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Hemolymph-Dependent and -Independent Responses in *Drosophila* Immune Tissue

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Abstract Insects possess an antimicrobial defense response that is similar to the mammalian innate immune response. The innate immune system is designed to recognize conserved components of microorganisms called pathogen-associated molecular patterns (PAMPs). How host receptors detect PAMPs and transmit the signals to mount the immune response is being elucidated. Using GFP-Dorsal, -Dif, and -Relish reporter proteins in ex vivo assays, we demonstrate that *Drosophila* fat bodies, a major immune tissue, have both hemolymph-dependent and -independent responses. Microbial preparations such as lipoteichoic acid (LTA) and peptidoglycan (PGN) can stimulate some responses from dissected and rinsed larval fat bodies. Therefore, at least some aspects of recognition can occur on fat body cell surfaces, bypassing the requirement of hemolymph. Our results also show that supernatants from bacterial cultures can stimulate the nuclear translocation of Dorsal in dissected fat bodies, but this stimulation is strictly hemolymph-dependent. Various biochemical assays suggest that the factors from bacterial supernatants that stimulate the hemolymph-dependent nuclear translocation are likely made up of proteins. We further show that Dorsal mutant larvae have much lower phenoloxidase activity, consistent with a more important role of Dorsal in innate immunity than previously shown. J. Cell. Biochem. 92: 849–863, 2004.

Key words: innate immunity; hemolymph; *Drosophila*; NF-kappaB; Dorsal

Insects protect themselves against microbial infection by mounting an efficient self-defense response. This insect antimicrobial response, which consists of humoral antimicrobial peptides and blood cells, is similar to the innate immune response of mammals [Hoffmann, 2003; Hultmark, 2003; Medzhitov and Biron, 2003]. During infection, insects produce many antimicrobial peptides, which function synergistically to neutralize the invading microorganisms. Without this signal-dependent synthesis of antimicrobial peptides insects succumb easily to microbial infection.

In *Drosophila*, the induced production of antimicrobial peptides depends on the Toll and the Immune deficiency (Imd) signaling pathways [Anderson, 2000; Imler and Hoffmann, 2002; Khush et al., 2002; Hultmark, 2003]. Toll is a transmembrane protein. The Toll signaling pathway controls the expression of a subset of antimicrobial peptide genes in response to fungal and Gram-positive bacterial infection. The signaling components in the *Drosophila* Toll pathway are highly homologous to those in the mammalian Toll-like receptor (TLR) pathways [Gilmore and Ip, 2003; Medzhitov and Biron, 2003]. Imd is an adaptor protein homologous to the tumor necrosis factor receptor (TNFR) interacting protein RIP. The Imd pathway governs the expression of another set of antimicrobial peptide genes in response to Gram-negative bacterial infection. The Imd

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pathway also bears many similarities to the TNFR signaling cascade in mammals [Georgel et al., 2001; Silverman and Maniatis, 2001].

Stimulation of Toll and Imd pathways leads to the nuclear transport of Dorsal, Dif, and Relish, the three regulatory proteins in *Drosophila* that belong to the NF-κB family of transcription factors [Anderson, 2000; Imler and Hoffmann, 2002; Khush et al., 2002; Hultmark, 2003]. Dif functions downstream of Toll to mediate the response to fungal and Gram-positive bacterial infections [Meng et al., 1999; Rutschmann et al., 2000, 2002]. Relish is critical for the Imd pathway to regulate the Gram-negative bacterial response [Hedengren et al., 1999]. Dorsal does not seem to be essential for the immune response in adult flies but appears to be redundant to Dif in larvae [Lemaitre et al., 1995b; Meng et al., 1999; Rutschmann et al., 2000]. These three *Drosophila* NF-κB proteins (thereinafter referred as to Dorsal, Dif, and Relish) can also form heterodimers in various assays [Gross et al., 1996; Han and Ip, 1999], but whether heterodimers are formed in vivo and how they are used during infection remains unclear.

How *Drosophila* tissues recognize pathogens and how the signal is relayed to cell surface receptors are important questions that are under investigation. Genetic evidence demonstrates that mammalian TLRs respond to pathogen-associated molecular patterns (PAMPs) by rather direct recognition [da Silva Correia et al., 2001; Medzhitov and Biron, 2003; Mizel et al., 2003; Smith et al., 2003; Takeda and Akira, 2003]. The *Drosophila* Toll, while critical for an antimicrobial response, probably requires the participation of more upstream proteins during stimulation by microorganisms. Fungal infection stimulates a serine protease cascade in which Necrotic acts as a protease inhibitor and Persephone acts as a serine protease [Levashina et al., 1999; Ligoxygakis et al., 2002b]. Gram-positive bacteria infection stimulates the peptidoglycan (PGN) recognition protein PGRP-SA [Michel et al., 2001; Leulier et al., 2003]. The stimulation of these various upstream components leads to proteolytic processing and activation of Spätzle, which likely binds to and activates Toll [Levashina et al., 1999; Weber et al., 2003]. Meanwhile, there are more than 10 different PGN recognition proteins in *Drosophila* [Werner et al., 2000]. One of these recognition proteins, PGRP-LC, acts upstream of Imd to mediate the Gram-negative bacterial response [Choe et al., 2002; Gottar et al., 2002; Leulier et al., 2003]. Even though many upstream components have been identified, further investigation is required to determine how they function to detect and signal infections.

Common methods used to study the *Drosophila* immune response involve septic injury and cell cultures. Septic injury may alter the physiology of the animals in addition to immune induction, and the use of cell cultures may introduce exogenous factors present in the growth media [Samakovlis et al., 1992; Han and Ip, 1999; Imler et al., 2000; Leulier et al., 2003; Werner et al., 2003]. We report here the development of fly strains bearing GFP-NF-κB reporter proteins to test whether *Drosophila* tissues can be used as an ex vivo system to study microbial stimulation. We demonstrate that several commercial preparations of pathogen-associated molecules can stimulate a response in dissected larval fat bodies. Moreover, rinsed fat bodies still exhibit NF-κB nuclear translocation revealing that at least some of the recognition complexes are associated with the fat body cell surface. Our assays also reveal that supernatants of bacteria cultures can induce Dorsal nuclear translocation in fat bodies, but this induction absolutely requires hemolymph. Various tests suggest that the bacterial supernatants contain protein factors that induce this hemolymph-dependent Dorsal response. Moreover, we show that Dorsal mutant larvae have much lower phenoloxidase activity and melanin deposition, demonstrating a more significant role of Dorsal in innate immunity. Together, our results suggest that the *Drosophila* innate immune system can use different mechanisms to recognize multiple components of microorganisms.

MATERIALS AND METHODS

Transgenic Lines

The cDNAs of Dif, dorsal, and Relish were used as templates for PCR amplification and the products were cloned into Bluescript vector containing the GFP coding sequence. The GFP sequence is at the 5′ end for the NF-κB clones. The fusion constructs were subcloned into the pUAST vector [Brand and Perrimon, 1993]. The

transformation constructs carrying the genes encoding the GFP-Dorsal, CFP-Dif, and YFP-Relish fusion proteins were injected into embryos as previously described [Brand and Perrimon, 1993]. Transgenic lines were obtained and maintained in a yeast/cornmeal/molasse/agar medium at 25°C. The lines were crossed to balancer flies and homozygous flies were used for the genetic crosses to the Gal4 driver. The Cg-Gal4 line, a collagen promoter-driven Gal-4 expression line, directs the expression of the transgenes in fat bodies and hemocytes of 3rd instar larvae [Asha et al., 2003]. OregonR, CantonS, or y w strains were used as controls.

Immune Challenge and Fat Body Dissection

Septic injury was performed by pricking 3rd instar larvae with a 33-gauge bevel point needle (Hamilton n° 90033) and soaking them in 50 μl of bacterial mixture on a concave microscope slide. The bacterial mixture was a mix of overnight cultures of *Micrococcus luteus*, *Erwinia carotovora carotovora* 15, *Escherichia coli* 055:B5 and *Enterobacter cloacae*. After septic injury, the larvae were transferred to a wet Whatman paper to allow recovery for 30–60 min. Dissection and isolation of fat body tissues were performed in staining dishes containing phosphate buffered saline (PBS, pH 7.2, 0.1 μm filtered, without calcium chloride, without magnesium chloride; Gibco, Invitrogen life technologies, Cat. No. 20012-027). The tissues were mounted onto glass slides for protein visualization under a fluorescence microscope.

Ex Vivo Fat Body Stimulation

Fat bodies were isolated from 3rd instar larvae in PBS and incubated with microbial preparations at 10 μg/ml concentration. Solutions of lipopolysaccharide (LPS) (*E. coli* O55:B5, Sigma L-4524, phenol extracted), lipoteichoic acid (LTA) (*B. subtilis*, Sigma L-3265), PGN (*S. aureus*, Fluka 77140), and laminarin (LMN), a soluble form of β-1,3-glucan (*L. digitata*, Sigma L-9634), were used in a final volume of 200 μl for 20 larvae. Incubation was for 60 min at room temperature. 0.001% hydrogen peroxide and 10 μM phorbol-12-myristate-13-acetate (PMA, Sigma P-8139) diluted in PBS were used for incubations with transgenic Dorsal fat bodies. For hemolymph-free experiments, fat bodies were thoroughly rinsed by three consecutive, gentle washes with sterile PBS prior to incubation in solutions that contained the specified compound. For the immunization transfer experiment isolated fat bodies from naïve transgenic GFP-Dorsal larvae were rinsed and mixed with a hemolymph extract from bacterial challenged wild type *Drosophila* larvae. This hemolymph extract was obtained from 30 wild type larvae previously induced by septic injury. The larvae were opened in 30 μl of PBS at 4°C, and the hemolymph/PBS mixture was collected and centrifuged at 5,000*g* for 30 min to eliminate fragmented tissues and hemocytes. The supernatant was then mixed with rinsed fat bodies expressing GFP-Dorsal. For bacterial induction experiments, individual bacteria cultures were grown in 2xYT medium for approximately 20 h until stationary phase was reached. Growth density was measured at OD590 and the cultures were all diluted to 0.3 OD with PBS. Equal volumes of the four individual bacterial cultures were then combined immediately before use as mixed bacteria. Mixed bacteria, individual bacteria, or supernatants were used by adding 80 μl of these cultures to 20 dissected larvae in 200 μl of PBS in a staining dish. The tissues were incubated for 60 min before examining the GFP transgenic proteins or fixing for antibody staining. For incubations of fat bodies with different microbial solutions, sterilized glass staining dishes were used and contact with air was minimized by covering each well with a layer of parafilm.

Immunostaining for Endogenous Dorsal

Wild type larval fat bodies were used for immunofluorescent staining to detect subcellular localization of Dorsal proteins. Whole larvae were subjected to septic injury and were allowed to recover for 1 h before dissection in PBS. Fat bodies dissected from 20 larvae in 200 μl of PBS were treated with 80 μl of diluted bacteria or supernatant and incubated for 1 h. The tissues were fixed in 4% formaldehyde-PBS solution for 10 min and then washed twice with PBS containing 0.1% Tween80 and 2% BSA. The fixed tissues were blocked in PBS containing 2% BSA at room temperature for 1 h. Dorsal monoclonal antibody (7A4, Developmental Studies Hybridoma Bank, University of Iowa) was used at 1:50 dilution. Incubation with antibody was carried out overnight at 4°C in Tris buffered saline (TBS) containing 0.1% Tween80 and 1%

casein (Sigma C-5890). The fat bodies were then washed twice with TBS/Tween, and incubated in secondary antibody for 2 h. A 1:200 dilution of goat anti-mouse IgG conjugated with green Alexa Fluor 488® ($F(ab^1)_2$ fragment, Molecular Probes, Inc., OR, Cat. No. A-11017) was used as the secondary antibody. After three washes, the fat bodies were mounted onto glass slides and visualized by epi-fluorescent microscopy using a FITC filter.

Phenoloxidase Assay

Larval cuticle melanization was visualized by dissecting wild type or $dorsal^{I5}$ larvae in PBS and allowing tissues to be exposed to air for 1 h. Cuticles were mounted onto slides and visualized under bright field microscopy. Liquid assay for phenoloxidase activity in the hemolymph was performed using the substrate dihydroxyphenylalanine (L-DOPA, Sigma D-9628). Hemolymph collected from 10 larvae was resuspended in 100 μl phosphate buffer, pH 6.8. The mixture was added immediately to 100 μl of freshly prepared saturated solution of L-DOPA. This hemolymph-substrate mix was added to 300 μl phosphate buffer and the absorbance was measured at 492 nm wavelength. After the reaction had been mixed, absorbance measurements were performed every 15 min for a period of 1 h.

RESULTS

In Vivo Response of GFP-NF-κB Fusion Proteins to Septic Injury

The Gal4-UAS transgenic expression system was employed to express fusion proteins for studying the immune response of live tissues. EGFP-Dorsal, ECFP-Dif, and EYFP-Relish were expressed under the control of the Cg-Gal4 driver, which contains the collagen gene promoter and directs the expression in tissues including fat bodies and hemocytes [Asha et al., 2003]. The nuclear translocation of the three GFP-NF-κB proteins was assessed in live larval fat bodies, a major immune tissue in *Drosophila*. The nuclear signals of GFP-Dorsal, CFP-Dif, and YFP-Relish (both GFP and YFP images were captured using a FITC green filter) reached their peaks around 60 min after bacterial challenge to whole larvae (Fig. 1A–I). The kinetics of nuclear accumulation is similar to those of endogenous proteins as reported previously [Ip et al., 1993; Lemaitre et al., 1995b; Stoven et al., 2000]. The GFP-Dorsal fusion protein had no obvious nuclear signal in naïve animals, but some nuclear fluorescence of Dif and Relish fusions was observed before septic injury. Low levels of nuclear localization in uninfected animals can be observed even with endogenous Dif and Relish [Stoven et al., 2000; Ligoxygakis et al., 2002a]. The nuclear localization prior to stimulation may be due to basal activation or inefficient cytoplasmic retention of these two NF-κB proteins. Nonetheless, the subcellular distribution of these NF-κB fusion proteins can serve as real-time reporters for microbial stimulation.

Fat Bodies can be Stimulated by Pathogen-Associated Molecules

We then addressed the question of whether *Drosophila* tissue could be stimulated directly by microbial compounds without the help of exogenous factors. Transgenic larvae were pulled open in staining dishes. While still associated with their own hemolymph, the dissected tissues were incubated with microbial compounds for 60 min. We tested commercial preparations of LPS, LTA, PGN, and laminarin (LMN), which are derived from cell membranes and cell walls of bacteria and algae. In control experiments using PBS alone, there was little increase of nuclear accumulation of the NF-κB reporter proteins (Fig. 2A,F,K). Incubation with the microbial compounds stimulated nuclear accumulation of Dif and Relish fusion proteins, concomitant with lower cytoplasmic signals. All four preparations stimulated Dif and Relish fusions to respond similarly (Fig. 2G–J and 2L–O). Based on the genetic results that Relish and Dif are essential for two independent signaling pathways, one might expect they respond differently. On the other hand, it has also been shown that Dif and Relish can form heterodimers [Han and Ip, 1999]. Moreover, recent reports demonstrate that other compounds present in highly purified preparations are responsible for the induction of an immune response [Leulier et al., 2003; Werner et al., 2003]. Therefore, extensive experiments are required to distinguish between the actual chemicals that induced the Dif and Relish responses in these ex vivo assays. Nonetheless, we can conclude that dissected *Drosophila* immune tissues can respond to various microbial preparations from diverse microorganisms without the help of exogenous factors.

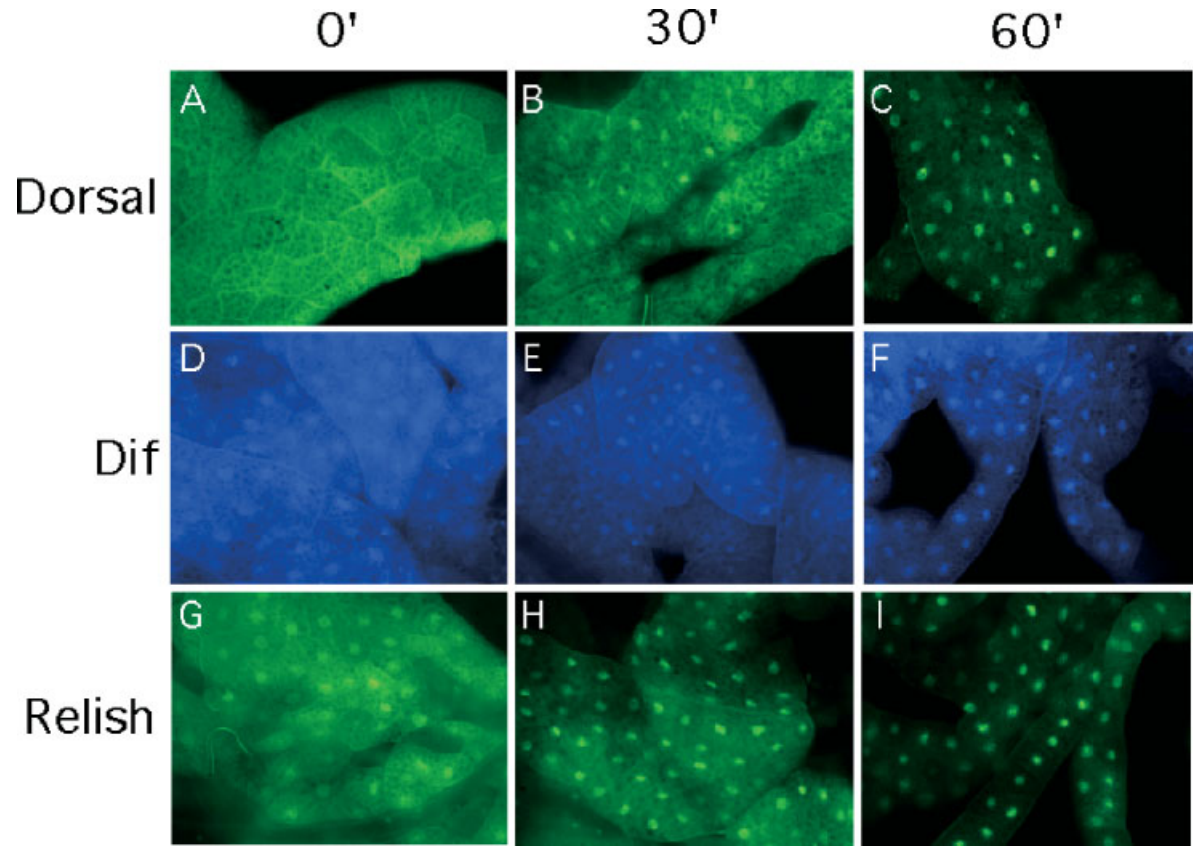

Fig. 1. Subcellular localization of GFP-NF-κB proteins in response to septic injury. Transgenic larvae expressing GFP-Dorsal, CFP-Dif, and YFP-Relish were used for the experiments. Third instar larvae were pricked with needles and were dipped into a culture of mixed bacteria, which includes *Escherichia coli*, *Erwinia carotovora*, *Micrococcus luteus*, and *Enterobacter cloacae*. The pricked animals were allowed to recover at room temperature for the indicated times. Fat bodies were dissected and the images of GFP or GFP-variants were captured immediately under fluorescent microscope. To show more intense fluorescence, both the GFP and YFP in this figure and all subsequent figures were captured using a FITC filter, therefore, both appear as green. The results show that the transgenic proteins can sense septic injury and change their subcellular localization within 30–60 min.

Hemolymph-Independent Response of Dissected Fat Bodies

To further investigate how the recognition of microbial compounds leads to stimulation of dissected fat bodies, we tested the hemolymph requirement for the responses of Dif and Relish. We performed similar experiments by using fat bodies that were previously rinsed three-times with PBS. The rinsing should eliminate hemolymph factors that are loosely associated with the fat tissue, as well as the hemocytes that are loosely attached. We found that the response to the microbial compounds in the rinsed fat bodies was similar to that in hemolymph-associated fat bodies. In both conditions, Dif and Relish responded to the stimulants but Dorsal did not show translocation (Fig. 2P–T for Relish, and data not shown for Dorsal and Dif). Therefore, some receptor complexes already assembled on the fat cell surface are sufficient for recognizing the added microbial compounds and signaling to Dif and Relish. These results indicate that at least part of the immune response can bypass free hemolymph factors or circulating hemocytes. However, hemolymph factors or hemocytes should be involved at the initial stage of recognition complex assembly on the fat cell surface. Furthermore, in intact animals the stimulation of Dif and Relish may also involve hemolymph. Nonetheless, the two mechanisms need not be mutually exclusive.

Stimulation of Dorsal Translocation Requires Additional Microbial and Hemolymph Factors

In dissected fat bodies the GFP-Dorsal did not respond to any of the microbial compounds tested (Fig. 2B–E). This result is drastically different from the apparently normal response of this fusion protein in whole animals

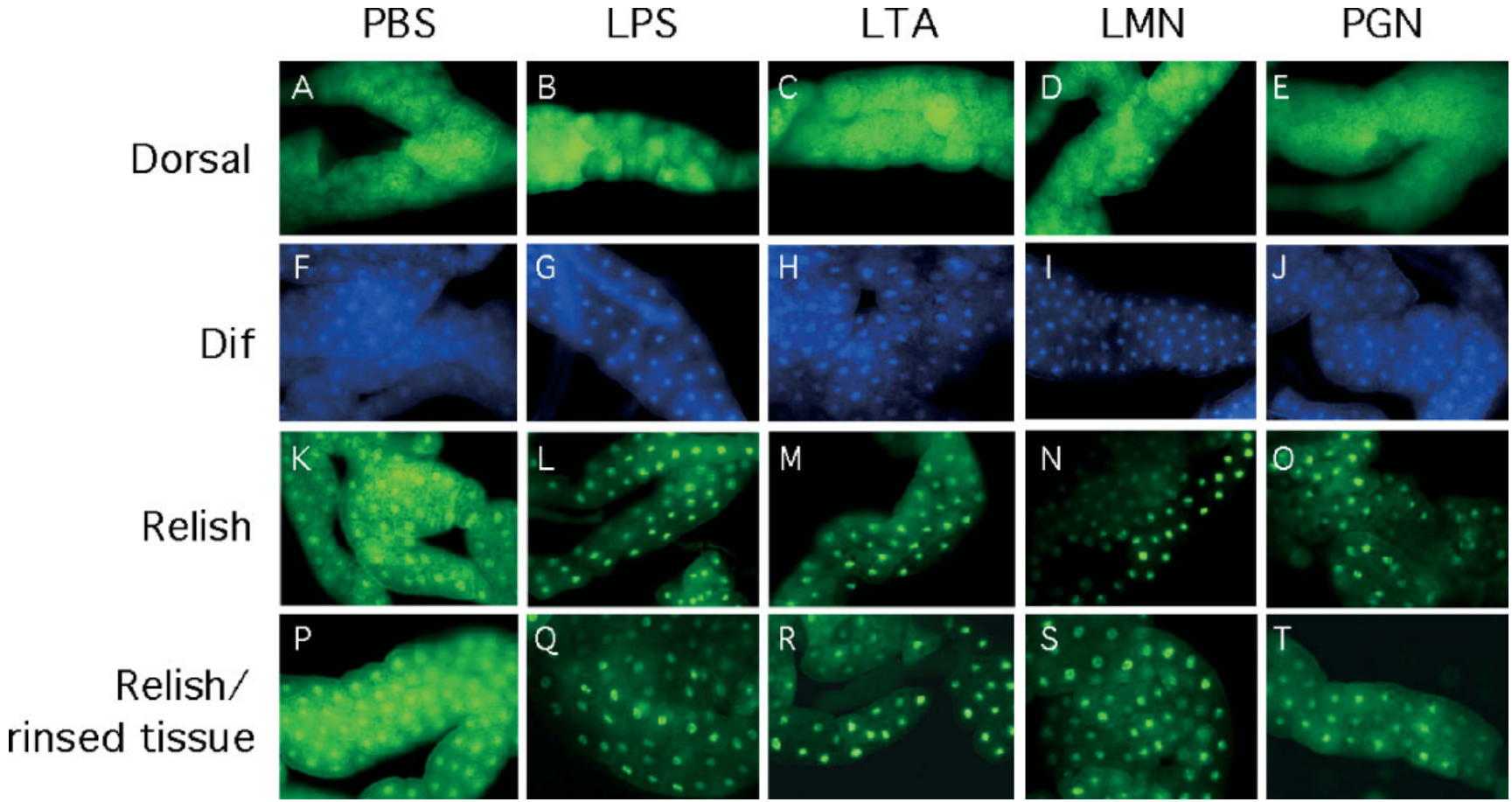

Fig. 2. Dissected fat bodies can respond to various preparations of microbial compounds. Ten larvae expressing the indicated GFP-fusion proteins were pull opened in staining dishes. The opened larval tissues including hemolymph were incubated for 60 min in the indicated compound preparations dissolved in PBS (**panels A–O**). The final concentration of microbial compounds used was approximately 10 μg/ml. The fluorescence of fat bodies was captured immediately at the indicated times. Dif and Relish fusion proteins showed significant changes in their nuclear localization (panels F–O), while the Dorsal fusion did not respond to any stimulant (panels A–E). The results show that all microbial substances can stimulate at least some response in dissected fat bodies. **Panels P–T**: Show similar experiments but using fat bodies that were dissected and rinsed three-times in PBS before incubation with the compounds. Even after rinsing, the nuclear translocation of Relish was clearly stimulated by all the reagents. The same response was observed for CFP-Dif, whereas GFP-Dorsal again did not respond to any of the compounds tested in rinsed tissues (data not shown).

(Fig. 1A–C). This rather different behavior of GFP-Dorsal prompted us to investigate the possibility of multiple recognition mechanisms in *Drosophila*. We first carried out experiments to ascertain that the GFP-Dorsal in dissected fat bodies can respond to diffusible compounds. The phorbol ester PMA can diffuse into cytoplasm and acts as a strong inducer of protein kinase C and nitric oxide production [Ron and Kazanietz, 1999]. The chemical H_2O_2 is an intermediate of oxidative stress and has been postulated to be a central inducer of NF-κB in mammalian cells [Baeuerle and Baltimore, 1996]. These two compounds clearly induced GFP-Dorsal to enter nuclei (Fig. 3B,C), dissipating the concern that this GFP-Dorsal is somehow defective or unresponsive. Thus, we speculated that Dorsal might respond to other bacterial components. To test this hypothesis, we stimulated dissected fat bodies by adding a diluted mixture of four bacterial cultures in the presence of hemolymph. Under this condition, GFP-Dorsal translocated to nuclei (Fig. 3D). These results demonstrate that GFP-Dorsal responds efficiently to components presented by whole bacteria but not to the microbial preparations that we have tested.

We further examined whether the response of GFP-Dorsal to whole bacteria is hemolymph-dependent. Using PBS-rinsed fat bodies, we found that GFP-Dorsal lost all the response to bacteria (Fig. 3E), thus demonstrating an absolute requirement of hemolymph for the recognition of the bacterial cultures or the stimulation of GFP-Dorsal. This hemolymph requirement was further examined by a transfer immunity experiment. In this experiment, wild type larvae were first induced by septic injury. Cell-free hemolymph from these induced larvae was collected and added to rinsed fat

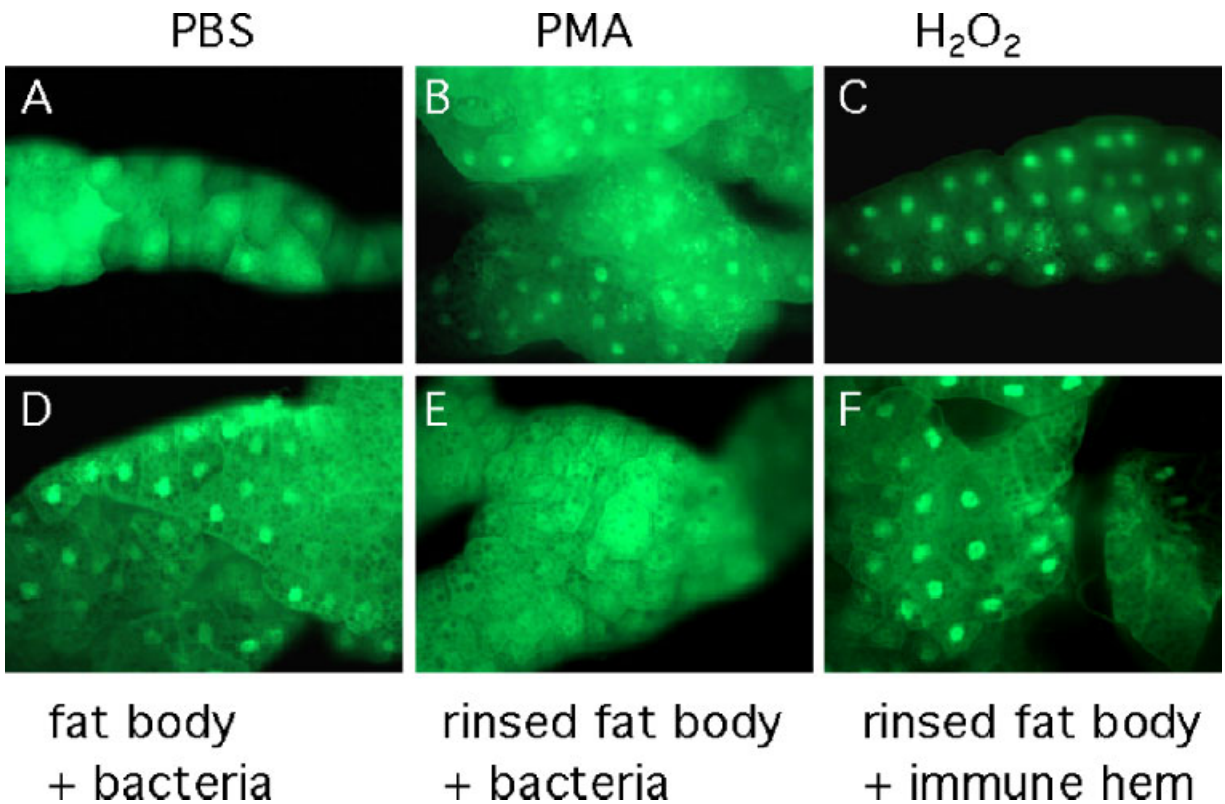

Fig. 3. Transgenic GFP-Dorsal exhibits a hemolymph-dependent response to whole bacteria. Larvae expressing GFP-Dorsal were dissected in staining dishes and incubated with 200 μl of solutions containing the indicated reagents for 60 min. The dissected fat bodies were incubated in (**A**) PBS, (**B**) 10 μM PMA, or (**C**) 0.001% hydrogen peroxide. The two compounds induced Dorsal nuclear localization (B and C). In **panel** (**D**) the dissected fat body was incubated with the whole bacterial mixture in the presence of hemolymph. This treatment clearly induced nuclear localization of GFP-Dorsal. **Panel E**: Shows a fat body that has been rinsed with PBS to eliminate the hemolymph before incubating with bacteria mixture. No Dorsal nuclear translocation was observed. However, when rinsed fat bodies were incubated with immunized wild type hemolymph, nuclear translocation was then observed (**panel F**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

bodies that expressed the GFP-Dorsal. This transferred immune hemolymph induced nuclear translocation of GFP-Dorsal in rinsed fat bodies (Fig. 3F). Together, our results demonstrate that GFP-Dorsal responds efficiently to some yet to be identified bacterial components and this process requires hemolymph.

To ascertain that the response of GFP-Dorsal represents an in vivo property of endogenous Dorsal, we performed similar experiments by staining for the endogenous protein. Fat bodies were stimulated by various methods and then fixed for antibody staining. Most of the staining remained cytoplasmic in unstimulated samples, and the lack of staining in dorsal mutant tissues suggests that the antibody has high specificity (Fig. 4A,C). As previously shown, septic injury of whole larvae induced nuclear translocation of the endogenous Dorsal (Fig. 4B) [Lemaitre et al., 1995b]. Similar to that observed in whole animal experiments, dissected fat bodies clearly showed nuclear transport of Dorsal when incubated with mixed bacteria in the presence of hemolymph (Fig. 4E). The medium is not the source of induction because incubation with the 2xYT medium alone did not show increased nuclear accumulation of Dorsal (Fig. 4D). Rinsing the fat body with PBS prior to the incubation with bacteria abolished the response (Fig. 4F), as was observed for GFP-Dorsal. Therefore, we conclude that Dorsal acts downstream of a hemolymph-dependent recognition mechanism, which can be stimulated by yet to be identified bacterial components.

Bacterial Proteins in Culture Media as Stimulants of Hemolymph-Dependent Dorsal Response

To characterize the yet to be identified bacterial components, we individually tested

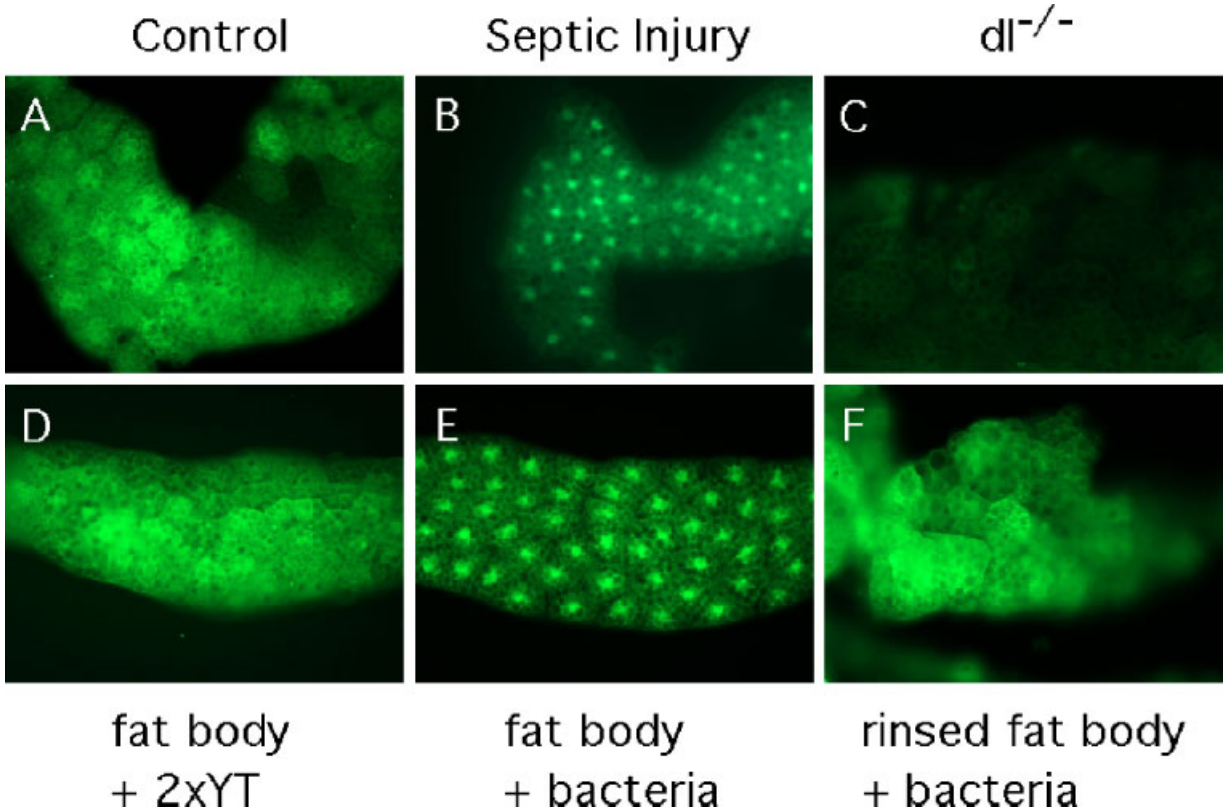

Fig. 4. Endogenous Dorsal responds to whole bacteria in a hemolymph-dependent manner. Wild type animals were used for these experiments except in panel C where dorsal^{I5} mutant was used. The tissues were stained for endogenous Dorsal using a monoclonal antibody. Images of fat bodies are shown using indirect immunofluorescent microscopy. **Panels A–C**: Show fat bodies isolated from whole larvae either without any treatment (A and C) or previously challenged with mixed bacteria (B). The dorsal^{I5} mutant is a protein null allele and served as negative control for staining (C). **Panels D–F**: Show the subcellular localization of Dorsal in dissected fat bodies after various treatments. Incubation with 2xYT medium alone did not cause nuclear translocation (D), while incubation with bacterial mixture induced nuclear accumulation (E). In contrast, this reaction is hemolymph-dependent because rinsed fat bodies did not respond to bacteria (F). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the four bacteria in the mixture used in the previous experiments. Diluted cultures of these bacteria were used directly, or separated into supernatants and pellets before use. All individual bacterial cultures were capable of inducing nuclear accumulation of the endogenous Dorsal (Fig. 5A,D,G,J,M). Therefore, it is likely that the inducing factors are some common components

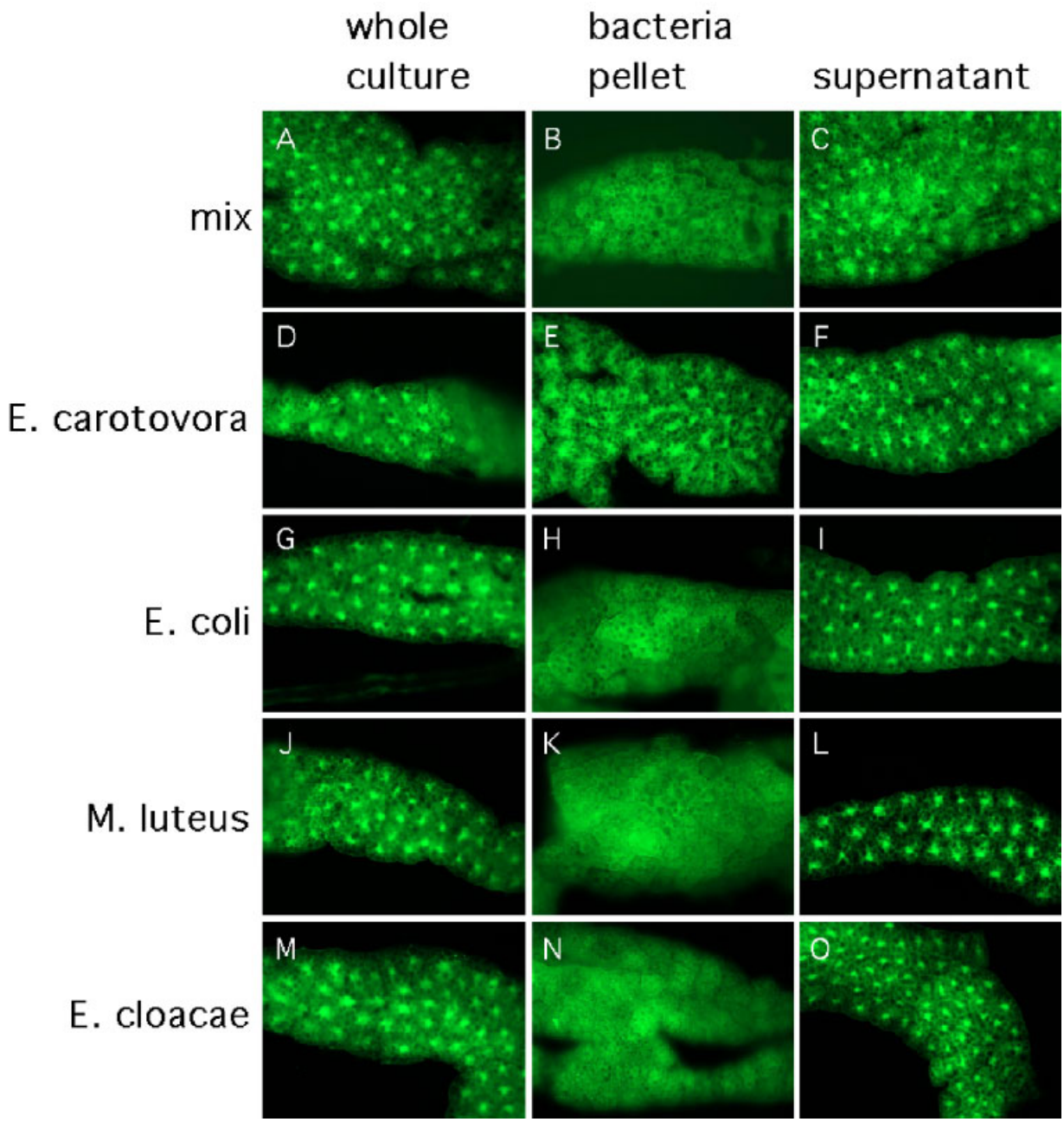

Fig. 5. Supernatants of bacterial cultures contain the factors that induce translocation of endogenous Dorsal. A mixture of all four bacterial cultures (**panel A**) or individual bacterial cultures as indicated (**panels D**, **G**, **J**, **M**) were added to dissected fat bodies from wild type larvae. Each bacteria culture could induce nuclear translocation of endogenous Dorsal, demonstrating that the inducing factors are not restricted to certain classes of microorganisms. The cultures were spun and the supernatants or bacterial pellets, which had been rinsed and resuspended, were added to the fat bodies to evaluate the effect on Dorsal translocation. All supernatants showed inducing activities (**panels C**, **F**, **I**, **L**, **O**), whereas the pellets had no such activities except that from *E. carotovora*, which still retained some residual activity (**panels B**, **E**, **H**, **K**, **N**). These results show that the inducing factors are either secreted or breakdown products of bacteria. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

not specifically produced by Gram-positive bacteria (*M. luteus*), Gram-negative bacteria (*E. carotovora*, *E. coli*, *E. cloacae*), or phytopathogens (*E. carotovora*).

By using separated culture media and resuspended bacteria pellets, we demonstrated that supernatants from the cultures were necessary and sufficient to induce the fat body to respond. All bacteria pellets were washed three-times in PBS and then resuspended and added to the dissected larvae. Except for the *E. carotovora* pellet, which induced some response (Fig. 5E), the washed bacteria largely lost the ability to stimulate Dorsal nuclear translocation. On the contrary, all the supernatants showed the ability to induce nuclear translocation of Dorsal in dissected fat bodies (Fig. 5C,F,I,L,O). Therefore, either secreted or breakdown products from the cultures act as the inducing factors.

As demonstrated in experiments described in Figures 1 and 2, none of the tested lipid- or polysaccharide-based microbial compounds induced the GFP-Dorsal nuclear translocation in dissected fat bodies. Further experiments as described below suggest that the inducing factors likely consist of bacterial proteins. First, the inducing activity is sensitive to extensive heat treatment. Incubation of the *E. carotovora* supernatant at 37°C for 1 h had no effect on the activity (Fig. 6C), suggesting that the factors

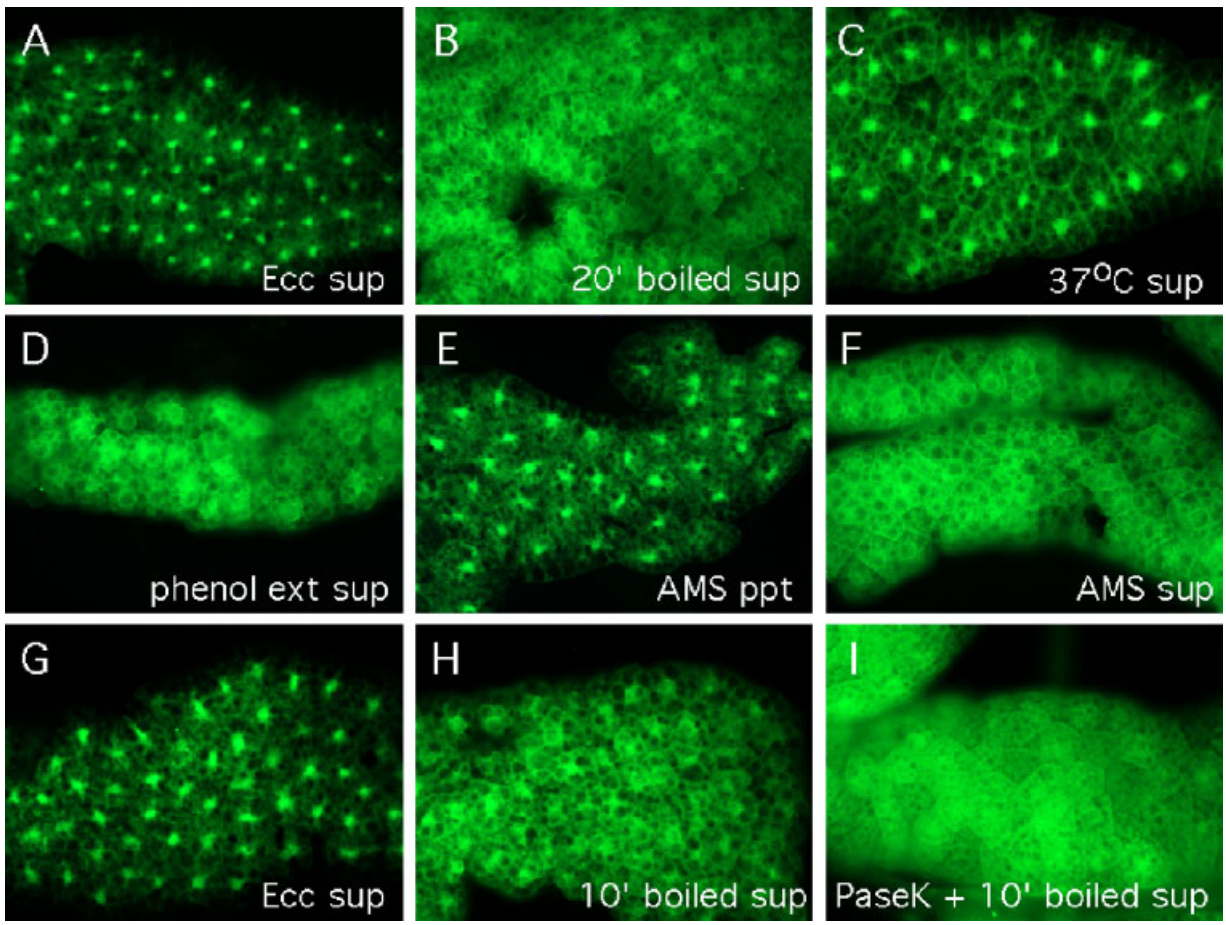

Fig. 6. The bacterial factor that induces Dorsal is protein-like. Supernatants from *E. carotovora* were used for all the experiments shown. Heat treatment of the supernatant for 20 min at boiling temperature abolished the activity (**panel B**), whereas incubation of this supernatant at 37°C for 1 h did not alter the inducing activity significantly (**C**). Extraction by phenol and chloroform of the supernatant prior to use for induction abolished the activity in the supernatant (**D**). Resuspended precipitate obtained by ammonium sulfate (80%) precipitation reconstituted the inducing activity, while the remaining supernatant after precipitation was not active (**panel E** and **F**). **Panel** (**G**) is another control fat body induced by untreated *E. carotovora* supernatant. Treatment of a similar supernatant at boiling temperature for 10 min caused significant reduction of, but did not abolish, the activity (**H**). Proteinase K digestion of the supernatant followed by 10 min of boiling further reduced the activity (**panel I**), suggesting that the inducing factors contain proteins as essential components. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

are relatively stable. However, boiling the supernatant for 10 min reduced the activity substantially and boiling the supernatant for 20 min eliminated the activity (Fig. 6B,H). Second, phenol and chloroform extraction eliminated the activity from the aqueous phase (Fig. 6D), suggesting that the factors are not nucleic acids or carbohydrates. Third, ammonium sulfate precipitation, which is a widely used method to concentrate proteins from an aqueous environment, recovered all the activity that induced translocation of endogenous Dorsal (Fig. 6E). No significant inducing activity remained in the ammonium sulfate treated supernatant (Fig. 6F). Together, these results suggest that the inducing factors are bacterial proteins present in the culture supernatants. Therefore, we tested whether proteinase K could eliminate the activity. Because direct proteinase K treatment of the fat bodies causes tissue damage and disrupts fat body morphology, we first incubated the bacterial supernatant with proteinase K, which should digest most of the proteins in the supernatant. Before adding to dissected fat bodies, the treated supernatant was heated to 100°C for 10 min to eliminate the proteinase K activity. The 10 min heat treatment alone should allow some inducing activity to remain (Fig. 6H). The combination of proteinase K treatment followed by 10 min boiling completely eliminated the inducing activity (Fig. 6I), suggesting that the factors responsible for stimulating Dorsal translocation are susceptible to proteinase K. In conclusion, we show that novel bacterial stimulants that are likely proteins can interact with hemolymph factors and stimulate Dorsal nuclear translocation in larval fat bodies.

Dorsal is Essential for Regulating Phenoloxidase Activity in Larval Hemolymph

On account of the results described above suggesting that Dorsal responds to the hemolymph-dependent recognition of bacterial proteins, we searched for additional biological functions of Dorsal in larvae. While nuclear translocation of Dorsal can be induced by microbial challenge or activation of the Toll pathway, dorsal mutants do not have detectable defects in antimicrobial peptide gene expression [Lemaitre et al., 1995b]. Dif and Dorsal may have redundant functions in larval immune response by reason of either protein can rescue the defective drosomycin expression in a deficiency strain that uncovers both dif and dorsal [Manfruelli et al., 1999; Rutschmann et al., 2000]. During dissection experiments, we observed that the hemolymph of dorsal mutant larvae displayed a slower rate of melanization when compared to other samples. In wild type animals, melanization of the hemolymph accumulates to high levels within 60 min and deposition onto dissected cuticles could be observed easily (Fig. 7A). However, in dorsal$^{-/-}$ mutant larvae, much lower melanization of hemolymph and deposition onto cuticles was observed (Fig. 7B). To quantify this observation, we carried out enzymatic assays for humoral phenoloxidase activity in the hemolymph of various combinations of dorsal mutant alleles. These alleles were generated in different laboratories at different times. Thus, it is unlikely that they contain the same secondary mutations in the chromosomes. All the trans-allelic combinations showed significantly lower phenoloxidase activity (Fig. 7C). Consequently, Dorsal has an additional function in innate immunity by regulating the expression of some components required for the phenoloxidase cascade in the larval hemolymph.

Discussion

We show that the use of freshly dissected fat bodies represents a suitable system to address the question of pathogen recognition. Our results demonstrate that fat bodies can respond in the absence of exogenous factors. Moreover, four different commercial preparations of microbial substances can generate a response using Dif and Relish as reporters. Because recent reports showed that contaminants but not the major constituents in microbial preparations can be accounted for the stimulation of a *Drosophila* immune response [Weber et al., 2003; Werner et al., 2003], we cannot implicate the specific compounds in each preparation that solicited the responses in dissected tissues. Highly purified compounds and additional experiments are required to address the specificity of the responses. Nonetheless, the results presented in this study show that dissected fat bodies can recognize microbial compounds.

Mutant mice of TLR4, TLR2, TLR9, and TLR5 have defects in LPS, PGN, bacterial DNA, and flagellin responses, respectively [Hoshino et al., 1999; Hemmi et al., 2000; Hayashi et al., 2001]. Accordingly, mammalian innate immune sys-

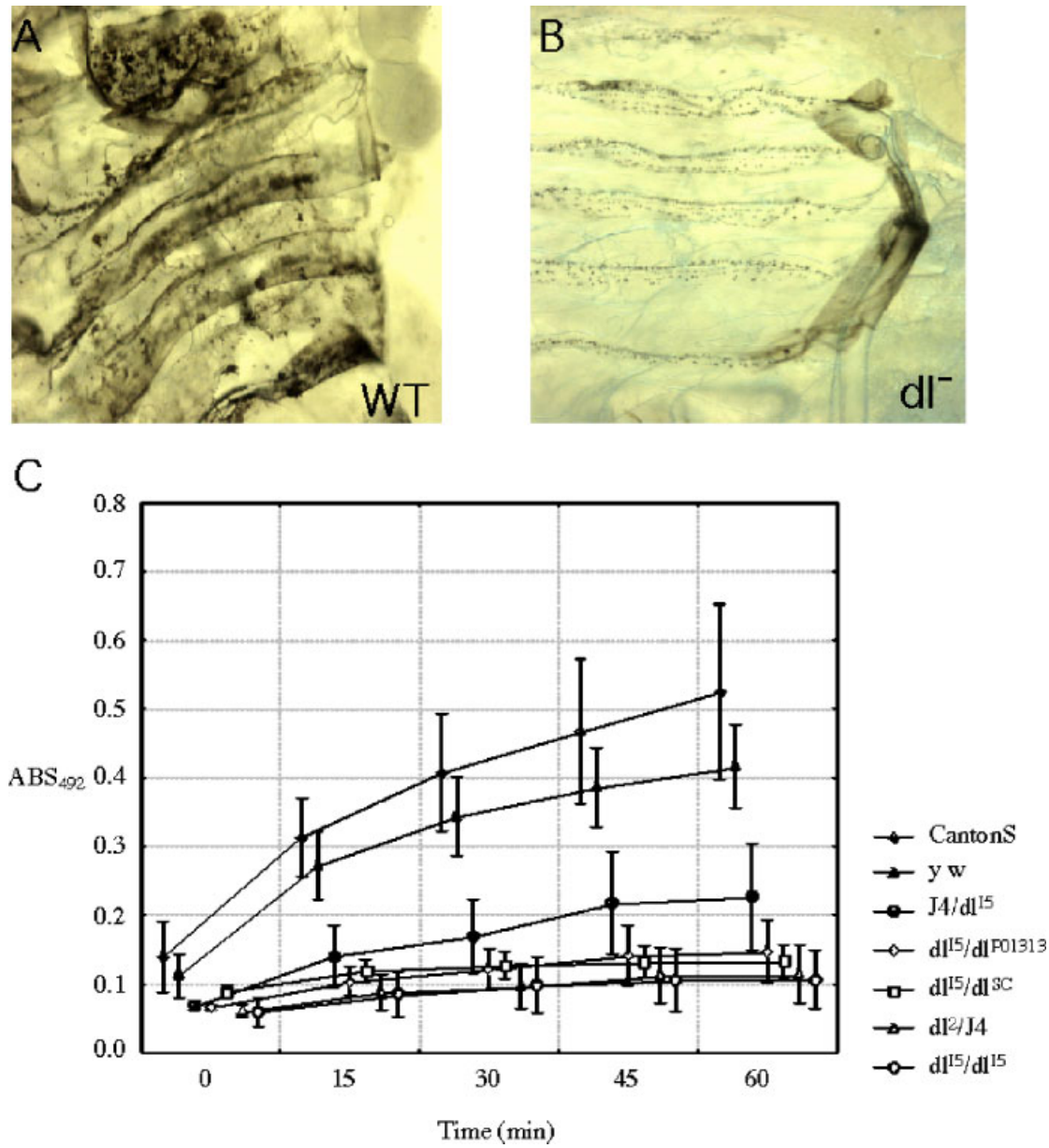

Fig. 7. Dorsal has an essential role in regulating phenoloxidase activity in larval hemolymph. Wild type (WT, **panel A**) larvae were dissected and exposed to air for 60 min. During this time melanization of hemolymph occurred and deposition of melanin on transparent cuticles could be observed. In dorsal^{15} homozygous mutant larvae (**panel B**), the melanization reaction and melanin deposition on cuticles was substantially reduced. Phenoloxidase activities in hemolymph were assayed using larvae from wild type and y w strains as controls. Substantial activities were detected within 60 min as measured by absorbance at 492 nm wavelength. As indicated, hemolymph from various combinations of dorsal mutants was assayed in parallel and plotted as shown. The results indicate that hemolymph of dorsal mutants all have significantly reduced phenoloxidase activity. Each data point is the means ± standard error of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tems have evolved different mechanisms to detect microbial compounds that are of lipid, polysaccharide, nuclei acid, and protein natures. Using dissected and rinsed fat bodies, we demonstrated that different recognition mechanisms also exist in *Drosophila* (Fig. 8). We have observed that *Drosophila* larval fat bodies can detect canonical compounds that are of polysaccharide and lipid nature, such as PGN and LTA. The interaction with these molecules does not require free hemolymph factors, and can take place directly on fat body cell surfaces. We have also defined a second detection mechanism, which is hemolymph-dependent

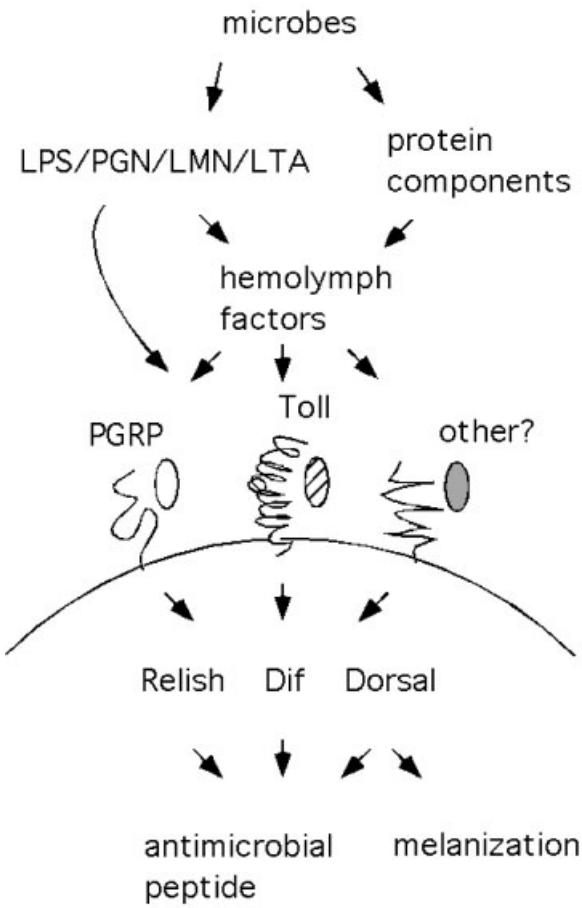

Fig. 8. Model describing recognition of microbial components by *Drosophila* innate immune system. Microbial products such as peptidoglycan can interact directly with fat body cell surface receptors, bypassing the hemolymph and hemocytes, to stimulate innate immune response. Our assays did not exclude the possibility that these canonical microbial substances can also interact with hemolymph factors and hemocytes to stimulate the response, particularly in whole animals. Thus, we indicate both possible routes by arrows. We speculate that the PGRP-LC and Toll pathways are involved. Our model suggests a second recognition mechanism that involves hemolymph factors interacting with protein components of many microorganisms. Dorsal may play a role in mediating the response to the hemolymph-dependent recognition. Further work is required to establish the identity of the microbial and host factors involved for this branch of recognition and signaling.

and may detect proteins that are produced by bacteria. This hemolymph-bacteria protein interaction stimulates the nuclear translocation of Dorsal in the fat bodies.

Characterization of the Dorsal inducing factors in bacterial supernatants suggests that the factors are likely proteins and that all four bacteria tested produce the inducing factors. We do not know whether the factors from the various bacteria are identical or homologous. We favor the possibility that the bacterial factors have similar properties and interact with a rather general sensing mechanism in the *Drosophila* innate immune system. For instance, it may be that secreted proteases in the supernatants from bacteria cultures are responsible for the activation of hemolymph factors through non-specific proteolysis. Once enough proteolysis occurs, the fat bodies sense the event and respond by activating the nuclear translocation of Dorsal. The characterization of the bacterial factors and the study of how they interact with hemolymph should provide significant insights into host defense mechanisms against microbial infection.

Even though we observed efficient nuclear translocation of the three NF-κB proteins after stimulation, further analysis revealed that the dissected tissues had lower responses with respect to antimicrobial peptide gene expression. Addition of microbial compounds to dissected tissues increased cecropin, diptericin, and drosomycin expression approximately two-fold after 2 h, while septic injury of whole larvae caused an approximately five-fold induction of these genes (data not shown). Therefore, dissected tissues, while capable of responding, have missed some components that synergize with nuclear NF-κB to activate target genes. Further investigation is required to optimize the fat body ex vivo assay system.

The five mammalian NF-κB factors have overlapping and distinct functions in response to a wide range of signals [Ghosh et al., 1998; Li and Verma, 2002]. In *Drosophila*, the three NF-κB factors appear to have distinct functions. Relish is the key transcription factor in the Imd pathway and Dif is critical in the Toll pathway [Hedengren et al., 1999; Meng et al., 1999; Rutschmann et al., 2000, 2002]. While the requirements of Dif and Relish are distinct at the genetic level, the two transcription factors may function together at least under some circumstances. It has been shown that Dif and Relish can form functional heterodimers in cultured cells [Han and Ip, 1999]. The formation of Dif/Relish heterodimers also increases the specificity in selecting target sequences in vitro [Senger et al., 2004]. The results presented in this study show that Dif and Relish respond similarly to an array of microbial compounds. Perhaps Dif and Relish cooperate or coordinate to perform some of their functions in vivo.

Dorsal has a critical function during dorsal-ventral embryonic development but is not essential for the induction of antimicrobial peptide genes [Lemaitre et al., 1995b; Meng et al., 1999; Rutschmann et al., 2000]. Nonetheless, Dorsal clearly responds to septic injury (Fig. 4) [Lemaitre et al., 1995b], thereby suggesting a role in other aspects of immunity. While septic injury experiments indicate that Dorsal can be induced to translocate, our ex vivo assays demonstrate yet another level of complexity. In dissected tissues, Dorsal can be stimulated only by bacterial supernatants but not by the commercially purified microbial compounds tested. More interestingly, the stimulation of Dorsal nuclear translocation strictly requires hemolymph. We also demonstrate that dorsal mutants have specific defects in phenoloxidase activity. Perhaps Dorsal has a wider role in innate immunity by responding to a specific subset of pathogen-associated molecules and regulating melanization as part of the immune response.

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