Immunity, Vol. 17, 575–581, November, 2002, Copyright ©2002 by Cell Press

The *Drosophila* Immune Defense against Gram-Negative Infection Requires the Death Protein dFADD

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Summary

Drosophila **responds to Gram-negative infections by mounting an immune response that depends on components of the IMD pathway. We recently showed that** ***imd*** **encodes a protein with a death domain with high similarity to that of mammalian RIP. Using a two-hybrid screen in yeast, we have isolated the death protein dFADD as a molecule that associates with IMD. Our data show that loss of** ***dFADD*** **function renders flies highly susceptible to Gram-negative infections without affecting resistance to Gram-positive bacteria. By genetic analysis we show that dFADD acts downstream of IMD in the pathway that controls inducibility of the antibacterial peptide genes.**

Introduction

Microbial infections in *Drosophila* activate a series of immune defense reactions that culminate in the production by the fat body (an equivalent of the mammalian liver) of a battery of small-sized cationic peptides with potent antifungal and antibacterial activities. Genetic analysis has shown that two distinct pathways, Toll and IMD, govern the challenge-dependent synthesis of these antimicrobial peptides (reviewed in Hoffmann and Reichhart, 1997, 2002). The Toll pathway controls the immune activation of the antifungal peptide *Drosomycin* and is required for resistance to fungi and Gram-positive bacteria (Lemaitre et al., 1996). The IMD pathway, which controls the inducibility of most of the antibacterial peptide genes, mediates the defense against Gram-negative infections (Lemaitre et al., 1995). Activation of both pathways largely depends on recognition of microbial cell wall components by distinct members of extracellular pattern recognition receptors, which belong to the family of the peptidoglycan recognition proteins (PGRPs) (Choe et al., 2002; Gottar et al., 2002; Michel et al., 2001; Ramet et al., 2002; Werner et al., 2000). The molecular mechanisms that lead to the challenge-dependent activation of the antibacterial peptide genes through the IMD pathway by Gram-negative sepsis have not been worked out. Expression of these effector genes requires signal-dependent cleavage and subsequent nuclear translocation of Relish, a member of the NF-κB family of latent transcriptional activators (Silverman et al., 2000; Stöven et al., 2000). Upstream of *Relish*, an IKK-signalosome equivalent, composed of proteins with structural similarities to mammalian IKKβ and IKKγ (encoded by the *ird5* and *kenny* genes, respectively), is required for survival to bacterial infections and for cleavage of Relish (Elrod-Erickson et al., 2000; Lu et al., 2001; Rutschmann et al., 2000a; Silverman et al., 2000). The upstream events that link Gram-negative infection to the signalosome-dependent cleavage of Relish are poorly understood at present.

We have recently identified the *imd* gene and shown that it encodes a protein with a death domain (DD) with high similarity to that of mammalian RIP (receptor interacting protein), a molecule which plays a role in both NF-κB activation and apoptosis (Georgel et al., 2001; Kelliher et al., 1998; Stanger et al., 1995). IMD acts upstream of two additional genes required for resistance to Gram-negative infections and antibacterial peptide gene expression: *dredd*, which encodes a *Drosophila* caspase-8 homolog (Chen et al., 1998; Leulier et al., 2000), and *dTAK1*, encoding a mitogen-activated protein 3 (MAP3) kinase with homology to mammalian TAK1 (Mihaly et al., 2001; Vidal et al., 2001). The precise functions of dTAK1 and DREDD as well as their reciprocal interactions in the IMD pathway have not yet been elucidated. As dTAK1 acts upstream of dIKKγ and dIKKβ, it has been proposed that this kinase may directly activate the IKK-signalosome equivalent in response to bacterial challenge. The presence in IMD of a DD with similarity to that of the adaptor protein RIP suggests that this molecule may be part of an extensive receptor-adaptor complex responsible to sense Gram-negative bacterial infections (Georgel et al., 2001).

To clarify the role of the *imd* gene and to identify new components of the IMD signaling pathway, we have used a two-hybrid protein interaction system in yeast. Using a construct encompassing the IMD death domain, we have isolated the death adaptor protein dFADD and shown that this molecule can interact in our experimental system with IMD through its conserved DD. We report that loss of *dFADD* function by double-stranded RNA interference (RNAi) and mutations in the *dFADD* gene impair inducible expression of the antibacterial peptide genes and render flies highly susceptible to Gram-negative bacterial infections, without affecting resistance to Gram-positive bacteria. Through genetic analysis we further show that dFADD acts downstream of IMD to activate the transcription of the antibacterial peptide

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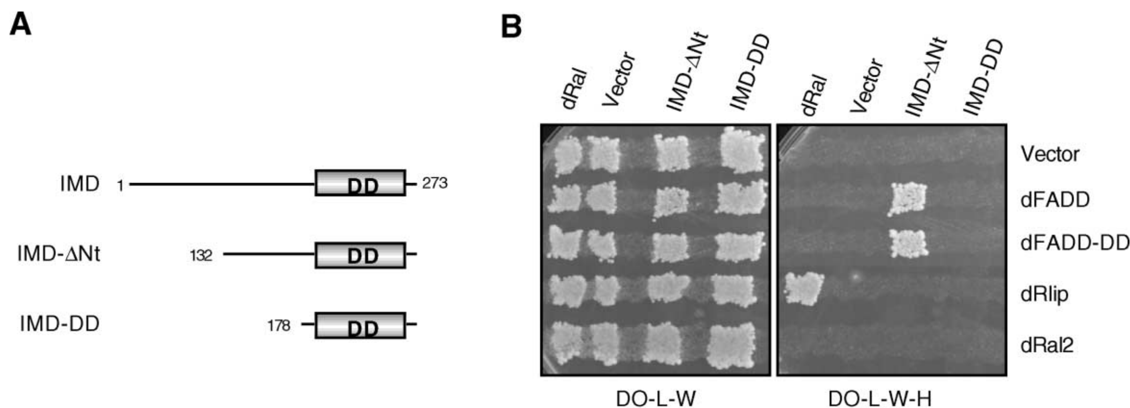

Figure 1. Specificity of IMD-dFADD Interactions in Yeast

(A) Schematic representation of the IMD protein and the bait constructs used in two-hybrid experiments. Amino acids at the boundaries are indicated.

(B) Histidine prototrophy tests. IMD-ΔNt, IMD-DD, and dRal were expressed in the L40 yeast two-hybrid reporter strain as fusions to LexA DNA binding domain, together with two of the dFADD clones isolated in the screen (dFADD and dFADD-DD) expressed as a fusion to GAL4 transcriptional activation domain (GAD). The dFADD clone encompasses the full-length *dFADD* coding sequence whereas dFADD-DD encodes only the death domain (DD). dRlip and dRal2 fused to GAL4 activation domain were used as positive and negative controls, respectively, for interactions with dRal. A plasmid expressing only the GAL4 activation domain was also used as a negative control. Growth on medium lacking histidine (dropout [DO]-Leu [L]-Trp [W]-His [H]) indicates a positive two-hybrid interaction.

genes. Taken together, these data indicate that dFADD functions as an essential component of the IMD pathway.

Results

Identification of dFADD as a Molecule that Interacts with IMD

We have screened a two-hybrid *Drosophila* embryonic cDNA library using as baits constructs corresponding to different portions of the IMD protein (Figure 1A). Out of seven million clones screened with IMD-ΔNt, a construct encompassing residues 132–273 of the IMD protein, we have isolated ten cDNAs corresponding to overlapping fragments of the *dFADD* coding sequence. Figure 1B shows that dFADD associates specifically with IMD-ΔNt, as evidenced by the lack of interaction with an unrelated protein, dRal (Jullien-Flores et al., 1995), and the empty vector. Sequencing analysis has shown that the minimal overlapping region among the *dFADD* clones corresponds to the C-terminal death domain of the dFADD molecule, indicating that this conserved domain is responsible for the interaction with IMD. The bait construct IMD-DD (178–273 aa), encompassing only the death domain of IMD, does not associate with dFADD, suggesting that residues between 132–176 of the IMD protein sequence might be required for interaction with dFADD or, alternatively, to allow a functional conformation of the IMD death domain. At present, however, we have reasons to favor the second possibility. In fact, in contrast to IMD-ΔNt, the IMD-DD construct showed in the two hybrid screen nonspecific interactions with several *Drosophila* proteins.

To confirm the ability of dFADD to interact with IMD, we have performed immunoprecipitation experiments in *Drosophila* S2 cells transfected with tagged versions of IMD and dFADD. Lysates from cells cotransfected with IMD and dFADD were immunoprecipitated with anti-IMD antibodies and blots revealed with antibodies directed against the cMyc-tag. The presence in these lysates of a strong band corresponding to dFADD indicates that dFADD can associate with IMD in this assay (Figure 2). In control experiments, no signal was detected in lysates from cells transfected with dFADD alone or with an empty vector. Due to the limitations of the experimental systems used in this study (i.e., two hybrid and IP assays from overexpressing cells), we cannot firmly conclude whether the interaction detected between IMD and dFADD is direct or if it is mediated by as yet unknown components.

Mutant Analysis Indicates that dFADD Is Required for the *Drosophila* Defense against Gram-Negative Bacteria

When this work started, fly lines with mutations in the *dFADD* gene were not available. To analyze the role of

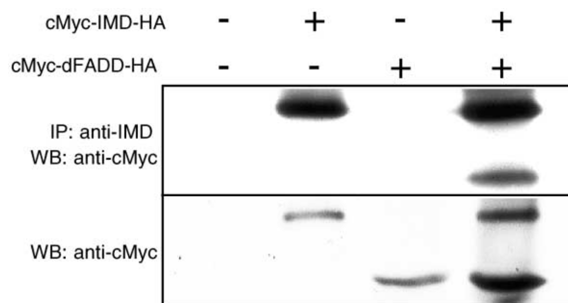

Figure 2. dFADD Specifically Associates with IMD in S2 Cells

Cells were transiently transfected with an empty vector, cMyc-IMD-HA, and cMyc-dFADD-HA either alone or together as indicated. Cell lysates were immunoprecipitated (IP) with beads coated with anti-IMD antibodies and proteins bound analyzed by Western blotting (WB) using anti-cMyc monoclonal antibodies. The bottom panel shows the correct expression of all the proteins in the cell lysates before immunoprecipitation.

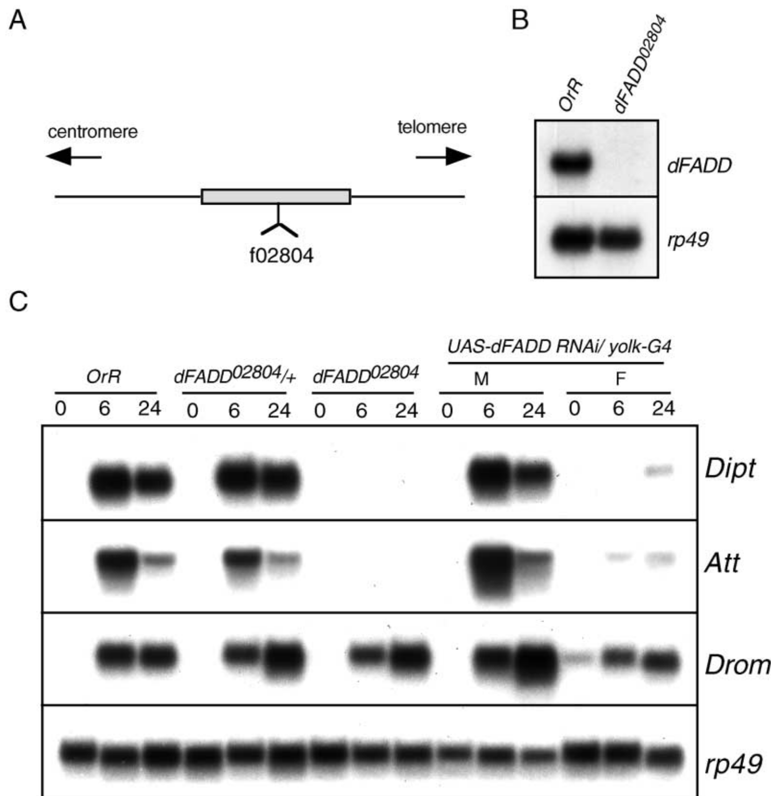

Figure 3. Expression of Antibacterial Peptides Is Affected in *dFADD* Mutant Flies

(A) Schematic representation of the *dFADD* locus. *dFADD* is encoded by a single exon (box) located in 93F14. The position of the transposon insertion in the *dFADD*^{02804} line is indicated (nt 464).

(B) Northern blot analysis shows that the *dFADD* gene is transcribed in wild-type adult flies (*OrR*) but is absent in the *dFADD*^{02804} mutant line. polyA+ RNA (10 μg) extracted from adult *OrR* and *dFADD*^{02804} flies was analyzed with the indicated probes (*rp49,* loading control).

(C) Northern blot analysis shows that inducibility of antibacterial peptide genes is compromised in *dFADD*^{02804} and UAS-dFADD RNAi/yolk-GAL4 flies. Total RNA (20 μg) extracted from unchallenged (0) and bacteria-challenged flies (6 and 24 hr after an infection with a mix of *E. coli* and *M. luteus*) was analyzed using the indicated probes (*Diptericin*, *Dipt*; *Drosomycin*, *Drom*; *Attacin*, *Att*; and *rp49* as a loading control). Similar results were obtained using different UAS-dFADD RNAi insertion lines. M, males; F, females.

dFADD in the *Drosophila* immune response, we have attempted to silence the *dFADD* gene by double-stranded RNA interference (RNAi). For this purpose, we have designed a RNAi construct predicted to form a double-stranded RNA after splicing, in which 500 bp of the *dFADD* cDNA were fused to the *chitin synthase* intron, followed by the same cDNA fragment in inverse orientation (see Experimental Procedures). This construct was cloned in the pUASp transformation vector (Rorth, 1998) and used to generate transgenic *UAS-dFADD RNAi* fly lines. Overexpression of the *UAS-dFADD RNAi* transgene in the fat body of female flies by the *yolk-GAL4* driver (Georgel et al., 2001) resulted in a destabilization of the *dFADD* transcript (data not shown). When challenged with a mixture of the Gram-negative bacteria *Escherichia coli* and the Gram-positive bacteria *Micrococcus luteus* (mix), *UAS-dFADD RNAi/yolk-GAL4* female flies showed a strongly reduced inducibility of the antibacterial peptide genes *Diptericin* and *Attacin*, as compared to wild-type flies (Figure 3C). Expression of the antifungal peptide gene *Drosomycin*, which is induced by infections with fungi and Gram-positive bacteria through the Toll pathway (Michel et al., 2001; Rutschmann et al., 2002), was not significantly affected in these flies. Parallel experiments with male flies yielded no reduction in *Diptericin* and *Attacin* expression in challenged flies, as expected by the lack of activity of the *yolk-GAL4* driver in males. To determine whether the compromised inducibility of the antibacterial peptide genes correlated with an increased susceptibility to Gram-negative bacterial infections, we have performed survival experiments. As shown in Figure 4A, *UAS-dFADD RNAi/yolk-GAL4* female flies showed a severe susceptibility to *E. coli* infections and close to wild-type resistance to Gram-positive bacteria *Streptococcus faecalis* (data not shown).

Recently, from a modified PiggyBac (Wart Hog) insertion library (S. Thibault, personal communication), we have isolated a mutant line, *dFADD*^{f02804}, with an insertion in the *dFADD* coding sequence, which results in the disruption of the gene (Figure 3A). Northern blot analysis confirmed the lack of the *dFADD* transcripts in the *dFADD*^{f02804} homozygous flies, which indicates that the transposon insertion generates a null mutation in the *dFADD* gene (Figure 3B). As illustrated in Figure 3C, analysis of antimicrobial peptide gene expression upon challenge with a mixture of *E. coli* and *M. luteus* showed that *Diptericin* and *Attacin* inducibility was completely abolished in *dFADD*^{f02804} homozygous flies. By contrast, *dFADD*^{f02804} heterozygous flies expressed wild-type levels of the *Diptericin* gene. Notably, inducibility of the antifungal peptide gene *Drosomycin* was not affected in this line. We have analyzed the survival rates of *dFADD*^{f02804} homozygous flies after immune challenge. As shown in Figure 4, *dFADD*^{f02804}/*dFADD*^{f02804} flies are highly susceptible to *E. coli* infections, and all the flies have died 3 days after infection. The same experiment showed that the survival curves of *dFADD*^{f02804} homozygous flies are similar to those of *key*^{1} flies, which carry a strong mutation in the gene encoding the *Drosophila* IKKγ homolog (*kenny*) (Rutschmann et al., 2000a). Comparison of survival curves of *dFADD*^{f02804} homozygous flies and *UAS-dFADD* RNAi/*yolk-GAL4* female flies, shows an increased susceptibility to Gram-negative bacterial infections in the *dFADD*^{f02804} mutant flies, indicating that the *dFADD* RNAi construct is not able to fully silence the *dFADD* gene. As shown in Figure 4B, survival rates of flies challenged with the Gram-positive bacteria

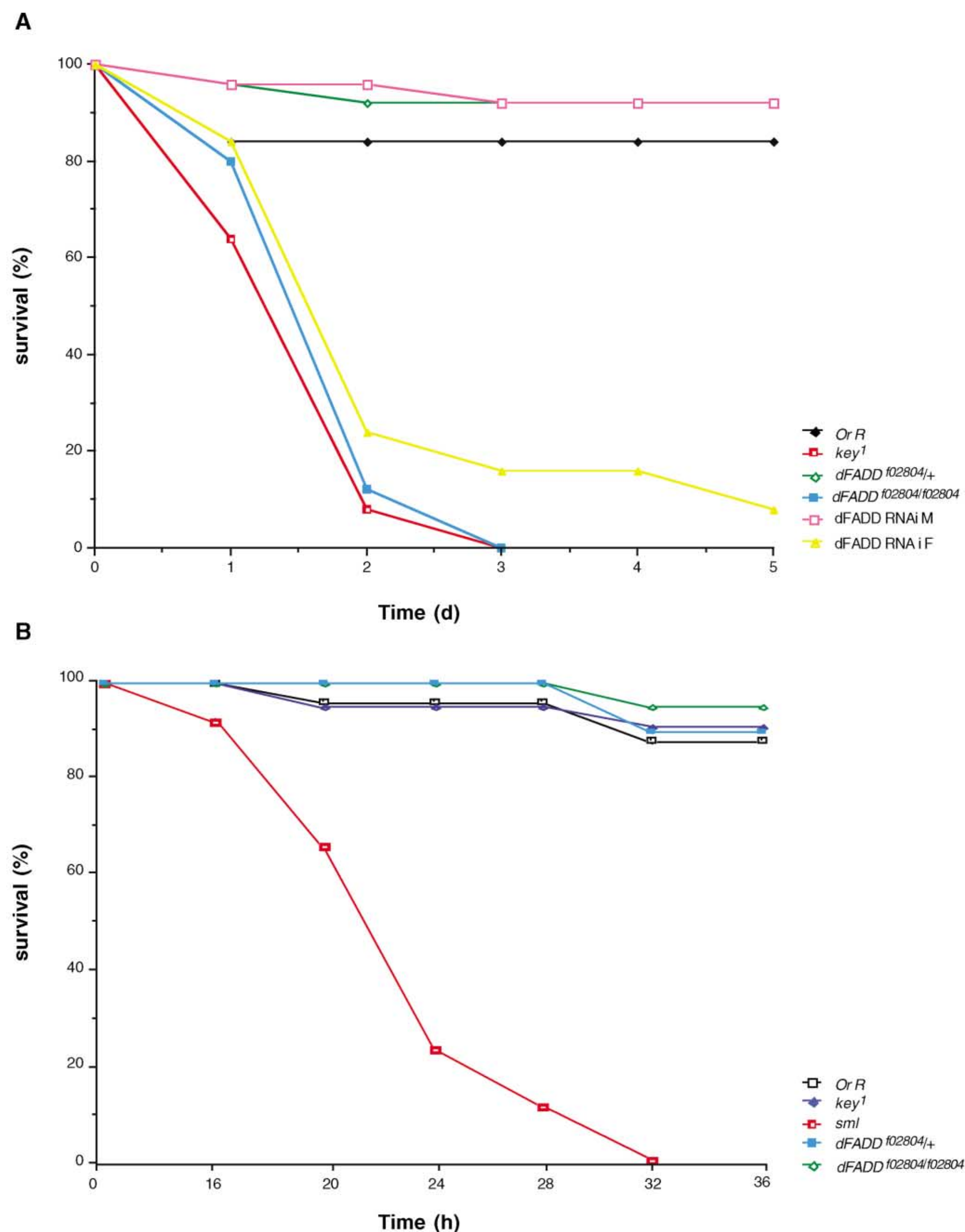

Figure 4. *dFADD* Mutant Flies Are Highly Susceptible to Infections by Gram-Negative Bacteria

For survival experiments, 25 flies of the indicated genotypes were immune challenged with (A) *E. coli* or (B) *Streptococcus faecalis*. Surviving flies were counted up to 5 days after infection. The results shown in the figure are representative of three independent experiments.

show that *dFADD*^{f202804} mutant flies are resistant to this type of infection. These results, together with the data presented above, demonstrate that the *dFADD* gene is a component of the IMD signaling pathway and is required for the fly host defense against Gram-negative bacteria.

dFADD Acts Downstream of IMD in the Activation of the Antibacterial Peptide Genes

We have previously shown that overexpression of the *imd* gene in flies leads to challenge-independent expression of the antibacterial peptide genes but not of that of the antifungal peptide gene *Drosomycin* (Georgel et al., 2001). We have used the dominant effect of *imd* overexpression to establish the epistatic relationship between the *dFADD* and the *imd* genes. As illustrated in Figure 5, when we overexpressed *imd* using the *yolk-GAL4* driver in flies carrying a copy of the *UAS-dFADD RNAi* transgene, we did not observe challenge-independent expression of the *Diptericin* gene. Inducibility of this peptide gene upon infection with a bacterial mix was also abolished in *UAS-IMD/UAS-dFADD RNAi/yolk-*

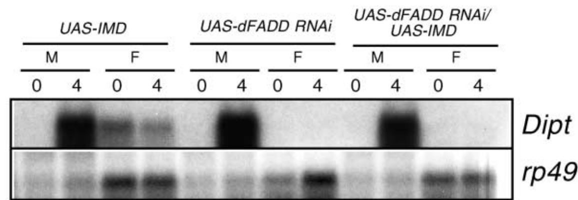

Figure 5. Epistatic Interactions of dFADD and IMD

Northern blot analysis of total RNA (20 μg) extracted from unchallenged (0) or 4 hr bacterial mix-challenged flies. Transcription of *Diptericin* (*Dipt*) was compared in UAS-IMD/yolk-GAL4, UAS-dFADD RNAi/yolk-GAL4, and UAS-dFADD RNAi/UAS-IMD/yolk-GAL4 female flies (F). Males flies (M) of the same genotype were used as control. *rp49* was used as a loading control. The expression of the *Drosomycin* gene was not affected in these experiments (data not shown).

GAL4 female flies. These results indicate that the *dFADD* gene acts downstream of *imd* in the pathway that controls expression of the antibacterial peptide genes.

Discussion

The major objective of this study was to clarify the role of IMD in the IMD signaling pathway through the characterization of IMD-interacting molecules. Here we report the identification by a two-hybrid screen of dFADD as an IMD-interacting molecule and demonstrate, through mutation analysis, that it plays a mandatory role in the fly defense against Gram-negative bacterial infections.

In mammals, the cytosolic adaptor protein FADD has a critical role in signaling from Fas and other members of the death receptor family, such as the TNF-R1 (Chinnaiyan et al., 1995; Strasser and Newton, 1999). FADD has two conserved domains that can act in homotypic protein-protein interactions. The C-terminal death domain mediates binding of FADD to the homologous domain present in the cytoplasmic portion of the death receptors. The N-terminal death effector domain (DED) is needed to recruit apical caspases, such as caspase-8, to the receptor-adaptor complex. This last event triggers the sequential activation of the executioner caspases, which in turn leads to the rapid induction of cell death by apoptosis (Nagata, 1997). The precise function of FADD is at the moment controversial. Studies on FADD knockout mice have proved the essential role of FADD in Fas- and TNFR1-dependent apoptotic cell death (Yeh et al., 1998; Zhang et al., 1998). Surprisingly, these reports together with studies on transgenic mice expressing a dominant-interfering mutant of FADD have also revealed that dFADD may play additional roles in embryonic development, cell survival, and T cell proliferation (Newton et al., 1998). Recently, it has also been reported that, depending on the experimental conditions and on the type of cell line tested, FADD can function as either a negative or a positive regulator of NF-κB function (Bannerman et al., 2002; Chaudhary et al., 2000; Hu et al., 2000; Schaub et al., 2000). How FADD might mediate distinct pathways of death and growth is at the moment the object of intense research.

In *Drosophila*, a homolog of FADD, dFADD, has recently been identified (Hu and Yang, 2000). This molecule shares an overall structure similarity with that of mammalian FADD, particularly within the C-terminal death domain. So far, the role of dFADD in *Drosophila* has not been fully elucidated. In cell culture assays, this molecule has been reported to interact with the caspase-8 homolog DREDD through a novel domain, the death-inducing domain (DID), present both at the N terminus of dFADD and in the prodomain of DREDD (Horng and Medzhitov, 2001; Hu and Yang, 2000). In experiments with mammalian cell lines, dFADD was shown to be able to induce DREDD cleavage and to enhance the cell death activity of this molecule. This is, however, in contrast with the finding that overexpression of DREDD and dFADD does not induce cell death in *Drosophila* cell lines (Hu and Yang, 2000).

The data reported in this study clearly show that dFADD plays a key role in the fly defense against Gram-negative bacterial infections. Flies in which the *dFADD* gene was silenced by double-stranded RNAi or disrupted by a transposon insertion were highly susceptible to infection by Gram-negative bacteria and failed to express the antibacterial peptide genes upon immune challenge. By contrast, Toll-dependent expression of the antifungal peptide gene *Drosomycin*, as well as survival against Gram-positive bacteria, was not affected in *dFADD* mutant flies. Thus, the phenotype of *dFADD* mutant flies recapitulates that of mutants of the IMD pathway, of which dFADD is an essential component.

We have clarified the epistatic relationship of *dFADD* with *imd* and shown that dFADD, which can interact with IMD through its C-terminal death domain, acts downstream of IMD to control antibacterial peptide gene expression. As previously mentioned, dFADD has also been reported to associate with DREDD (Horng and Medzhitov, 2001; Hu and Yang, 2000). We propose here that IMD, dFADD, and DREDD act as components of an upstream receptor-adaptor complex that is involved in sensing Gram-negative bacterial infections.

A new component of the IMD signaling pathway, namely PGRP-LC, has been recently identified and shown to act upstream of *imd* to activate transcription of the antibacterial peptide genes in response to Gram-negative infections (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002). PGRP-LC is a member of a diversified family of pattern recognition proteins, which are involved in sensing microbial infections in *Drosophila* and probably also in mammals (Liu et al., 2001; Werner et al., 2000). Overexpression of PGRP-LC induces challenge-independent expression of the antibacterial peptide genes. These data together with the presence in this molecule of a putative transmembrane domain have prompted the hypothesis that PGRP-LC may act as the receptor of Gram-negative infections. How PGRP-LC-mediated sensing of Gram-negative bacteria is connected to the adaptor complex composed by IMD, dFADD, and DREDD remains at the moment a challenging question. The identification of additional molecules in the IMD pathway will help to clarify how Gram-negative bacterial infections activate the fly immune response and may influence our current understanding of the mechanisms regulating innate immune responses in general.

Experimental Procedures

Plasmids and Reagents

For two-hybrid screens, the coding region of the *imd* gene corresponding to amino acids 132–273 and 178–273 was cloned into the pLexATet12 vector to generate, respectively, the bait constructs pLexA-IMD-ΔNt and pLexA-IMD-DD, which are C-terminal fusions to LexA DNA binding domain. RNA interference vector was constructed by inserting a PCR fragment encoding the *Drosophila chitin synthase* gene intron (CG7464, Flybase) into the transformation vector pP{UASp} (Rorth, 1998). This vector was shown to mediate highly efficient RNA interference-mediated gene suppression in vivo (Reichhart et al., 2002). The *dFADD* RNAi plasmid was constructed by inserting a 500 bp PCR fragment of the dFADD coding sequence flanked by BamHI and NheI sites between the NheI-BamHI sites (sense) and the XbaI-BglII sites (antisense), respectively. The P{UAS-dFADD.RNAi.SN} transgene was introduced into *w* flies by P-element-mediated transformation (Spradling and Rubin, 1982). For transfection of S2 cells, the coding sequences of the *dFADD* was cloned by PCR in pPac. c-Myc- and HA-epitope tags were introduced, respectively, at the N terminus and at the C terminus of the expression construct to generate the expression plasmids pPac-Myc-*dFADD*-HA. The construct pPac-Myc-*imd*-HA has been previously described (Georgel et al., 2001). For the production of anti-IMD polyclonal antibodies, the full-length *imd* coding sequence was cloned in the *E. coli* expression vector pDS56/RBII, 6xhis. His-tagged-*imd* was expressed in the M15 *E. coli* strain and purified by nickel chelate chromatography. Purified proteins were used to immunize rabbits. The nucleotide sequence of all the constructs was confirmed by sequencing. Primer sequences are available upon request.

Fly Strains and Procedures

Flies were grown on standard medium at 25°C, except for specific experiments. For RNAi experiments, several independent lines carrying the P{UAS-dFADD.RNAi.SN} transgene, *w*; *P{UAS-dFADD RNAi.SN}* (UAS-dFADD.RNAi) were crossed with the driver line *w*; *P{GAL4-YP$_1$.JMR}20* (*yolk-GAL4*) (Georgel et al., 2001). For epistasis experiments, the previously described *w*; *P{w+, UAS-imd.SN}F32* line (UAS-IMD) was crossed to *w*; *P{UAS-dFADD* RNAi.SN*}* lines and to the driver line *w*; *P{GAL4-YP$_1$.JMR}20*. The effect of transgene overexpression was analyzed in the progeny of adult flies kept at 29°C. *dFADD^{f02804}* flies carry a WartHog transposon insertion in the coding sequence of *dFADD*. *key^1* and *sml* flies were described elsewhere (Michel et al., 2001; Rutschmann et al., 2000a). Survival experiments were performed with *E. coli* and *S. faecalis* as previously described (Michel et al., 2001; Rutschmann et al., 2000a). Immune challenge for Northern blot analysis was carried out by pricking adult flies with a mixture of *E. coli* and *M. luteus*.

Two-Hybrid Screens

Two-hybrid screens and assays were carried out using a LexA-based system (Vojtek et al., 1993) and yeast strains L40ΔGAL4 (a gift of Drs. P. Legrain and M. Fromont-Racine) and Y187 (Clontech). A 0–24 hr *Drosophila* embryo cDNA library was a generous gift of Dr. S. Elledge. Standard techniques in yeast handling were used.

Transfection and Immunoprecipitation Experiments

S2 cells (InVitrogen) were grown and transiently transfected as previously described (Tauszig et al., 2000), using 0.1 μg of the cMyc-*dFADD*-HA and the cMyc-*imd*-HA expression plasmids. At 48 hr after transfection, cells were collected, washed in PBS, and lysed for 10 min on ice in 150 μl of lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 μg/ml pepstatin A, 1 μg/ml antipain, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cell debris was cleared by centrifugation, and 50 μl of supernatant was saved for Western blot analysis of the specific transgene expression. One hundred microliters of the cell lysates were incubated overnight at 4°C with sepharose beads (Sigma, St. Louis, MO) coated with protein A (Sigma) immunopurified rabbit anti-IMD polyclonal antibodies. After extensive wash in lysis buffer, immunoprecipitated proteins were eluted from the beads by boiling in Laemmli buffer, separated by 7.5% denaturing SDS-PAGE, and electroblotted onto a nitrocellulose filter. The membrane was probed with the monoclonal c-Myc antibody (Roche), and proteins were revealed by enhanced chemiluminescence (Amersham, ECL).

RNA Preparation and Northern Blot Analysis

RNA were prepared and Northern blots carried out as previously described (Rutschmann et al., 2000b). The *ribosomal protein 49* (*rp49*) probe was used as loading control.

Acknowledgments

We are grateful to A. Meunier, M.E. Moritz, and R. Sillas for technical assistance. This work was supported by the Centre National de la Recherche Scientifique and by grants from the NIH program project (1PO1 AI44220) and the European Commission Training and Mobility of Researchers Programme (ERB4061PL95). We also thank EntoMed and Exelixis for financial support. S.N. is the recipient of a Marie Curie fellowship.

Received: June 3, 2002
Revised: September 13, 2002

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