The Role of Ubiquitination in Drosophila Innate Immunity*

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Infection of Drosophila by Gram-negative bacteria triggers a signal transduction pathway (the IMD pathway) culminating in the expression of genes encoding antimicrobial peptides. A key component in this pathway is a Drosophila IκB kinase (DmIKK) complex, which stimulates the cleavage and activation of the NF-κB transcription factor Relish. Activation of the DmIKK complex requires the MAP3K dTAK1, but the mechanism of dTAK1 activation is not understood. In human cells, the activation of TAK1 and IKK requires the human ubiquitin-conjugating enzymes Ubc13 and UEV1a. Here we demonstrate that the Drosophila homologs of Ubc13 and UEV1a are similarly required for the activation of dTAK1 and the DmIKK complex. Surprisingly, we find that the Drosophila caspase DREDD and its partner DADD are required for the activation of DmIKK and JNK, in addition to their role in Relish cleavage. These studies reveal an evolutionarily conserved role of ubiquitination in IKK activation, and provide new insights into the hierarchy of signaling components in the Drosophila antibacterial immunity pathway.

The fruit fly Drosophila melanogaster has evolved multiple mechanisms to combat infectious microorganisms, including both cellular and humoral innate immune responses, and several proteolytic cascades (1–4). Activation of the humoral insect immune response leads to the rapid production of a battery of potent antimicrobial peptides such as Cecropin, Diptericin, Defensin, Drosocin, Mechnikowin, and Drosomycin (5). Considerable progress has been made in understanding the signal transduction pathways that govern the Drosophila humoral immune response. In particular, two distinct pathways, Toll and IMD, have been characterized (6, 7). Infection by fungi or Gram-positive bacteria triggers the processing of Spätzle (SPZ) by an extracellular pro-peptidase (8), which stimulates the cleavage and activation of the NF-κB transcription factor Relish. Activation of the DmIKK complex requires the MAP3K dTAK1, but the mechanism of dTAK1 activation is not understood. In human cells, the activation of TAK1 and IKK requires the human ubiquitin-conjugating enzymes Ubc13 and UEV1a. Here we demonstrate that the Drosophila homologs of Ubc13 and UEV1a are similarly required for the activation of dTAK1 and the DmIKK complex. Surprisingly, we find that the Drosophila caspase DREDD and its partner DADD are required for the activation of DmIKK and JNK, in addition to their role in Relish cleavage. These studies reveal an evolutionarily conserved role of ubiquitination in IKK activation, and provide new insights into the hierarchy of signaling components in the Drosophila antibacterial immunity pathway.

A distinct signaling pathway, the IMD pathway, is activated in response to infection by Gram-negative bacteria and culminates in the production of antibacterial peptides. Both genetic- and cell-based studies have identified several components of this pathway. Inactivation of most of the genes encoding these components results in the complete and specific inactivation of the IMD pathway. The IMD pathway is induced by the recognition of Gram-negative peptidoglycan (PGN) via the receptor PGRP-LC/ird7 (8–12). This receptor interacts with the IMD protein (13), which is homologous to mammalian RIP1, a critical adaptor protein in the TNFR complex (14). In turn, IMD is required for the activation of the Drosophila MAP kinase kinase kinase dTAK1, although the mechanisms of dTAK1 activation are unknown. In humans, the TRAF proteins are required for the activation of TAK1 in the TNFR and TLR signaling pathways. The Drosophila TRAF6 homolog, dTRAF2, may serve a similar function in the IMD pathway, because dTRAF2 has recently been shown to be required for maximal levels of antimicrobial gene expression in response to Escherichia coli infection in flies (15). dTAK1, in turn, is necessary for the activation of the Drosophila IKK complex, as well as for activation of JNK signaling (12, 16–18).

Relish is the critical NF-κB/rel transcription factor homolog activated by the IMD pathway. In unstimulated cells Relish is present in the cytoplasm as a full-length precursor. Upon activation of the IMD pathway, Relish is cleaved, and the N terminus, NF-κB-like cleavage product translocates to the nucleus where it drives transcription of antimicrobial peptide genes (19). IMD pathway-mediated Relish activation requires the Drosophila FADD homolog and its partner caspase DREDD, as well as dTAK1 and the DmIKK complex (20). It is believed that Relish is phosphorylated by DmIKK, and this phosphorylation is required for its DREDD-mediated cleavage (19–23). Consistent with this model, the Drosophila IKK can directly phosphorylate Relish, and Relish cleavage is likely mediated by a caspase. However, DREDD has not been shown to directly cleave Relish.

Recent biochemical studies have revealed a novel mechanism of action for the TRAF6-mediated activation of IKK and NF-κB in mammals. TRAFs are required for NF-κB activation following IL-1R, TLR, or TNFR stimulation. TRAF proteins contain an N-terminal RING domain, a signature domain for ubiquitin E3 ligases. In vitro, TRAF6-mediated activation of the 1κB kinase requires the synthesis of polyubiquitin chains linked through Lys-63 of ubiquitin. This conjugation requires the E2 proteins Ubc13 and UEV1a (24). Interestingly, this ubiquitination does not lead to proteasome-mediated degradation of TRAF6. Rather, it activates TRAF6, which in turn activates TAK1 (25). This activation of TAK1 requires an associated protein, TAB2, which specifically binds to Lys-63 polyubiquitin chains through a conserved zinc finger domain (26).

At present, the mechanism by which the DmIKK complex is activated by IMD and dTAK1 is not understood. In this study we address the question of whether ubiquitination is required for this process. We also investigate the role of the adaptor protein dFADD and the apical caspase...
DREDD in the activation of IMD signaling. Using RNAi in immune responsive Drosophila cells in culture, we demonstrate that the Drosophila homologs of Ubc13 and UEV1a, dFADD, and DREDD are all required for IKK activation.

**MATERIALS AND METHODS**

**DNA Constructs and Stable Cell Lines—Bendless (C87A) mutant was generated using the QuikChange Site-directed Mutagenesis Kit (Stratagene). DNA fragments encoding FLAG-tagged wild-type and C87A Bendless, FLAG-tagged IMD, and dTAK1Δ (amino acids 1–284) were generated by PCR and cloned into pRmHa-3 by standard cloning techniques and verified by sequencing. The pRmHa-3-SPZ-C106 construct was generated by inserting pJM856-Spa-C106 fragment into pRmHa-3 by standard cloning techniques and verified by sequencing. The pRmHa-3-SPZ-C106 construct generated by PCR and cloned into pRmHa-3 by standard cloning techniques and verified by sequencing.**

**SPZ-containing Conditioned Medium Preparation—pRmHa-3-SPZ-C106 cell line was plated at the density of 10⁶ cells/ml in 10 ml tissue culture plates. Cells were treated 24 h later with 20-hydroxy-ecdysone (Sigma) at 10⁻⁶ M and copper at 0.5 mM. After 48 h, cells were spun down, and the SPZ-containing conditioned medium was harvested.

**dsRNA Synthesis, Cell Transfection, Cell Induction, RNA Isolation, and Northern Blotting—dsRNA synthesis was performed as described before (21).** S2 cells were plated at the density of 10⁶ cells/ml in 6-well tissue culture plates (3 ml of cells per well). Cells were transfected 24 h later with 5 μg of each dsRNA along with 100 ng of a LacZ reporter plasmid. Cells were split 24 h after transfection to 10⁶ cells/ml in 6-well tissue culture plates (3 ml of cells per well). After another 24 h, cells were treated with 20-hydroxy-ecdysone at 10⁻⁶ M for 24 h. Cells were then either stimulated with peptidoglycan (Sigma, crude E. coli 055:B5 LPS, 10 μg/ml) or stimulated with equal volume of SPZ-containing conditioned medium for 48 h. In experiments involving FLAG-IMD stable cells, cells were first plated at the density of 10⁶ cells/ml in 6-well tissue culture plates (3 ml of cells per well). Cells were transfected 24 h later with 5 μg of each dsRNA along with 100 ng of a LacZ reporter plasmid. Cells were split 24 h after transfection to 10⁶ cells/ml in 6-well tissue culture plates (3 ml of cells per well). After another 24 h, cells were treated with 20-hydroxy-ecdysone at 10⁻⁶ M for 24 h. Then cells were stimulated for 8 h with either peptidoglycan or copper sulfate (0.5 mM).

Cells were then harvested, and RNA isolation and Northern blotting were performed as described previously (21). dTAK1Δ stable cells were treated the same way as IMD cells except that the duration of copper sulfate treatment was 12 h.

**Kinase Assay—His-tagged full-length Relish protein was expressed in S9 cells using a baculovirus expression system and purified on Ni-NTA.** S2 cells were first transfected with various dsRNAs as described above. Cells were split 24 h after transfection to 10⁶ cells/ml in 6-well tissue culture plates (3 ml of cells per well). After an additional 24 h, cells were stimulated with peptidoglycan for 15 min and harvested. Cell extract preparation, immunoprecipitation, and kinase assays were performed as described previously except purified Relish protein was used as substrate in the kinase reactions (21).

**RESULTS**

We have established a cell culture system to study the IMD and Toll signaling pathways in S2 cells. The IMD pathway is activated by treating the cells with Gram-negative peptidoglycan (which is present in crude preparations of lipopolysaccharides) (12), whereas activation of the Toll pathway is achieved by treating the cells with the Spätzle ligand (27). Active Spätzle is produced from a cell line stably transfected with a plasmid containing the copper-inducible metallothionein promoter driving the expression of active Spätzle C-106. When these cells are treated with copper, active SPZ is secreted into the medium, and this conditioned media can be used to activate naïve cells. Using the RNAi-mediated gene inactivation method (28), we found that SPZ-induced Drosomycin gene activation in S2 cells requires the Drosophila Rel proteins Dif and Dorsal, as well as Toll, DmMyD88, Tube, Pelle, and Slimb, as expected (data not shown). In sharp contrast, these dsRNAs do not block the expression of antimicrobial peptide genes induced by peptidoglycan. Instead, RNAi studies demonstrate that peptidoglycan-induced gene expression requires all known components of the IMD pathway (data not shown). We have therefore used this RNAi approach to determine the roles of candidate signaling components in the IMD and Toll signaling pathways.

The **Ubiquitination-conjugating Enzymes Bendless and dUEV1a Are Required for the Induction of Antibacterial Peptides**—Previous studies have shown that activation of the mammalian IKK complex requires a ubiquitination step (29). In particular, TRAF6-mediated IKK activation was shown to require a dimeric ubiquitin-conjugating enzyme composed of the Ubc13 and UEV1a proteins (24). Bendless and dUEV1a are the Drosophila homologs of Ubc13 and UEV1a, respectively (this study and Refs. 30, 31). We have found that Bendless and dUEV1a, like their mammalian counterparts, associate with each other in vivo (data not shown). To investigate whether Bendless and dUEV1a are required for antibacterial gene expression in response to peptidoglycan, we used the RNAi-mediated gene inactivation method. S2 cells were transfected with various dsRNAs (as labeled on top of each panel of Fig. 1). After 48 h, cells were first treated with 20-hydroxy-ecdysone for 24 h to enhance their competence to induce antimicrobial genes in response to immune challenge (21, 32), and then treated with peptidoglycan or SPZ to activate the IMD or Toll signaling pathways, respectively. Total RNA was isolated from these cells and subjected to Northern blotting analysis using cDNA fragments corresponding to Diptericin or Drosomycin as probes, to examine the activation of the IMD and Toll pathways, respectively. As shown in Fig. 1A, both Bendless and dUEV1a are required for maximal levels of antibacterial peptide gene expression in response to peptidoglycan treatment (Fig. 1A, compare lanes 8 and 10 to lane 4). In fact, when both Bendless and dUEV1a are both targeted by RNAi, Diptericin induction is reduced to near background levels (lane 12). (The partial effect of Bendless or dUEV1a RNAi alone is likely because of the fact that RNAi often does not generate a complete null phenotype.) By contrast, the induction of Drosomycin by Toll activation is unaffected by the RNAi-mediated knock-down of Bendless and dUEV1a (Fig. 1B, lanes 8, 10, and 12. Note that the same cells were stimulated with either peptidoglycan or SPZ in Fig. 1A, B.) As a control, S2 cells treated with DmIkKα or Toll dsRNA showed significantly reduced peptidoglycan-induced Diptericin or SPZ-induced Drosomycin gene expression, respectively. (Fig. 1, A and B, compare lanes 6 and 14 with lanes 2 and 4). As a control, mRNA and/or protein levels of targeted genes were examined by RT-PCR and/or Western blotting to confirm the effectiveness of RNAi (data not shown and supplementary Fig. S1).

To provide further evidence that Bendless is involved in the IMD pathway, we used a dominant negative mutant Bendless to determine whether peptidoglycan-induced antibacterial gene activation can be blocked. Stable S2 cell lines were generated that express either wild-type or C87A Bendless under the control of the metallothionein promoter.
Ubiquitination Regulates the IMD Pathway

The cysteine to alanine mutation at position 87 creates a dominant-negative mutant because this residue, located in the catalytic pocket in ubiquitin-conjugating enzymes, is crucial for the catalytic activity of E2s. These cells were then treated with various combinations of peptidoglycan and copper (as indicated on top of Fig. 1C), and Northern blotting was employed to examine the expression of antibacterial genes including Attacin, Cecropin and Diptericin. Overexpression of wild-type Bendless has no effect on peptidoglycan-induced antibacterial gene activation, as similar levels of antibacterial peptide gene expression were detected in cells treated with or without copper (compare lanes 2 and 4). In contrast, overexpression of Bendless C87A leads to a significant reduction in peptidoglycan-activated expression of antibacterial peptide genes (compare lanes 6 and 8).

Bendless flies have been identified which carry a proline to serine substitution at position 97 within the strictly conserved active site region of E2s (30). In order to determine whether bendless flies are defective in response to Gram-negative bacterial infection, we subjected both wild-type and bendless flies to E. coli infection and examined Diptericin gene activation by Northern blotting. As shown in Fig. 2, bendless flies display significantly weaker Diptericin gene activation compared with wild-type flies (compare lanes 4 and 5 with lanes 9 and 10). These results indicate that the Bendless-dUEV1a E2 complex is required for signaling by the IMD pathway.

**Bendless and dUEV1a Are Required for Drosophila IKK Activation**—We also carried out experiments to determine whether Bendless and dUEV1a are required for peptidoglycan-induced activation of the DmiKK complex. Previous studies have shown that peptidoglycan treatment induces the kinase activity of the endogenous DmiKK complex in S2 cells (21). Cells were transfected with dsRNAs corresponding to various mRNAs. After 48 h, these cells were treated with peptidoglycan for 15 min, and the endogenous DmiKK complex was immunoprecipitated and subjected to in vitro kinase assays using recombinant Relish protein as substrate. As shown in Fig. 3, Bendless or dUEV1a dsRNA treatment leads to a significant decrease in peptidoglycan-induced DmiKK kinase activity (lanes 8, 10, and 12), suggesting that Bendless and dUEV1a are required for peptidoglycan-induced DmiKK activation. As a control, DmiKK’ dsRNA treatment completely abolished peptidoglycan-induced DmiKK activation (lane 6). We conclude that the ubiquitin-conjugating enzymes Bendless and dUEV1a are specifically involved in the Drosophila IMD pathway, and they play a role upstream of the DmiKK complex.

**Bendless and dUEV1a Function Downstream of IMD but Upstream of dTAK1**—Overexpression of IMD in Drosophila results in the activation of antibacterial genes in the absence of bacterial infection (14). We show here that IMD overexpression, under control of the copper-inducible metallothionein promoter, can also strongly activate expression of the Diptericin gene in S2 cells. This stable cell line therefore provides a useful tool to perform an epistatic analysis to determine the position of Bendless/dUEV1a complex relative to IMD in the Drosophila antibacterial signaling pathway. The IMD stable cells were first transfected with dsRNAs derived from various genes and then stimulated with copper or peptidoglycan, and IMD- and peptidoglycan-induced Diptericin gene activation was examined. As shown in Fig. 4, overexpression of IMD, via the addition of copper, leads to strong activation of the Diptericin gene. In fact, copper-induced IMD expression is as potent as peptidoglycan in driving diptericin expression (compare lanes 7 and 13). Cells transfected with LacZ dsRNA show a similar Diptericin expression profile compared with cells that were mock-treated (compare lanes 7 and 8 with lanes 13 and 14). Consistent with the observation that the DmiKK complex functions downstream of IMD, cells transfected with DmiKK’ dsRNA are severely defective in both IMD- and peptidoglycan-induced Diptericin expression (lanes 9 and 15). Furthermore, cells treated with dsRNAs derived from Bendless and dUEV1a genes display a significant reduction in both peptidoglycan- and IMD-mediated Diptericin gene activation (compare lanes 10–12 and lanes 16–18 with lanes 7 and 8 and lanes 13 and 14, respectively). These results indicate that the ubiquitin conjugating enzymes Bendless and dUEV1a function downstream of IMD in this signaling pathway.

It has been shown that overexpression of dTAK1 in Drosophila results in infection-independent Diptericin gene activation (16). We also observed that overexpression in S2 cells of truncated dTAK1 (dTAK1Δ), containing just the N-terminal kinase domain, led to both DmiKK activation and Diptericin gene expression. We therefore employed this cell line to determine the order of signaling components relative to dTAK1. As shown in Fig. 4B, in cells treated with Bendless dsRNA, peptidoglycan-induced Diptericin gene activation was significantly reduced (compare lane 8 with lanes 5 and 6). In contrast, dTAK1Δ-induced Diptericin activation was not significantly affected in these cells (compare lane 12 with lanes 9 and 10), suggesting that the Bendless is not required for dTAK1Δ-mediated antibacterial peptide
gene activation. These results suggest that the Bendless/dUEV1a complex functions upstream, or in parallel, to dTAK1.

dFADD and DREDD Function Upstream of the Drosophila IKK Complex—dFADD is the Drosophila homolog of mammalian FADD, an adaptor protein in the TNFR signaling complex. Recent studies have implicated dFADD in the Drosophila IMD pathway (33, 34). The dFADD protein has been shown to interact with DREDD, an apical caspase also required in the IMD signaling pathway (35, 36). To further examine the role of dFADD and DREDD in the Drosophila antibacterial pathway we studied the consequences of their inactivation in S2 cells. As expected, dFADD and DREDD dsRNA treatment caused a significant reduction in peptidoglycan-activated Diptericin expression (data not shown). Surprisingly, dFADD and DREDD were also required for activation of the IKK complex. dFADD dsRNA treatment abolished peptidoglycan-induced DmIKK activation (Fig. 5A, lane 8), and similar results were observed with DREDD dsRNA (lane 16). Taken together, these results show that dFADD and DREDD are essential components in the Drosophila antibacterial signaling pathway required for peptidoglycan-induced DmIKK activation.

dFADD and DREDD Function Downstream of IMD but Upstream of dTAK1—We next carried out experiments to place dFADD and Dред relative to IMD and dTAK1 in the IMD pathway, taking advantage of the observation that overexpression of either IMD or dTAK1Δ activates DmIKK in S2 cells. dTAK1Δ stable cells were first transfected with various dsRNAs, then treated with copper to induce transgene expression, and the endogenous DmIKK complex was then immunoprecipitated and its kinase activity examined. As shown in Fig. 5B, dTAK1Δ overexpression caused a significant activation of the DmIKK complex (lanes 1 and 2). Neither dFADD nor DREDD dsRNA affects dTAK1Δ-induced DmIKK activation (compare lanes 4 and 6 with lane 2). In sharp contrast, in a parallel experiment, either dFADD or DREDD dsRNA treatment was sufficient to inhibit IMD-induced DmIKK activation (Fig. 5C). We therefore conclude that similar to Bendless and dUEV1a, dFADD and DREDD function downstream of IMD but upstream of dTAK1.

DREDD Is Required for Peptidoglycan-induced JNK Activation—Recently it has been shown that peptidoglycan-induced activation of Drosophila innate immunity bifurcates downstream of dTAK1, leading to the activation of both NF-κB and JNK signaling pathways (17, 18, 37). The data presented above demonstrate that DREDD is required for peptidoglycan-induced DmIKK activation at a step upstream of dTAK1. This predicts that DREDD will also be required for peptidoglycan-induced activation of the JNK signaling pathway. To address this question, S2 cells were first treated with various dsRNAs to knockdown the expression of corresponding genes, and then treated with peptidoglycan. Total cell extracts were prepared and subjected to Western blotting analysis to examine the levels of full length Relish and phosphorylated JNK to monitor the activation of NF-κB and JNK signaling pathways, respectively. As shown in Fig. 6, IMD, DREDD, or DmIKKΔ RNAi block peptidoglycan-induced Relish processing, as indicated by the persistence of full-length Relish (upper panel, compare lanes 5, 8, and 11 with lane 2). Furthermore, in cells treated with GFP dsRNA, peptidoglycan stimulation leads to rapid and transient appearance of phosphorylated JNK (lower panel, lane 2), and DmIKKΔ RNAi causes a further increase of phosphorylated JNK levels in response to peptidoglycan treatment (lower panel, lane 11). This is consistent with a recent report that Relish negatively regulates the JNK pathway (38). In contrast, IMD RNAi leads to a significant reduction of phosphorylated JNK levels (lower panel, lane 5). This observation is in good agreement with the notion that DmIKKΔ functions downstream of dTAK1 in the NF-κB branch of the signaling pathway, whereas IMD functions upstream of dTAK1 and is required for both NF-κB and JNK activation in response to peptidoglycan. Most importantly, DREDD RNAi also abolishes peptidoglycan-induced JNK phosphorylation (lower panel, lane 8), showing that DREDD functions at a step upstream of dTAK1 in the peptidoglycan response and thus is required for peptidoglycan-induced JNK activation. We therefore conclude that DREDD functions upstream of dTAK1.

dFADD and DREDD Also Play a Role Downstream of dTAK1—DREDD has been shown to interact with Relish in S2 cells. Furthermore, a caspase cleavage site within Relish is required for signal-induced Relish processing (20). Thus, DREDD has been proposed to directly cleave Relish in response to peptidoglycan. We show here that DREDD plays a role upstream of dTAK1 in the IMD pathway; however, this does not exclude the possibility that DREDD also functions downstream of dTAK1 and contributes to Relish cleavage. We therefore examined whether DREDD or dFADD is required for dTAK1Δ-induced Diptericin activation. Various dsRNAs were first transfected into dTAK1Δ cells to allow for the knock-down of specific gene expression. Cells were then treated with copper or peptidoglycan, and Diptericin gene activation was examined by Northern blotting analysis. As shown in Fig. 7, dFADD or DREDD dsRNA treatment significantly reduces both peptidoglycan- and dTAK1Δ-mediated Diptericin activation (compare lanes 13, 14, 20, and 21 with lanes 8, 9, 15, and 16). In contrast, neither PGRP-LC nor IMD dsRNA significantly affected dTAK1Δ-mediated Diptericin expression (compare lanes 18 and 19 with lanes 15 and 16), although both gene products are absolutely required for peptidoglycan-induced Diptericin gene activation (compare lanes 11 and 12 with lanes 8 and 9). We therefore conclude that dFADD and DREDD play two distinct roles in the IMD pathway; these experiments show that they function downstream of dTAK1 in the IMD pathway, while the IKK and JNK assays demonstrate a role for DREDD and dFADD upstream of dTAK1 (Figs. 5 and 6).

DISCUSSION

Here we report the results of biochemical and epistatic analyses of the Drosophila immune response pathways using cultured Drosophila cells...
and RNAi. By differentially activating the IMD and Toll signaling pathways in Drosophila S2 cells, we show that Bendless (dUbc13) and dUEV1a are required for the Drosophila IMD signaling pathway. Using RNAi to target Bendless and/or dUEV1a significantly reduces the levels of peptidoglycan-induced antibacterial peptide gene expression and activation of the Drosophila IKK complex. This mechanism of IKK activation is highly conserved; in mammals Ubc13 and UEV1a are required for TNFα-, IL-1β-, and TCR-mediated IKK and NF-κB activation (24, 39).

This ubiquitin-dependent kinase activation does not involve proteasome-mediated degradation. Proteasome inhibitors do not block IKK activation, in flies or humans (20, 40, 41). Moreover, ubiquitination without degradation has previously been shown to activate the human IKK complex (29), and a similar Drosophila activity has been identified.³

³ N. Silverman and T. Maniatis, unpublished data.
can make only K63-linked polyubiquitin chains (42). Thus, it is likely that the Drosophila Bendless/dUEV1a E2 catalyzes the formation of K63-linked ubiquitin chains.

In a cell-free system, human TRAF6 was shown to be an E3 ligase that auto-ubiquitinates in conjunction with Ubc13/UEV1a. This results in the activation of TRAF6 and, in turn, the activation of TAK1. Activated TAK1 phosphorylates key serine residues in the activation loop of IKKβ, resulting in the activation of IKKβ. We suspect similar mechanisms are involved in the Drosophila IMD pathway. Here, we demonstrate that the Drosophila TAK1 homolog functions downstream of Bendless and dUEV1a. Furthermore, we observed that a Drosophila homolog of TAB2/TAB3 is also required for the IMD pathway.5 Interestingly, the C-terminal zinc finger domain of TAB2, which is conserved in the Drosophila protein, has recently been shown to bind specifically to K63-polyubiquitin chains (26). Strikingly, the Ferrandon group has found that a galere/dTAB2 mutant, which is defective in the IMD pathway, carries a mutation in this zinc finger domain.5

The Drosophila E3 ligase, analogous to human TRAF2 or TRAF6, which functions with Bendless and dUEV1a in the activation of dTAK1 and DmIKK remains to be identified. In Drosophila, the dTARF2 protein is the closest homolog of mammalian TRAF6, and it is the only Drosophila TRAF protein that contains the RING domain, typical of E3 ligases. However, RNAi knockdown and dominant-negative studies suggest that dTARF2 is not involved in either the IMD or the Toll signaling pathways in S2 cells (data not shown). In fact this gene is expressed at undetectably low levels in S2 cells (data not shown). In one previous study dTARF2 was reported to interact physically and functionally with Pelle, a key signaling component in the Toll signaling pathway that controls the antifungal immune response (43). However, these studies were based on overexpression experiments and in vitro binding assays, which might not reflect the physiological role of dTARF2. On the other hand, a recent publication demonstrated that dTARF2 mutants are not fully able to induce antimicrobial peptide genes following E. coli infection (15). However, these studies did not clearly determine whether dTARF2 is involved in the Toll or IMD pathways. Our data suggest that dTARF2 is not a critical component of the IMD pathway in S2 cells. Further studies, in cells and in flies, are necessary to elucidate the role of dTARF2 in Drosophila immunity.

We considered the possibility that other Drosophila RING-containing proteins might be involved in Drosophila immunity. However, RNAi knockdown studies with 10 different RING domain-encoding genes failed to block either the Toll or IMD pathways (data not shown). Finally, since the structure of the RING domain of Rad5 has been successfully modeled to fit into the structure of the dimeric ubiquitin-conjugating enzyme complex Ubc13/UEV1a (42), we reasoned that the potential ubiquitin ligase involved in the IMD pathway might physically interact with Bendless and dUEV1a. We therefore performed yeast two-hybrid screens using Bendless and dUEV1a as baits in an effort to identify their protein interaction partners. A Drosophila RING protein, CG14435, was identified in such screens. CG14435 interacts robustly with both Bendless and dUEV1a in yeast two-hybrid assays. Furthermore, the CG14435-Bendless and CG14435-dUEV1a interaction was confirmed by co-immunoprecipitation of overexpressed proteins in S2 cells (data not shown). However, RNAi-based studies suggest that CG14435 is not involved in the Drosophila innate immunity signaling pathways (data not shown). It has been shown that bendless flies display defective synaptic connectivity and abnormal morphology within the visual system, suggesting Bendless functions in a variety of developmental processes (30, 31). In addition, the Saccharomyces cerevisiae homologs of Bendless and dUEV1a have been implicated in DNA damage repair (44). Therefore it is possible that CG14435 is involved in some cellular processes other than immunity which require Bendless and dUEV1a. Further studies are necessary to elucidate the physiological role of CG14435 and to identify the ubiquitin ligase activity required for ubiquitination-dependent DmIKK activation.

As in the TRAF6 pathway, dTARF2 and/or other E3 ligases that function with Bendless and dUEV1a in the IMD pathway may be the target(s) of K63 polyubiquitination. Another possible target of Bendless/dUEV1a-mediated ubiquitination is the Drosophila IKKγ subunit (also known as NEMO in mammals) of the IKK complex. In mammals, it has recently been shown that NEMO is K63 polyubiquitinated by the Ubc13/UEV1a complex in response to Bcl10 expression or T-cell activation (39, 48). Other possible targets of ubiquitination by Bendless and dUEV1a in the IMD pathway include the Drosophila TAB2 homolog and IMD. Recently, it was shown that the two mammalian homologs of TAB2 and TAB3 were ubiquitinated or associated with other ubiquitinated proteins (45). In addition, the mammalian RIP1, which is homologous to IMD protein especially in its death domain, has recently been shown to be K63 polyubiquitinated and associated with TAB2 in a TNFα-dependent manner (39, 46). In any case, K63 polyubiquitin chains likely function to recruit the Drosophila TAK1/TAB2 complex, via the TAB2 K63 polyubiquitin binding domain (26), to either (or both) the upstream activators, such as IMD, and/or the downstream target of dTAK1 kinase activity, the Drosophila IKK complex.

As expected, the epistatic analyses presented here demonstrate that IMD functions upstream of all other components in the pathway except the receptor PGRP-LC, and is required for IKK activation. Moreover, Bendless and dUEV1a function downstream of IMD and upstream of dTAK1, as predicted from the model for Ubc13 and UEV1a in mammals. As shown previously, dTAK1 is required for activation of the Drosophila IKK complex and likely functions as the IKK kinase (18). Although it is established that dFADD and DRED2 are required for

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5 V. Gobert and D. Ferrandon, personal communication.

FIGURE 7. dFADD and DRED2 are required for dTAK1Δ-mediated antibacterial peptide gene activation. dTAK1Δ stable cells were transfected with various dsRNAs, then treated with peptidoglycan or copper. Northern blotting analysis was performed to examine the expression of the antibacterial gene Diptericin.
the IMD pathway, previous experiments have suggested that they function downstream of the DmIKK complex. For example, DREDD overexpression in flies leads to Dipterin gene expression in the absence of Gram-negative bacterial infection, and DREDD-mediated Dipterin gene activation requires neither the DmIKK complex nor dFADD (16, 33). Also, recent studies have shown that DREDD interacts with Relish, and that a caspase-cleavage site within Relish is required for peptidoglycan-induced Relish activation (20). Therefore, it was speculated that DREDD functions downstream of the DmIKK complex by directly cleaving DmIKK-phosphorylated Relish. This possibility is consistent with our observation that DREDD and dFADD are required for dTAK1Δ-mediated Dipterin gene activation. Surprisingly, we also demonstrate that dFADD and DREDD are required for peptidoglycan-induced DmIKK activation, arguing that DREDD and dFADD function upstream in the pathway. In addition, we show that DREDD is also required for peptidoglycan-induced JNK activation, but neither DREDD nor dFADD are required for dTAK1- or dTAK1Δ-mediated DmIKK activation, suggesting that dFADD and DREDD act at a step upstream of dTAK1 in response to peptidoglycan. Based on these observations, we propose that dFADD and DREDD play dual roles in the Drosophila antibacterial signaling pathway. On the one hand, dFADD transduces signals from IMD to DREDD, resulting in DREDD activation and enabling Relish cleavage; on the other hand, dFADD and DREDD contribute to peptidoglycan-induced DmIKK activation through a mechanism that remains to be elucidated. DREDD may function similarly to human Caspase-8, which is a DED-containing apical caspase similar to DREDD. Caspase-8 has recently been shown to be required for NF-κB activation in response TCR-signaling. This role of Caspase-8 requires the enzymatic activity of full-length protein Caspase-8 and is involved in recruiting the IKK complex to the upstream signaling complex of CARMA1, Bc110, and MALT1 (47). Interestingly MALT1 is also a caspase-like gene (sometimes referred to as a paracaspase), and it is thought to function as an E3-ligase accessory factor with Ubc13 and UEV1a in TCR-mediated NF-κB activation (39, 48). DREDD may similarly function as E3-ligase accessory factors with Bendless and dUEV1a as the E2, in the IMD pathway.

We propose the following scheme for the Drosophila antibacterial signaling pathway (Fig. 8). Peptidoglycan treatment or Gram-negative bacterial infection leads to the activation of the membrane-bound peptidoglycan-recognition protein, PGRP-LC. Activated PGRP-LC in turn transduces signal to IMD. IMD, in turn, interacts with dFADD and subsequently DREDD. We propose that IMD, dFADD and DREDD form a complex that contributes to dTAK1 activation, perhaps as part of an E3 ligase. This complex is likely to function in conjunction with Bendless/dUEV1a to activate dTAK1 and then DmIKK. Once activated, the Drosophila IKK complex phosphorylates Relish, which is subsequently cleaved. The N-terminal Relish cleavage product, an NF-κB transcription factor, then translocates to the nucleus where it activates antimicrobial peptide gene expression. In addition to their role in IKK activation, DREDD and dFADD are also proposed to function downstream in this pathway, in the signal-induced cleavage of phospho-Relish.

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