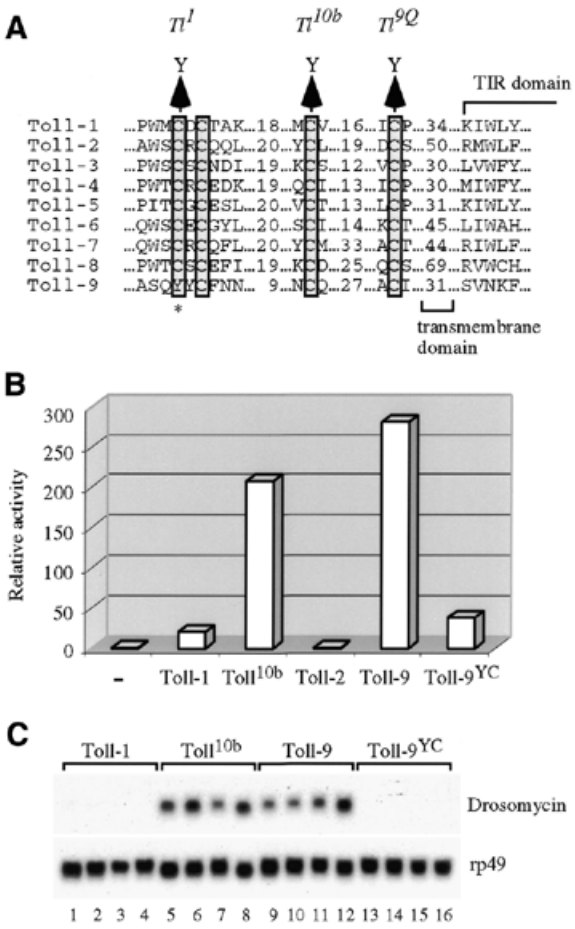

Fig. 3. Sequence alignment and mutagenesis reveal a constitutive activating potential of Toll-9. (**A**) Sequence alignment of Toll-1 to -9 in the cysteine-rich region. The four conserved cysteine residues proximal to the transmembrane domain are boxed. Toll-9 has four cysteine residues in this region but they are scattered and clearly do not form a CXC motif. One of the possible alignments as shown in (A) indicates a tyrosine residue in place of the first conserved cysteine residue. This change is similar to an induced gain-of-function mutation in Toll-1, named Toll[1]. (**B**) Changing the tyrosine to cysteine causes loss of activity of Toll-9. The Toll-9^{YC} mutant activity was examined in a transient transfection assay using the *drosomycin* reporter. The Y to C mutation allows Toll-9 to behave similar to Toll-1 and has much lower activity than that of Toll^{10b} and wild-type Toll-9. (**C**) Stably transfected cells containing Toll-1, Toll^{10b}, Toll-9 or Toll-9^{YC} were established in parallel and the endogenous *drosomycin* expression examined. Four individual lines of each construct exhibit the same results, such that Toll^{10b} and Toll-9 activates the endogenous target gene efficiently but Toll-1 and Toll-9^{YC} do not have such ability. The lower panel is the rp49 loading control.

Toll-9^{YC}) and examined the activity in a transfection assay. The result showed that this mutation made Toll-9 inactive in regulating all three luciferase reporters (Figure 3B for *drosomycin*; data not shown for *defensin* and *cecropin*). The tyrosine to cysteine substitution lowered the activity of Toll-9 to resemble that of Toll-1, rather than that of Toll^{10b}.

To further demonstrate the importance of the tyrosine/cysteine change in Toll-9, we established cell lines stably transfected with various Toll constructs including Toll-9^{YC}. Four independent transfection of each construct were analyzed for the expression of the endogenous *drosomycin* gene (Figure 3C). While the Toll-1 containing cells exhibited no activation of *drosomycin*, all four lines of Toll^{10b} showed obvious activation. The Toll-9 containing cells established in parallel had expression levels of *drosomycin* similar to those of the Toll^{10b} lines. All four Toll-9^{YC} lines had no elevated expression of the target gene, similar to that of Toll-1. These results strongly support the idea that Toll-9 is a constitutively active receptor protein.

Signaling components in the Toll-9 pathway

In both dorsal–ventral development and antifungal response, activated Toll-1 recruits Tube and Pelle to initiate signaling (Imler and Hoffmann, 2001; Kimbrell and Beutler, 2001). Both Tube and Pelle contain death domains, and Pelle is a kinase.

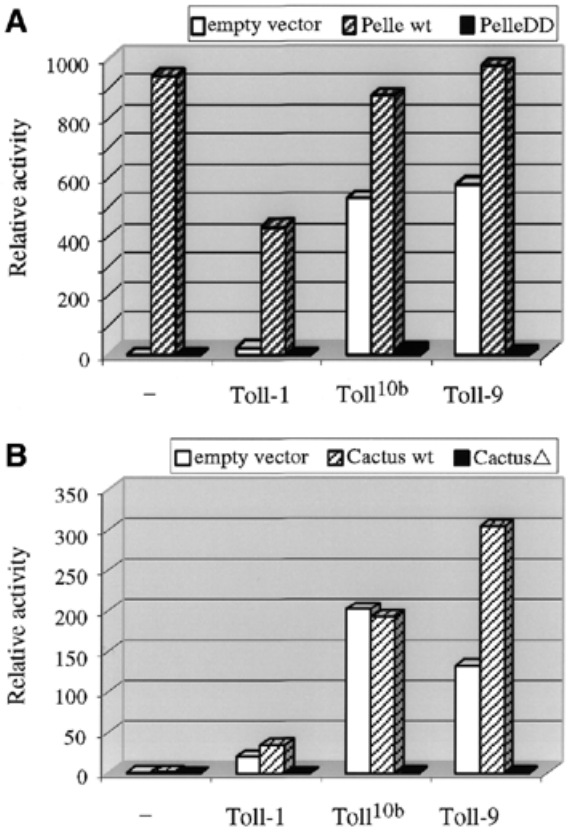

Fig. 4. Toll-9 activation is mediated by dorsal–ventral signaling components. (**A**) The Actin5C vector alone, Actin5C–Pelle or Actin5C–Pelle^{DD} were co-transfected with different Toll expression plasmids. Wild-type (wt) Pelle activates strongly the *drosomycin-luciferase* reporter. Pelle^{DD} cannot activate the reporter. In the presence of wt Pelle, the activation by the Toll proteins remain high. However, in the presence of Pelle^{DD}, activation by the Toll constructs is abolished. (**B**) Similar experiments were performed using the Actin5C–Cactus and Actin5C–Cactus$\Delta^{125}\Delta^{PEST}$ mutants. The results reveal that while wt Cactus has little effect on the activation of *drosomycin*, the Cactus$\Delta^{125}\Delta^{PEST}$ (CactusΔ) mutant blocks the signaling.

Recruitment of Pelle somehow leads to degradation of the inhibitor Cactus and release of the transcription factors, Dorsal and Dif. We examined whether Toll-9 employs the same signaling components to activate *drosomycin* expression. A construct for Pelle containing only the death domain (Pelle^{DD}), but lacking the kinase domain, was generated. This mutated Pelle protein should function as dominant negative by binding to the death domain of Tube but cannot phosphorylate downstream substrates (Xiao *et al.*, 1999). Transfection of wild-type Pelle activated the reporter gene efficiently, consistent with an important role of the protein in antifungal response (Figure 4). As expected, Pelle^{DD} did not activate the reporter. On the other hand, the Pelle^{DD} construct inhibited all the Toll-1-, Toll^{10b}- and Toll-9-mediated *drosomycin* reporter activities.

Cactus uses its ankyrin repeats to bind to the Rel homology domains of Dif and Dorsal (Tatei and Levine, 1995). The Cactus protein degradation is regulated both by signal dependent and signal independent mechanisms, through the N-terminal serine residues and C-terminal PEST sequence, respectively (Belvin and Anderson, 1996). Therefore, we utilized a construct Cactus$\Delta^{125}\Delta^{PEST}$ that contained only the ankyrin repeats. This mutant Cactus should stably bind to and inhibit Dif and Dorsal, even when the signaling pathway is stimulated. Co-transfection of wild-type Cactus did not lead to significant changes in the activation of *drosomycin* by Toll-1, Toll^{10b} and Toll-9. In contrast, the Cactus$\Delta^{125}\Delta^{PEST}$ construct abolished all these Toll signaling activities (Figure 4B). Therefore, Cactus and Pelle, and probably the binding partners Dif and Tube, are likely signaling components that mediate the activation of *drosomycin* by Toll-9.

Expression of Toll-9 in whole animals and during infection

If Toll-9 has a constitutive function in activating antimicrobial defense, there should be detectable expression at the larval and adult stages, when the animals are free moving in the environment and need continuous protection. Thus, we isolated RNA from wild-type flies and wandering third instar larvae to analyze the *Toll-9* gene expression. There are two detectable *Toll-9* transcripts on the northern blot, with approximate sizes 3 and 5 kb (Figure 5A). The 3 kb transcript is present in both larval and adult stages, and did not seem to change upon bacterial challenge. The 5 kb transcript was detected only in larvae, and also had no significant change on infection. The two bands on the northern blot may represent the mature and the non-spliced transcripts, based on the annotated sequence prediction (CG5528, Gadfly; Berkeley *Drosophila* Genome Project). Other possible explanations include alternative promoter usage and alternative splicing. Regardless of these, there is constitutive expression of *Toll-9* at relevant stages. This constitutive expression of Toll-9 correlates with an easily detectable level of *drosomycin* mRNA at the same stages even in the absence of microbial challenge (Figure 5B), as observed previously (Lemaitre *et al.*, 1996; Meng *et al.*, 1999). The exposure time for the *drosomycin* blot shown in Figure 5B was 1.5 h, compared with 3 days for that of *Toll-9*, highlighting the relatively high level of constitutive *drosomycin* expression. These results support the idea that Toll-9 may function to maintain a constant production of antimicrobial substances in whole animals.

DISCUSSION

We have demonstrated that the *Drosophila* Toll-9 protein is a potent regulator of the antifungal gene *drosomycin*. The results corroborate with a biological function of continuous stimulation of antimicrobial peptide production by Toll-9. As illustrated in Figure 5, *drosomycin* has both constitutive and inducible expression in whole animals (Lemaitre *et al.*, 1996; Ferrandon *et al.*, 1998; Meng *et al.*, 1999). Toll-1 is required for the inducible expression of *drosomycin*, but in Toll-1 loss-of-function mutants the constitutive expression persisted (Lemaitre *et al.*, 1996; Ferrandon *et al.*, 1998). Therefore, Toll-9 may be a key factor in maintaining the constitutive expression in whole animals.

Toll proteins have been demonstrated to function as parts of pathogen recognition receptors to signal infection (Imler and Hoffmann, 2001; Takeuchi and Akira, 2001). Our results suggest

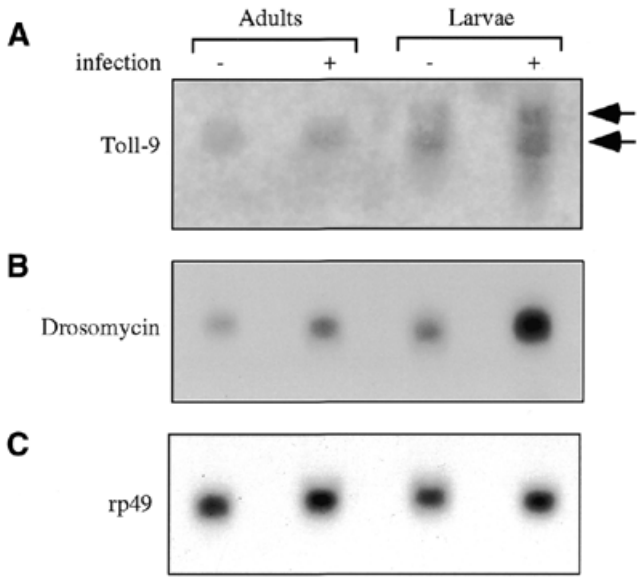

Fig. 5. Expression of *Toll-9* correlates with constitutive *drosomycin* in larvae and adults. Poly(A) mRNA was isolated from uninjected and injected (left to recover for 3 h) wild-type animals at larval and adult stages. The samples were analyzed by northern blotting and hybridization. (**A**) The autoradiograph is shown. The upper band resulting from *Toll-9* hybridization is ~5 kb and the lower band is ~3 kb. The larval and adult mRNA both contain the 3 kb band and the signal does not change significantly after *Escherichia coli* injection. The upper band is only detected in larvae, and also has no significant change upon injection. (**B**) The same blot was hybridized with a *drosomycin* probe, revealing an ~0.5 kb band. The *drosomycin* mRNA can be detected easily in uninduced animals and have higher levels in induced animals (**C**). The blot was re-hybridized with rp49 as the loading control.

a novel function for Toll proteins: some of them may provide constant protection prior to systemic infection. The digestive tracts and epidermis of complex organisms are in constant contact with microbes. The epithelial tissues besides serving as a passive barrier, also provide active protection against infection by secreting antimicrobial molecules (Ferrandon *et al.*, 1998; Tzou *et al.*, 2000). Therefore, proteins such as Toll-9 would function as a constitutive stimulator of the regulatory pathways to achieve this. The *in vivo* requirement of Toll-9 in the self defense process can be examined once mutant alleles of *Toll-9* are available. In addition, we have tested *pelle*^{385} loss-of-function mutant flies but found no change in the basal expression level of *drosomycin* (data not shown). Further genetic experiments are required to examine whether null alleles of *pelle* may have a defect or whether other signaling components, such as the newly identified DMyD88 (Horng and Medzhitov, 2001), may provide redundant functions.

We have not been able to unveil a functional Toll receptor that could mediate the LPS response in *Drosophila*. A possible explanation is that hetero-multimerization of different Toll proteins is required to form a functional receptor. Alternatively, it is possible that the Toll protein that functions in LPS recognition is not among the nine different genes we tested. It is also noteworthy that the mammalian LPS receptor complex includes at least three proteins: TLR4, CD14 and MD2 (da Silva Correia *et al.*, 2001). The *Drosophila* genome sequence has not revealed obvious CD14 and MD2 homologs so far. LPS recognition in insects may utilize different mechanism because of an independent evolutionary course.

While our experiments reveal a constitutive function of Toll-9, whether it can be stimulated by molecules derived from pathogens remains unknown. The Toll-9 protein contains the characteristic leucine-rich repeats in the extracellular domain and therefore may function similarly to other Toll proteins in pathogen recognition. Furthermore, since Toll-1 and Toll-9 both activate the antifungal defense and may share signaling intermediates, whether Toll-9 dimerizes with Toll-1 or with other Toll proteins to signal infection, is another interesting question that remains to be answered.

METHODS

Molecular cloning. The *Toll-1* and *Toll*^{10b} cDNA clones in the Actin5C vector have been described previously (Norris and Manley, 1996). The *Toll-2* to *Toll-9* clones were PCR amplified using wild-type genomic DNA as template, thus some clones contain intron sequences. The accession numbers of the *Toll* genes have been published previously (Tauszig *et al.*, 2000). The PCR products were first cloned into the Bluescript KS vector and sequenced to confirm the identity. The fragments were then subcloned into the pGem3–Actin5C vector for subsequent transfection assays.

To generate the *Actin5C–Pelle*^{DD} construct, the *Pelle* cDNA was digested with *Bsp*EI enzyme and the 700 bp fragment (from position –58 to +642, where +1 is the translation start site) was purified. The fragment was blunt-end cloned into the *Eco*RV site of the pGem3–Actin5C vector. The *Actin5C–Pelle* wild-type clone was as described previously (Norris and Manley, 1996). To generate *Actin5C–cactus*, a *Hin*dIII–*Not*I fragment from the *pNB40–cactus* cDNA was subcloned into the same sites of the Bluescript KS vector. A *Kpn*I–*Sac*I fragment was then excised and cloned into the same sites of the Actin5C vector. The *cactus*$\Delta^{125}\Delta^{PEST}$ cloning started with a *cactus* cDNA that has the first 125 amino acids deleted and has a stop codon in front of the PEST sequence. A *Hin*dIII–*Not*I fragment was excised and cloned into the Bluescript KS vector. Next, a *Kpn*I–*Sac*I fragment was similarly subcloned into the Actin5C vector.

Site-directed mutagenesis was used to generate the *Toll-9*^{YC} construct. A *Not*I–*Sal*I fragment of the Bluescript *Toll-9* clone was excised and subcloned into the same sites of the Bluescript KS vector. Mutagenesis was conducted using double-stranded plasmid and QuikChange mutagenesis kit from Stratagene. Two oligonucleotides were utilized to change codon 656 from TAT to TGT. The region was fully sequenced to confirm the targeted mutation and the lack of the other undesired mutation. *Actin5C–Toll-9*^{YC} was generated by swapping the *Not*I–*Sal*I fragment with that of the wild-type clone.

Cell culture and transfection assay. The *Drosophila* Schneider-2 and the various Toll-expressing cell lines were maintained at 25°C in Schneider's medium (Life Technologies) supplemented with 10% heat-treated fetal bovine serum (Hyclone), 100 U/ml of penicillin and 100 mg/ml of streptomycin sulfate. Transient transfections were performed as described in the protocol for the Lipofectin transient transfection (Life Technologies). The cells were transfected with 1 µg of the *Actin5C* constructs, along with

0.1 µg of *luciferase* reporter plasmids and 1 µg of *copia–lacZ* plasmids. The *cecropin-*, *defensin-* and *drosomycin*-luciferase reporters contained 0.8, 0.9 and 2.4 kb of upstream sequences, respectively. The transfected cells were grown for 48 h and harvested. The cells were lysed in 100 µl lysis buffer containing 1% Triton, 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM dithiothreitol. Ten microliters of the lysate were used for luciferase assays while 50 µl were used for β-galactosidase assay. Stable transfectants were generated as described previously (Han and Ip, 1999). The cells were assayed for RNA expression of the respective *Toll* genes to confirm the integration and expression (data not shown). The RNA preparation and northern blot analysis were performed as described previously (Han and Ip, 1999).

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