Critical evaluation of the role of the Toll-like receptor 18-Wheeler in the host defense of *Drosophila*

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Essential aspects of innate immune responses to microbial infections appear to be conserved between insects and mammals. In particular, in both groups, transmembrane receptors of the Toll superfamily play a crucial role in activating immune defenses. The *Drosophila* Toll family member 18-Wheeler had been proposed to sense Gram-negative infection and direct selective expression of peptides active against Gram-negative bacteria. Here we re-examine the role of 18-Wheeler and show that in adults it is dispensable for immune responses. In larvae, *18wheeler* is required for normal fat body development, and in mutant larvae induction of all antimicrobial peptide genes, and not only of those directed against Gram-negative bacteria, is compromised. 18-Wheeler does not qualify as a pattern recognition receptor of Gram-negative bacteria.

INTRODUCTION

The host defense of *Drosophila* shares significant similarities with mammalian innate immunity (Hoffmann *et al.*, 1999). A hallmark of the *Drosophila* immune reaction is the infection-induced transcription in the fat body of a battery of genes encoding small cationic peptides with potent activities against fungi and/or bacteria (Bulet *et al.*, 1999). In the mid-1990s, it was proposed that distinct signaling pathways control the resistance against fungi and bacteria: Toll, a transmembrane receptor protein, and several additional genes of the dorsoventral regulatory cassette of *Drosophila* regulate the antifungal response (Lemaitre *et al.*, 1996), whereas resistance to Gram-negative bacteria depends on the *imd* gene (for immune deficiency). This gene has since been shown to act upstream of a regulatory pathway independent of Toll (for a review, see Hoffmann and Reichhart, 2002). The discovery of the role of Toll in the host defense of *Drosophila* was shortly followed by the cloning of a Toll-like receptor (TLR) in mammalian cells and the demonstration

of its role in innate immunity (Medzhitov *et al.*, 1997). To date, 10 mammalian TLRs have been reported (Takeuchi and Akira, 2001).

Drosophila expresses, in addition to Toll, eight genes coding for transmembrane receptors with significant similarities to Toll (Tauszig *et al.*, 2000). One of these, *18-Wheeler* (*18w*), was shown to serve, like Toll, as an adhesion molecule during various processes of morphogenesis (Eldon *et al.*, 1994). In 1997, loss-of-function mutant *18w* larvae were reported to exhibit severely reduced inducibility of the *attacin* gene in response to Gram-negative infection (Williams *et al.*, 1997). Attacin is considered to be predominantly active against Gram-negative bacteria and the report raised the interesting prospect that the Toll-like 18-Wheeler sensed Gram-negative infection and signaled to express an anti-Gram-negative peptide. This situation appeared to parallel that observed with Toll, which responds to fungal infection by directing expression of the antifungal peptide drosomycin. From these studies, the attractive paradigm emerged that distinct Toll receptors discriminate between different groups of microorganisms and control the selective expression of antimicrobial peptides with activity spectra directed against the corresponding invading microorganisms. This paradigm was indirectly supported by the accumulating evidence that mammalian TLRs are activated by distinct microbial patterns, e.g. TLR2 by peptidoglycan, TLR4 by lipopolysaccharide, TLR5 by bacterial flagellin, etc. (for a review, see Imler and Hoffmann, 2001; Takeuchi and Akira, 2001).

The observation that the *Drosophila* genome encodes nine distinct transmembrane receptors of the Toll family raised the tantalizing prospect that this battery of Toll receptors can serve to discriminate between a plethora of microbial patterns. This laboratory has taken up this question (Tauszig *et al.*, 2000), which has also led to the re-evaluation here of the role of *18w* in

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Fig. 1. Immune inducibility of the antimicrobial peptides is not affected in *18w* mutant adult flies. **(A)** *Df(2R)017* is a deficiency that uncovers the *18w* locus. We have designed three primers in the genomic region of the *18w* gene. P1 and P2 flank the region deleted in the *18w*⁷⁻³⁵ P-element excision mutant. Primer P3 was designed inside the 7-35 deleted region. If the genomic region of the *18w* gene is intact, a 650 bp fragment (*) between P1 and P3 can be amplified. The distance between P1 and P2 does not permit DNA amplification in standard conditions. If the 7-35 deletion chromosome is present, a 1 kb fragment between primers P1 and P2 can be amplified (**). In WT and *Cyol/Df(2R)017* strains (lanes 1 and 2), a 650 bp fragment can be detected. In *Cyol/18w*⁷⁻³⁵ flies (lane 3), the 1 kb fragment appears in addition to the 650 bp fragment. Standard PCR reaction results show that the *18w*⁷⁻³⁵/*Df(2R)017* combination in adult flies (lane 4) is molecularly identical to the *18w*⁷⁻³⁵ homozygous condition in larvae (lane 5). In both genetic backgrounds, only the 1 kb fragment is present. Lane 6 represents a control reaction without DNA. Details for primer sequence and reaction conditions are described in Supplementary data. **(B)** Wild-type (WT) or mutant strains were subjected to a septic injury with a sharp needle previously dipped in *E. coli* as a Gram-negative bacterial strain (G-) [or *M. luteus* as a Gram-positive bacterial strain (G+)], total RNA was isolated and northern blot experiments were carried out. Probes used are indicated at the top of each graph. Results obtained by northern blotting were quantified and analyzed as described in Methods. Each bar represents the result for one experiment. The abbreviation *key1* is for the allele of the *Drosophila* IKK γ homologue, *kenny1* (Flybase, <http://flybase.bio.indiana.edu/>).

Fig. 2. AttacinC is present in *18w* flies. Comparison of MALDI-TOF mass spectra from hemolymph from a wild-type fly (WT 24h) and an *18w⁷⁻³⁵/Df* mutant fly (18W 24h) following a 24 h bacterial infection in the mass range from 2250 to 3150 in *m/z*. The mass spectra were recorded in a linear positive mode using the sandwich sample preparation described previously (Kusmann *et al.*, 1997). The peak at 3027 (*m/z*) corresponds to metchnikowin. The peak at 2972 (*m/z*) corresponds to the pro-domain of attacin C (2785 Da) carrying two post-translational modifications. The corresponding peaks are not detected in the unchallenged flies (WT C or 18W C).

the antimicrobial response. The data that we present provide different pictures for larvae and adults. A functional 18-Wheeler product appears to be required for the larval fat body to develop into a fully responsive immunocompetent tissue. Mutant larvae are unable to express wild-type levels of any of the antimicrobial peptide genes in response to immune challenge. In contrast, *18w* mutant adults have a wild-type response to bacterial challenge, and in particular express the *attacin* genes at wild-type levels. These data are not in favor of a selective role for the 18W receptor in sensing Gram-negative infection to mount an appropriate response.

RESULTS

The initial studies, which involved the *18w* gene as a receptor of Gram-negative sepsis, were based on the *18w⁷⁻³⁵* mutation (Eldon *et al.*, 1994). This *18w* allele had been generated by imprecise excision of a P element inserted 23 bp upstream of the transcription start site. The excision had removed a DNA fragment of ~2.2 kb, comprising 1.7 kb of N-terminal sequence of the *18w* open reading frame. In molecular terms, the *18w⁷⁻³⁵* allele generates an N-terminally truncated transcript, which results in expression of a protein lacking the extracellular and transmembrane domains of the wild-type receptor and effectively corresponding to an N-terminally truncated TIR (Toll-interleukin-1 receptor homology) domain. This protein is detected in the cytoplasm and is not localized to the cell membrane (Williams *et al.*, 1997). *18w⁷⁻³⁵* mutants develop up to the third larval instar, and after a prolonged duration of this stage (up to 4 days, see also Methods) die prior to pupariation. Very few escapers are occasionally observed, which reach adulthood.

To minimize potential interferences of the truncated TIR domain with other signaling pathways and to eliminate possible contributions of other mutations in the genetic background of the *18w⁷⁻³⁵* allele, we combined this mutation with a chromosomal deletion (FlyBase: the database of the *Drosophila* genome projects and community literature; <http://flybase.bio.indiana.edu/>), *Df(2R)017*, which uncovers the 56F6-9 locus to which *18w* maps (Eldon *et al.*, 1994) (see also Figure 1A). This combination produces viable adults (although in numbers significantly lower than the expected Mendelian ratios), indicating that the lethality of the *18w⁷⁻³⁵* larvae did not entirely result from the lack of a functional 18W product. In the following, we will address separately the possible roles of *18w* in the antimicrobial response in adults and in larvae.

18wheeler adults have a wild-type antimicrobial response

We have subjected *18w⁷⁻³⁵/Df(2R)017* flies to infection by the Gram-negative bacteria *Escherichia coli* and have observed, as illustrated in Figure 1B, that all antimicrobial peptide genes are expressed as in wild-type flies. In keeping with results obtained previously by this and other groups, the predominant reactants of this Gram-negative challenge were the *cecropin*, *dipteracin*, *defensin*, *metchnikowin*, *drosocin* and *attacin* genes. Induction of *drosomycin* was less marked. In contrast, infection with the Gram-positive bacteria *Micrococcus luteus* resulted in a stronger induction of *drosomycin*, but again the levels induced for the various peptide genes were not significantly different between *18w* mutants and wild type.

We were intrigued by a recent report suggesting that the *attacinC* gene, rather than the *attacinA* gene, was dependent on

an input from the 18W receptor (Hedengren *et al.*, 2000). This report was based on $18w^{7-35}$ homozygous adult escapers. In our hands, this mutation did not give enough adult flies to perform

northern blots and we therefore addressed the $18w^{7-35}/Df(2R)017$ hemizygous strains. Four northern blots performed on total RNA extracted from these flies did not show any difference between the inducibility of *attacinC* in wild type and mutants. AttacinC was recently shown by Bulet and colleagues in this laboratory to give rise to a cleaved Pro-domain, which is post-translationally modified and stable (P. Bulet *et al.*, unpublished results). This Pro-domain form has a molecular weight of 2972 Da and is easily detected in hemolymph samples from single flies by MALDI-TOF mass spectrometry. We have found that this molecule is present after Gram-negative infection in the hemolymph of wild-type and $18w^{7-35}/Df(2R)017$ adults (Figure 2). Interestingly, it is totally absent in mutants for the Imd pathway, but is present in *Dif* mutants (data not shown).

Flies mutant for the Toll pathway are highly susceptible to fungal infection, while survival of Gram-negative infection is severely reduced in flies mutant for the Imd pathway. We have subjected $18w^{7-35}/Df(2R)017$ flies to fungal and Gram-negative infection, but did not observe any difference in survival rates compared with wild-type flies (data not shown).

18W has been suggested to regulate immune responses through infection-induced nuclear translocation of the Rel protein DIF. However, in mutants for *Dif*, *attacin* induction is not impaired (Manfrulli *et al.*, 1999; Meng *et al.*, 1999; Rutschmann *et al.*, 2000). As illustrated in Figure 3B, in adult fat body cells, bacteria-induced translocation of DIF in $18w^{7-35}/Df(2R)017$ mutants is similar to that observed in wild type. As described previously (Rutschmann *et al.*, 2000), we find that in adult fat body cells, DIF translocation following immune challenge is controlled by the Toll pathway, since in *spz* mutants DIF cannot be targeted to the nuclei (data not shown).

18wheeler larvae exhibit an abnormal development of their fat body, which affects its immune function

We next analyzed the induction of antimicrobial peptides in third instar larvae lacking a functional copy of the *18w* gene. We confirmed the earlier observation that in an $18w^{7-35}$ homozygous context, *attacin* induction is reduced compared with wild-type larvae (Williams *et al.*, 1997). However, as illustrated in Figure 4, this reduction affected all antimicrobial peptides, including the antifungal peptide Drosomycin. We obtained similar results with $18w^{7-35}/Df(2R)017$ hemizygous larvae.

Earlier studies from this laboratory had shown that the expression levels of the *dipteracin* gene in bacteria-challenged third instar

Fig. 3. *18w* is not required for nuclear uptake of the Rel protein DIF. DIF is shown in red and an antibody against histones (green) is used to visualize the nuclei. In left panels, co-localization following infection is shown in yellow. In all panels, the red and green channels are merged. Immune challenge was achieved by infection with a mixture of Gram-positive and Gram-negative bacteria. (A) Immunolocalization of DIF in the fat body of adult flies. DIF staining is not nuclear in the absence of immune challenge in both WT and *18w* mutant flies. There is no difference in the translocation of DIF between WT and *18w* flies after bacterial challenge. These data correlate with results showing that, in adult flies, DIF is under the control of the TOLL signaling pathway (Rutschmann *et al.*, 2000). (B) Immunolocalization of DIF in the fat body of *18w* mutant larvae. Although in these larvae antimicrobial peptide expression is impaired (see Figure 4), we observed no effect on DIF nuclear uptake. Note that, in SPZ-deficient larvae, DIF is not able to translocate to the nuclei of fat body cells after immune challenge.

Fig. 4. Immune inducibility of all the antimicrobial peptides is affected in *18w* larvae. WT and *18w* mutant larvae were staged during the third instar moult as early, 72 h after egg laying (AEL), mid (96 h AEL) and late (120 h AEL). As judged by independent developmental markers such as *Fbp1* (see Figure 5), the *18w*⁷⁻³⁵ homozygous larvae never reached the late stage and this is why in our experiments we categorized them as ‘mid-late’ (see also Supplementary data). Each group of larvae was subjected to immune challenge by *E. coli*. Total RNA was isolated 4 h after the bacterial challenge and northern blot experiments were carried out. These blots were quantified and analyzed as described in Methods. Probes used are indicated at the top of each graph.

larvae progressively increase during the instar (Reichhart *et al.*, 1992), and that this increase is ecdysone dependent (Meister and Richards, 1996). We were unable to monitor a similar increase

either in *18w*⁷⁻³⁵ homozygous larvae or in *18w*⁷⁻³⁵/*Df*(2R)017 hemizygous larvae. We reasoned that the low levels of inducibility of all antimicrobial peptide genes reflected developmental

Fig. 5. Fat body signaling is perturbed in *18w* mutant larvae. All times are given after egg laying (AEL) and for development at 25°C. (A) A time-related schematic representation of *Drosophila* larval development. Ecdysone-induced events are shown. The timing of each hormone wave is derived from previous studies (Andres *et al.*, 1993; Laval *et al.*, 1993; Huet *et al.*, 1995). The timing of *Fbp1* transcriptional activation is based on Brodu *et al.* (1999). (B) An ecdysone-responsive fragment of the promoter of the *Fbp1* gene linked to a *lacZ* reporter was used as a marker for time and tissue specificity of fat body signaling and larval maturation. In WT larvae, β -galactosidase activity staining is detected from 110 h AEL and promoter expression reaches a peak at 120 h AEL. However, in *18w* mutant larval fat body, *Fbp1* promoter expression cannot be detected.

retardation of the fat body, and addressed this question with a fat body-specific gene independent of the immune response, i.e. the larval fat body gene *Fbp1*. This gene encodes a receptor that mediates the uptake of hexamerins from the hemolymph (Brodu *et al.*, 1999). It is exclusively transcribed in response to ecdysone in fat body cells during the second half of the third larval instar (Laval *et al.*, 1993). We based our analysis on an *Fbp1* promoter-*lacZ* reporter transgene, which has an ecdysone-responsive enhancer conferring a fat body-specific ecdysteroid response (Laval *et al.*, 1993). We observed that in wild-type flies,

expression of the transgene starts in third instar larvae 108 h after egg laying and reaches a maximum 12 h later, at the very end of the third instar. In contrast, we detected no reporter activity in *18w⁷⁻³⁵* larvae at similar time points and noted only a weak expression in *18w⁷⁻³⁵/Df(2R)017* larvae. These data confirm that *18w*-deficient larvae do not have a normal fat body physiology (Figure 5B). The result that the *18w⁷⁻³⁵/Df* larvae do not express *fbp1*, but proceed to metamorphosis, suggests that ecdysone-dependent signaling is perturbed only in the larval fat body. However, the fact that ultimately the *18w⁷⁻³⁵/Df* adults are recovered in lower numbers than expected shows that the *18w⁷⁻³⁵* mutation influences viability. Taking in conjunction the facts that (i) expression of antimicrobial peptide genes is reduced, (ii) ecdysone-dependent fat body maturation is affected, (iii) antimicrobial peptide gene expression maturation is ecdysone dependent and (iv) DIF is normally targeted to the nuclei of fat body cells in *18w⁷⁻³⁵* and *18w⁷⁻³⁵/Df(2R)* larvae, we suggest that the reduction of peptide expression in these larvae is caused by a general problem of fat body maturation and is not due to impaired NF- κ B signaling.

DISCUSSION

The data presented in this study confirm the proposal made in 1997 that a functional *18wheeler* gene is required for larvae to mount a wild-type antimicrobial response (Williams *et al.*, 1997). Rather than restricting this effect to the induction of *attacin*, our data show that the inducibility of all antimicrobial peptides is affected. As exemplified by the expression of the unrelated *Fbp1* gene in mutant fat body, the data point to a general developmental delay of the fat body as a result of the mutation. A developmental role of 18W has already been postulated (Chiang and Beatchy, 1994; Eldon *et al.*, 1994). It is an open question whether the effects that we observe in the fat body directly result from the absence of functional copies of the 18W protein in *18w⁷⁻³⁵* homozygous or *18w⁷⁻³⁵/Df(2R)* hemizygous larvae. Indeed, the *18w⁷⁻³⁵*-encoded N-terminal truncated TIR domain could have a dominant-negative effect on a neighboring pathway by analogy with the effect of over-expression of the MyD88 TIR domain on interleukin-1 receptor-induced NF- κ B activation (Muzio *et al.*, 1997). This would account for the less drastically affected development in *18w⁷⁻³⁵/Df(2R)* hemizygous larvae, in which the concentration of the truncated protein is half that of *18w⁷⁻³⁵* homozygous larvae. The *18w⁷⁻³⁵* allele affects viability since *18w⁷⁻³⁵/Df(2R)* flies are recovered in higher numbers than *18w⁷⁻³⁵* homozygous flies. This could designate *18w⁷⁻³⁵* as a neomorphic mutation indirectly affecting the larval immune response. Alternatively, other unrelated mutations on the *18w⁷⁻³⁵* chromosome, which lie outside of the deficiency used, could contribute to this difference.

We conclude that there is no stringent evidence to consider the transmembrane receptor 18Wheeler as a sensor of Gram-negative infection. In fact, three independent studies point to a paramount role of the peptidoglycan recognition protein PGRP-LC in sensing Gram-negative infection (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002). Data on the other receptors of the Toll family indicate that they do not signal to any of the bacterial peptide genes, and that only Toll-5 and Toll-9 are able to signal to the antifungal peptide gene *drosomycin* (Tauszig *et al.*, 2000).

In the context of the valuable cross-talk between the studies on *Drosophila* host defense and on mammalian innate immunity, it is relevant that we realize that we may not fully equate Toll signaling with that of the TLRs. The data available in *Drosophila* indicate that Toll is not activated by direct (or even indirect) interaction with microbial ligands, but rather responds to the cleavage product of the cytokine Spaetzle (Levashina *et al.*, 1999). Cleavage of Spaetzle, in turn, depends on a proteolytic amplification cascade that is triggered when upstream proteins interact with microbial patterns in the hemolymph. This is in stark contrast to the situation described for the TLR family, whose members appear to directly interact with and discriminate between distinct microbial patterns. Furthermore, our data, taken in conjunction with those on other members of the Toll family, lead one to question whether Tolls are involved at all in the defense against Gram-negative sepsis in *Drosophila*.

METHODS

Fly strains and procedures. See Supplementary data.

RNA preparation and northern blot analysis. RNA was prepared and northern blots carried out as described previously (Lemaitre *et al.*, 1996). The ribosomal protein 49 (rp49) probe was used as a loading control.

Mass spectrometry. Mass spectrometry was performed as in Kussmann *et al.* (1997).

Immunocytochemistry. Flies or larvae immunized for 1 h or non-immunized were dissected and stained as described previously (Rutschmann *et al.*, 2000). Adult or larval fat body was incubated with a 1:500 dilution of a specific DIF rabbit antibody described previously (Rutschmann *et al.*, 2000). Visualization of the nuclei was achieved with the use of a specific histone mouse antibody (Chemicon International Inc.). Secondary antibodies used were an anti-rabbit coupled with the dye Alexa546 (red signal) and an anti-mouse coupled with the dye Alexa448 (green signal), both at a dilution of 1:500 (Molecular Probes). Histochemical detection of β -galactosidase activity was performed as previously described (Ashburner, 1989).

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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