

# *Drosophila* Immune Deficiency (IMD) Is a Death Domain Protein that Activates Antibacterial Defense and Can Promote Apoptosis

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## Summary

We report the molecular characterization of the *immune deficiency (imd)* gene, which controls antibacterial defense in *Drosophila*. *imd* encodes a protein with a death domain similar to that of mammalian RIP (receptor interacting protein), a protein that plays a role in both NF- $\kappa$ B activation and apoptosis. We show that *imd* functions upstream of the DmIKK signalosome and the caspase DREDD in the control of antibacterial peptide genes. Strikingly, overexpression of *imd* leads to constitutive transcription of these genes and to apoptosis, and both effects are blocked by coexpression of the caspase inhibitor P35. We also show that *imd* is involved in the apoptotic response to UV irradiation. These data raise the possibility that antibacterial response and apoptosis share common control elements in *Drosophila*.

## Introduction

Over the past decade, *Drosophila* has evolved as a highly attractive model for the study of innate immunity (Hoffmann et al., 1999). Like other insects, *Drosophila* lacks adaptive immune responses, but is remarkably resistant to microbial infections. Prominent among the defense reactions are the phagocytosis and/or encapsulation of invading microorganisms by hemocytes (blood cells) and the massive synthesis of antimicrobial peptides by the fat body (a functional equivalent of the liver). This synthesis is induced within a few hours following an immune challenge and the peptides are released into the hemolymph, where their combined concentrations reach 300  $\mu$ M by 24 hr after a challenge. The peptides are mostly small in size and cationic, with a relatively broad spectrum of activity. For convenience, the *Drosophila* peptides are grouped into peptides with antifungal activities, namely Drosomycin and Metchnikowin, and peptides exhibiting predominantly, but not exclusively, antibacterial activities, namely Cecropin, Diptericin, Defensin, Attacin, and Drosocin (see review in Hoffmann and Reichhart, 1997). Barrier epithelia, such

as the gut, genital tract, tracheal epithelium, and epidermal cell layer, also participate in host defense, by producing various combinations of antimicrobial peptides when challenged by microorganisms (Ferrandon et al., 1998; Tzou et al., 2000).

In the mid 1990s, it became apparent that two distinct pathways control the antifungal and antibacterial responses (Lemaître et al., 1995, 1996). Indeed, it was found that the dorsoventral regulatory gene cassette *spätzle/Toll/cactus* directs the potent antifungal response in *Drosophila* adults, whereas the antibacterial defense is largely independent of these genes (Lemaître et al., 1996). A mutation was discovered at that time, and referred to as *imd* (*immune deficiency*) in which the antibacterial, but not the antifungal response, was compromised (Lemaître et al., 1995). Since the initial description of the *imd* mutation, four additional genes have been shown to participate in the antibacterial defense of *Drosophila*: (1) *ird5*, a gene encoding a homolog of mammalian IKK $\beta$  (Lu et al., 2001; Silverman et al., 2000); (2) *kenny*, a homolog of IKK $\gamma$ /NEMO (Rutschmann et al., 2000a; Silverman et al., 2000); (3) *dredd*, which is structurally related to vertebrate caspase-8 (Elrod-Erickson et al., 2000; Leulier et al., 2000); and (4) *Relish*, a member of the NF- $\kappa$ B family (Hedengren et al., 1999).

We now report the identification of the *imd* gene. We show that this gene encodes a 30 kDa protein with a death domain which exhibits significant similarity with the death domain of mammalian RIP (receptor interacting protein), a protein associating with the TNF $\alpha$  receptor 1 and with the Fas receptor (Hsu et al., 1996; Stanger et al., 1995). Epistasis experiments indicate that *imd* acts in the antibacterial defense upstream of the four genes mentioned above. Overexpression of the *imd* gene in wild-type and *imd* mutant flies results in forced transcription of all genes encoding antibacterial peptides, but not of that coding for the antifungal peptide Drosomycin. Similar to the dual function of RIP in vertebrates (Kelliher et al., 1998; Lin et al., 1999), our data also suggest a possible role for *imd* in apoptosis. Overexpression of *imd* induces transcription of the *reaper* gene (White et al., 1996) and causes the massive appearance of TUNEL-reactive cells in the adult fat body. The effects of ectopic expression of *imd* on the transcription of antibacterial peptides and on the generation of TUNEL-reactive cells are blocked by coexpression of the baculovirus antiapoptotic protein P35 (Hay et al., 1994). Finally, we observed that *imd* mutant flies are more resistant than control flies to UV irradiation, suggesting that *imd* may be involved in the apoptotic response to DNA damage (Brachmann et al., 2000; Zhou et al., 1999).

## Results

### Mapping of the Immune Deficiency Gene

The initial reports of the *imd* mutation (*imd*<sup>1</sup>; Corbo and Levine, 1996; Lemaître et al., 1995) included complementation analysis based on available deficiencies,

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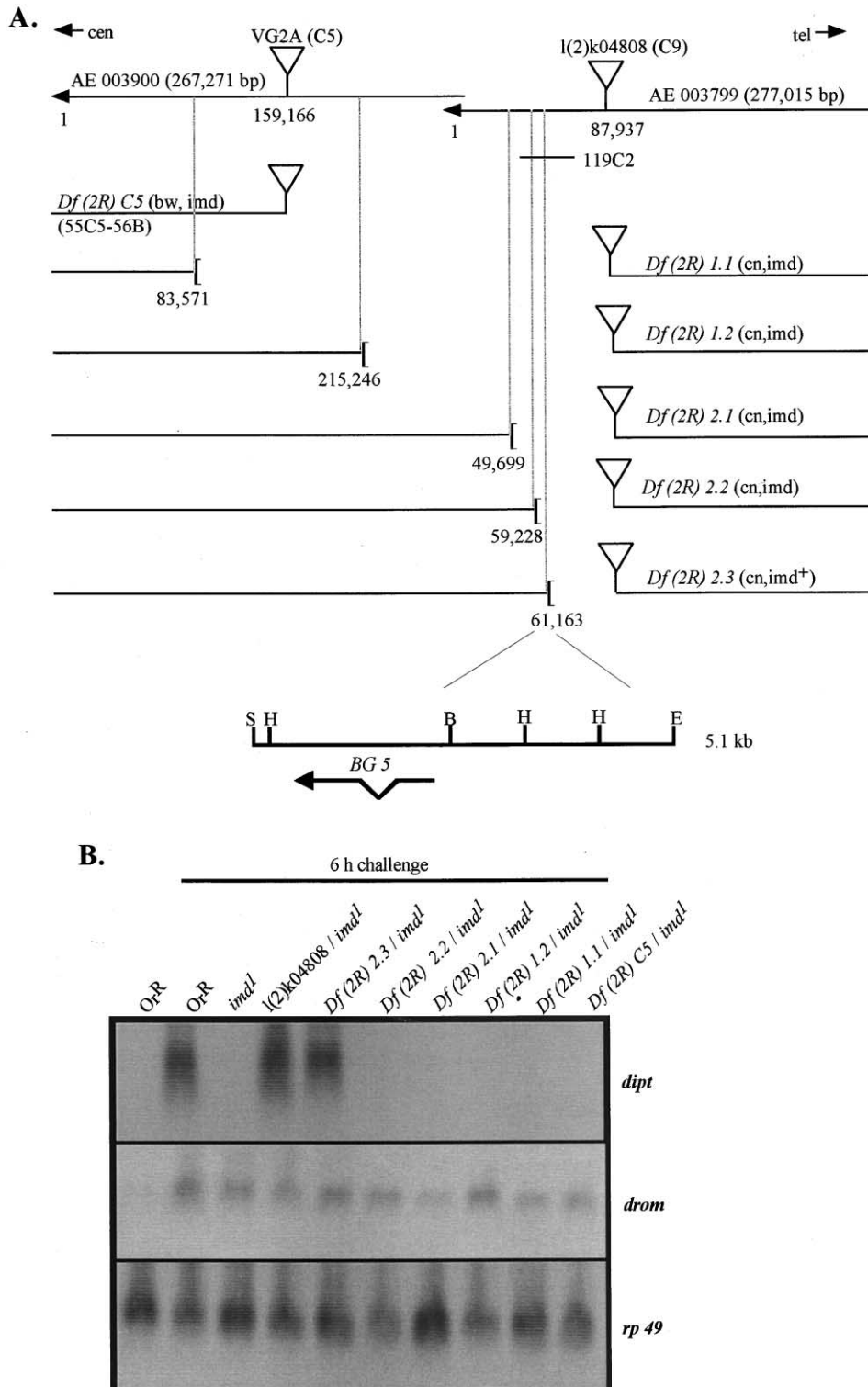


Figure 1. Genetic Mapping of the *imd* Gene

(A) Two P element-containing lines, VG2A at 55C5 and I(2)k04808 at 55C9, were used for the generation of deficiencies by male recombination. The position of the P elements is indicated on contigs of GenBank accession numbers AE003900 and AE003799 from the *Drosophila* genome sequence. Cosmid 119C2 served to subclone the 5.1 kb *Stu*I (S)-*Eco*RI (E) fragment used for phenotypic rescue of the *imd*<sup>1</sup> phenotype. H, HindIII; B, BamHI. The deficiencies used in this study are shown and the positions of the breakpoints are indicated according to the contigs. The phenotypes of the deficiencies over a *cn bw imd*<sup>1</sup> chromosome are indicated in parentheses.

(B) Deficiencies were tested for their capacity to complement the *imd*<sup>1</sup> mutation by Northern blot analysis. Total RNA (20  $\mu$ g) was extracted from unchallenged or bacteria-challenged flies (6 hr after an infection). Transcription of the *dip*tericin (*dip*t), *dros*omyacin (*drom*), and *rib*osomal protein 49 (*rp*49, loading control) genes was analyzed by hybridization with the indicated probes.

which mapped the mutation to the 55C–55E interval on the right arm of the second chromosome. To refine the mapping of the *imd* gene, we generated additional deficiencies by transposase-induced male recombination (Preston et al., 1996; see Experimental Procedures). The newly generated deficiencies were analyzed for complementation of the *imd*<sup>1</sup> mutation by monitoring the immune inducibility of the gene encoding the antibacterial peptide dipterin. Out of 60 deficiencies, two were of particular interest (see Figures 1A and 1B); one, *Df* (2R) 2.2, was the smallest deficiency which did not complement *imd*<sup>1</sup> (cn,*imd*), and the other, *Df* (2R) 2.3, was the largest deficiency complementing *imd*<sup>1</sup> (cn,*imd*<sup>+</sup>). These two deficiencies define a 1,935 bp region which contains at least part of the *imd* transcription unit. The genome annotation database of *Drosophila* (GadFly) predicts the presence of one gene in this region, *BG5*. *Df* (2R) 2.2 uncovers the upstream promoter region of this gene, since the breakpoint of the deficiency corresponds to the second nucleotide of the corresponding cDNA (EST GH20785). The nucleotide sequence of *BG5* codes for a 273-residue protein with an apparent 80-residue death domain (DD) in the C-terminal part (Figure 2A). This domain shows marked sequence similarity (33%) with the death domain of the mouse and human 75 kDa RIPs (receptor interacting proteins; Stanger et al., 1995). In contrast to mammalian RIP, the N-terminal region of the protein deduced from the *BG5* sequence has no apparent kinase domain. Significant similarity was also observed between the DD encoded by *BG5* and those of the death receptors TNFR1, DR3, and DR5, and the TNFR1- and Fas-associated DD proteins TRADD and FADD (Figure 2B).

We amplified the genomic region surrounding *BG5* in *imd*<sup>1</sup> homozygous and wild-type flies by PCR. The comparison of the two DNA sequences revealed a single nucleotide substitution, changing amino acid Ala31 in the wild-type to Val31 in the *imd*<sup>1</sup> mutant flies (Figure 2A; see Discussion). We also investigated the transcription profile of *BG5* and observed high expression levels in 6- to 24-hr-old embryos and at the time of pupariation. Note that the *imd* gene is also upregulated by immune challenge (Figure 2C).

#### Rescue of the Immune Deficiency Phenotype

We next generated *imd*<sup>1</sup> transgenic fly lines carrying a 5.1 kb genomic DNA fragment which contains the *BG5* open reading frame, 3 kb of upstream and 0.5 kb of downstream sequences, and no other predicted genes (see Figure 1). As shown in Figure 3A, immune inducibilities of the antibacterial peptide genes *dipterin*, *cecropin*, *attacin*, and *drosocin* were fully restored in these flies.

The induction of antimicrobial peptide genes is correlated in *Drosophila* to the binding of Rel proteins to  $\kappa$ B-related responsive elements in their promoter sequences (Engström et al., 1993; Georgel et al., 1993; Ip et al., 1993; Kappler et al., 1993). This type of binding does not occur in *imd*<sup>1</sup> mutant flies (Lemaitre et al., 1995). We have analyzed by gel shift experiments the ability of protein extracts from immune-challenged *imd*<sup>1</sup> and transgenic flies to bind to a labeled oligonucleotide containing a  $\kappa$ B-related motif from the *dipterin* promoter. As illustrated in Figure 3B, the binding capacity was rescued in the transgenic flies and was specific for

the nucleotide sequence, as evidenced by incubation with a mutated probe.

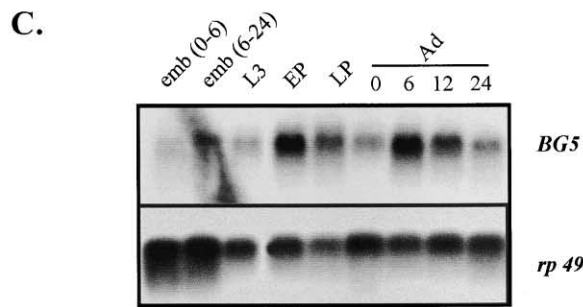
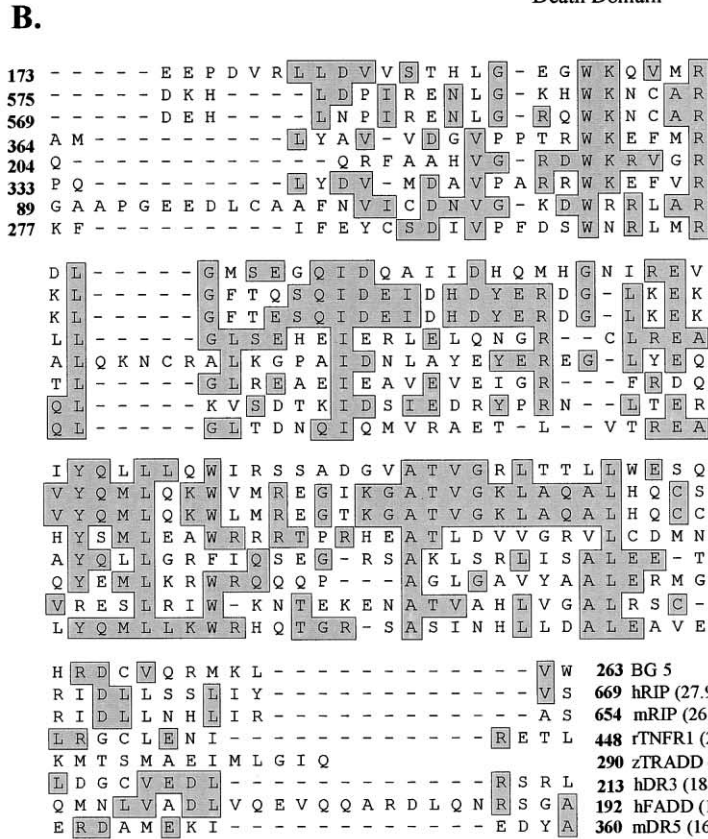
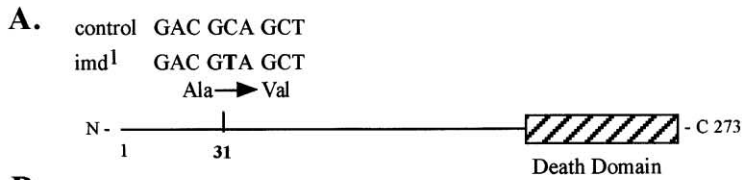
*imd*<sup>1</sup> flies are susceptible to infection with bacteria (Lemaitre et al., 1995, 1996; Leulier et al., 2000; Rutschmann et al., 2000a). We have analyzed the survival to infection with *E. coli* and *M. luteus* (mixture) of *imd*<sup>1</sup> and transgenic flies carrying a wild-type copy of *BG5*. As expected, *imd*<sup>1</sup> flies were susceptible to infection in these experiments. However, the transgenic flies had a survival rate similar to that of wild-type flies (Figure 3C), demonstrating that *BG5* can rescue the *imd*<sup>1</sup> survival phenotype. We will therefore refer to *BG5* as the *imd* gene. The data presented in Figure 3C further show that the death rates of hemizygous *imd*<sup>1</sup>/*Df* (2R) 2.1 flies, which are similar to those of *dredd* and *kenny* alleles (Leulier et al., 2000; Rutschmann et al., 2000a), are lower than those of *imd*<sup>1</sup> homozygous flies. These data indicate that *imd*<sup>1</sup> is a hypomorphic mutation. The deficiency introduced in this context had no effect by itself, as evidenced by the survival curve of the hemizygous + / *Df* (2R) 2.1 flies.

#### Overexpression of *imd* Has a Dominant Effect on Antibacterial Peptide Genes

We generated wild-type and *imd*<sup>1</sup> mutant fly lines carrying a *UAS-IMD* transgene and analyzed the effects of overexpression of *imd* using different *GAL4* drivers (Brand and Perrimon, 1993). Expression of *imd* directed by the *hs-GAL4* driver in *imd*<sup>1</sup> mutant flies restored the immune inducibility of all the antibacterial peptides to levels comparable to wild-type (Figure 4A). Remarkably, in these flies, the transcription of all the antibacterial peptide genes occurred even in the absence of immune challenge, indicating that overexpression of *imd* has a dominant effect on the activation of the pathway controlling the antibacterial peptide genes. Note that *drosomycin* expression was not affected in these experiments, which is consistent with previous observations that *drosomycin* expression is independent of *imd* (Lemaitre et al., 1996). We also observed in *UAS-IMD/hs-GAL4* flies a constitutive expression of antibacterial peptide genes even in the absence of heat shock (Figures 4A and 4B). However, we did not detect spontaneous expression of these genes in any of the original fly lines carrying the *UAS-IMD* insertion. We therefore propose that the basal level of *GAL4* expression from the *hsp* promoter accounts for the *UAS-IMD*-dependent transcription of the antibacterial peptide genes. This assumption is supported by our observation that the *hs-GAL4* driver is able to sustain a basal level of expression of a *UAS-GFP* and a *UAS-lacZ* reporter gene in the absence of heat shock (data not shown). Overexpression of *imd* by *hs-GAL4* also had a dominant effect on antibacterial genes in wild-type flies (see Figure 4B).

We further analyzed the effect of overexpression of *imd* by selecting a tissue-specific driver, namely *yolk-GAL4*, which directs transcription of *UAS*-dependent genes in the fat body of adult females starting 3 to 5 days after hatching (see Experimental Procedures). As illustrated in Figure 4B (right panel), *dipterin* is strongly expressed in these flies in the absence of immune challenge, but not in unchallenged males of the same genotype.

Expression of most antimicrobial peptide genes is



induced by LPS in the *Drosophila* macrophage-like S2 cell line (Dimarcq et al., 1997). We transfected these cells with an *imd* expression vector and observed strong expression of *attacin*- (and *drosocin*-) *luciferase* reporter genes (Tauszig et al., 2000) in the absence of LPS (Figure 4C, panel i). In contrast, transfection with mutated *imd* (*imd*<sup>1</sup>) was unable to induce the *attacin-luciferase* reporter gene (Figure 4C, panel ii). Lack of induction of the *attacin* reporter gene by the *imd*<sup>1</sup> construct did not reflect the inability of the cells to synthesize the recombinant protein, as the wild-type and mutated IMD proteins were expressed at comparable levels in transfected

Figure 2. *BG5* Encodes a Novel Death Domain-Containing Protein

(A) Schematic representation of the protein encoded by the *BG5* gene showing the C-terminal death domain (DD). Sequence analysis of *BG5* in control, namely *OregonR* (*Or*<sup>R</sup>) or *cn bw*, and *imd*<sup>1</sup> flies reveals a single nucleotide modification (C to T, bold) changing Ala to Val at position 31 in the coding sequence.

(B) Sequence alignment of the DD of *BG5*, human RIP (GenBank accession number Q13546), mouse RIP (Q60855), rat TNFR1 (P22934), zebrafish TRADD (AAF66959), human DR3 (BAB40662), human FADD (Q13158), and mouse DR5 (NP064671). Identical residues are boxed in gray and the percentage of identity with *BG5* is given in brackets. The alignment was performed using the Ballast program (<http://igbmc.u-strasbg.fr:8080/ballast.html>).

(C) Expression profile and immune inducibility of the *BG5* gene. polyA<sup>+</sup> RNA (8 μg) extracted from early (0–6) or late (6–24) embryos, third instar larvae (L3), early (EP), or late (LP) pupae and adult (Ad) flies, unchallenged (0) or 6, 12, or 24 hr after a septic injury, was analyzed by Northern blotting with the indicated probes (*rp49*, loading control).

cells (see Figure 4C, panel iii). These results corroborate the in vivo data indicating that the Ala31 to Val31 substitution in the *imd*<sup>1</sup> allele fully accounts for the immune-deficient phenotype.

**The *imd* Gene Acts Upstream of DmIKKγ and the Caspase DREDD to Activate the Expression of Antibacterial Peptide Genes**

The dominant effect on antibacterial peptide genes of overexpression of *imd* driven by the *hs-GAL4* driver (even in the absence of heat shock) served to establish the epistatic relationships of *imd* with other genes in the

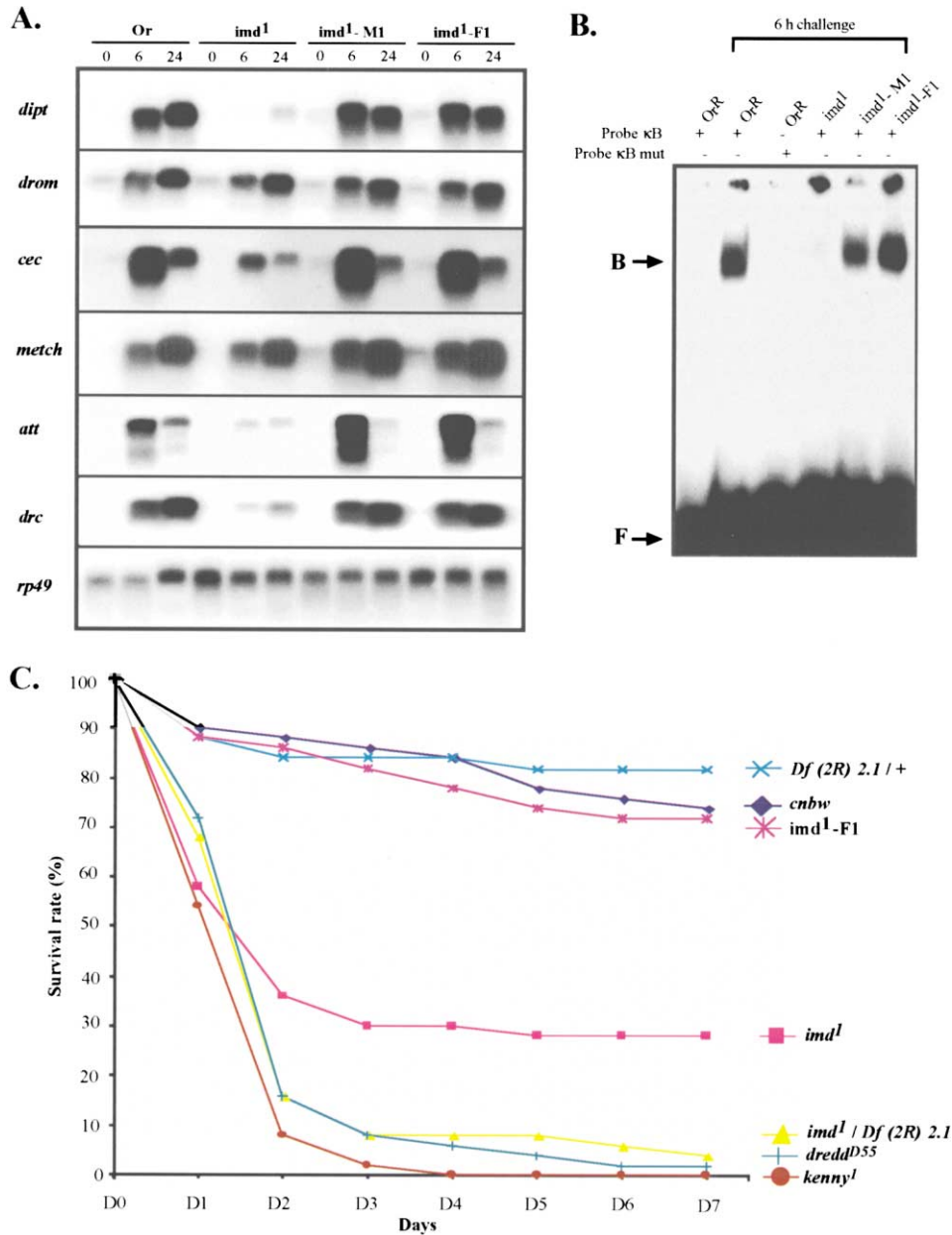


Figure 3. A Transgene Containing 5.1 Kb Genomic Sequences Harboring the *BG5* Gene Rescues *imd'* Phenotypes

(A) Total RNA (20 μg) extracted from unchallenged (0) and bacteria-challenged flies (6 and 24 hr after an infection) was analyzed by Northern blotting using the indicated probes (*dipteracin*, *dipt*; *drosomycin*, *drom*; *cecropin*, *cec*; *metchnikowin*, *metch*; *attacin*, *att*; *drosocin*, *drc*; and *rp49* as a loading control). *imd'-M1* and *imd'-F1* are two independent transgenic lines carrying the P{w+, *imd.5.1*} transgene. The immune inducibility of the *defensin* gene is also restored in these lines, as revealed by the expression of a *defensin-GFP* reporter gene (Tzou et al., 2000; data not shown).

(B) Gel shift experiments were performed with protein extracts from *OregonR* (*Or<sup>R</sup>*), *imd'* (*imd'*), and transgenic lines (*imd'-M1* and *imd'-F1*) 6 h after bacterial challenge or in unchallenged flies as a control. After incubation with radiolabeled oligonucleotides containing a wild-type or mutated κB binding site, bound (B) and free (F) probes were resolved on a nondenaturing polyacrylamide gel.

(C) For survival experiments, 50 flies of the indicated genotypes were immune challenged and placed at 29°C. Surviving flies were counted up to 7 days after infection. The results shown in the figure are representative of three independent experiments.

pathway. In *dredd* and *kenny* mutant flies, expression of all antibacterial peptide genes in response to bacterial challenge, as well as survival to infection, are strongly impaired (Elrod-Erickson et al., 2000; Leulier et al., 2000; Rutschmann et al., 2000a; Figure 2C). As illustrated in Figure 4D, the overexpression of *imd* was unable to

confer challenge-independent expression of *dipteracin* in *dredd<sup>D55</sup>* and *kenny<sup>1</sup>* mutant backgrounds. Furthermore, it did not restore immune inducibility of the *dipteracin* gene in these mutants. It is noteworthy that *drosomycin* expression is not affected in these flies. These results demonstrate that *imd* acts upstream of both

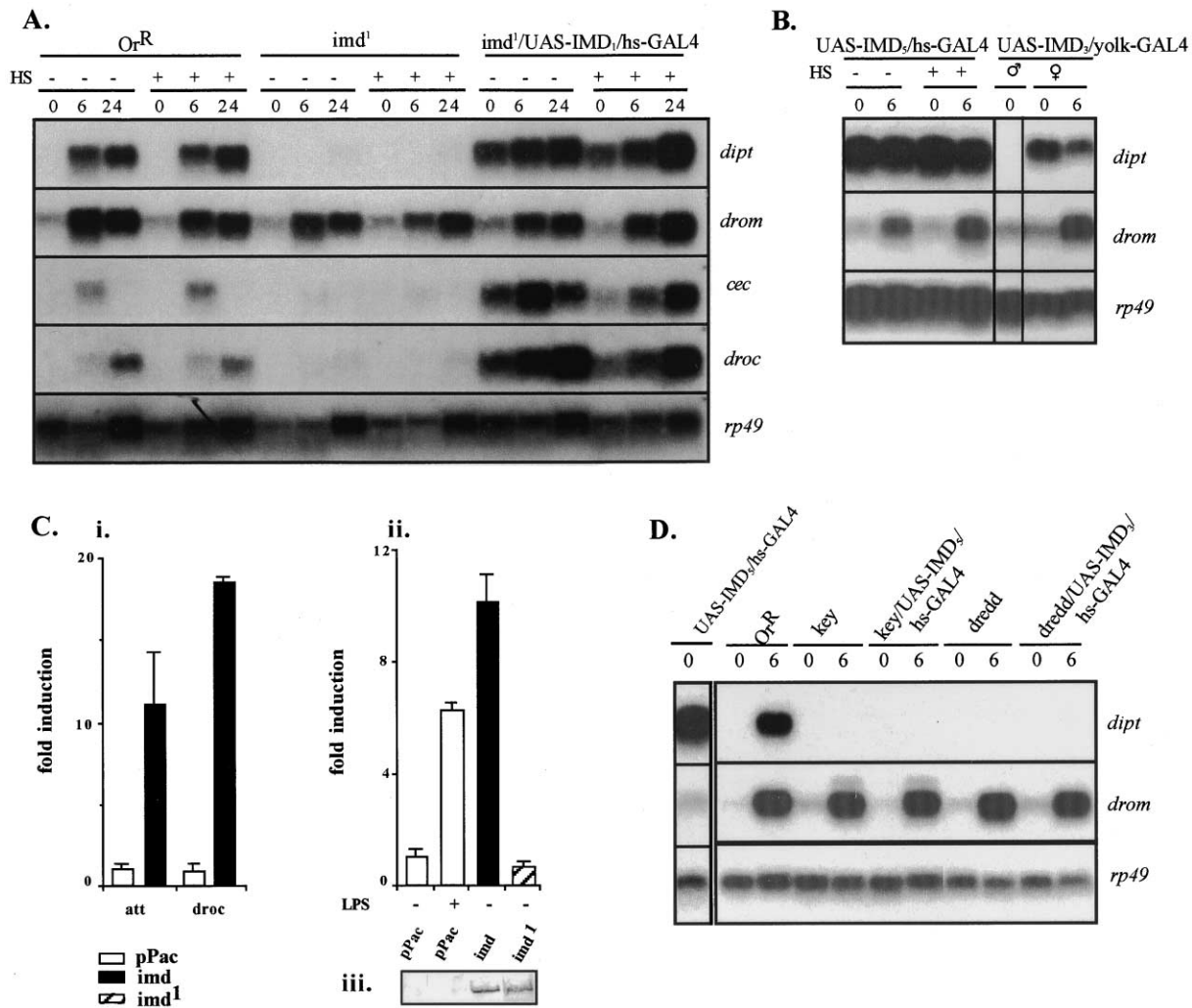


Figure 4. Dominant Effect of *imd* on Antibacterial Peptide Gene Expression and Epistatic Analysis

(A) Total RNA (20  $\mu$ g) extracted from unchallenged (0) and bacteria-challenged flies (6 and 24 hr after infection) was analyzed by Northern blotting and hybridized with the indicated probes. Transcription of *dip*tericin (*dipt*), *drosomycin* (*drom*), *cecropin* (*cec*), *drosocin* (*droc*), and *rp49* was compared in *OregonR*, (*Or<sup>R</sup>*), *imd<sup>1</sup>* (*imd<sup>1</sup>*), and *imd<sup>1</sup>/UAS-IMD<sub>2</sub>/hs-GAL4* flies in the absence of (–) or after heat shock (HS) treatment (+). (B) Northern blot analysis of total RNA (20  $\mu$ g) extracted from *UAS-IMD<sub>2</sub>/hs-GAL4* (left panel) and *UAS-IMD<sub>2</sub>/yolk-GAL4* (right panel). Transcription of *dip*tericin is detected in the absence of challenge in these flies. Unchallenged *UAS-IMD<sub>2</sub>/yolk-GAL4* males do not express *dip*tericin. Note that transcription of the antibacterial peptide genes in *imd<sup>1</sup>/UAS-IMD<sub>2</sub>/hs-GAL4* and *UAS-IMD<sub>2</sub>/hs-GAL4* flies is observed in the absence of HS ([A], and [B], right panel).

(C) Transfection of *imd* expression constructs in S2 cells. (i) An *imd* expression construct (*imd*) was compared to empty vector (pPac) for its ability to induce expression of *attacin-luciferase* (*att*) and *drosocin-luciferase* (*droc*) reporters (Tauszig et al., 2000) in the absence of LPS. (ii) S2 cells transfected with empty vector (pPac) or constructs expressing wild-type (*imd*) or mutated *imd* (*imd<sup>1</sup>*). LPS was added to the cells as indicated (+/–). The reporter gene was *attacin-luciferase*. Error bars represent the standard deviation of triplicate values. (iii) Western blot analysis of S2 transfected cells from (ii), using an anti-c-Myc mAb. Protein extracts from cells transfected with *imd* or *imd<sup>1</sup>* c-Myc-tagged expression constructs show a similar level of recombinant proteins. *imd*, black bars; *imd<sup>1</sup>*, hatched bars; pPac, white bars.

(D) Northern blot analysis of total RNA (20  $\mu$ g) extracted from unchallenged (0) or 6 hr bacteria-challenged flies without heat shock. Transcription of *dip*tericin, *drosomycin*, and *rp49* was compared in *UAS-IMD<sub>2</sub>/hs-GAL4*, *OregonR* (*Or<sup>R</sup>*), *key<sup>1</sup>* (*key*), *key/UAS-IMD<sub>2</sub>/hs-GAL4*, *dredd<sup>d955</sup>* (*dredd*), and *dredd/UAS-IMD<sub>2</sub>/hs-GAL4*. Note that two signals are detected with the *drosomycin* probe in *key* and *key/UAS-IMD<sub>2</sub>/hs-GAL4* flies, the upper one corresponding to the RNA of the *drom-GFP* reporter gene (Rutschmann et al., 2000a). Similar results were obtained when flies were subjected to heat shock or using a different UAS-IMD insertion line (data not shown).

*dredd* and *kenny* to activate the antibacterial peptide genes during an immune response. In keeping with these results, transcription of the *attacin-luciferase* reporter gene induced by overexpression of *imd* in S2 cells was abolished by an *ird<sup>5</sup>* (IKK $\beta$  homolog) dominant-negative expression construct (data not shown).

#### Overexpression of *imd* Can Induce Apoptosis

In the experiments described above, we also used transgenic flies in which *imd* expression was under the control of the ubiquitous *da-GAL4* driver. We were surprised to observe 100% lethality in these flies during early larval development. The lethality was partially rescued by

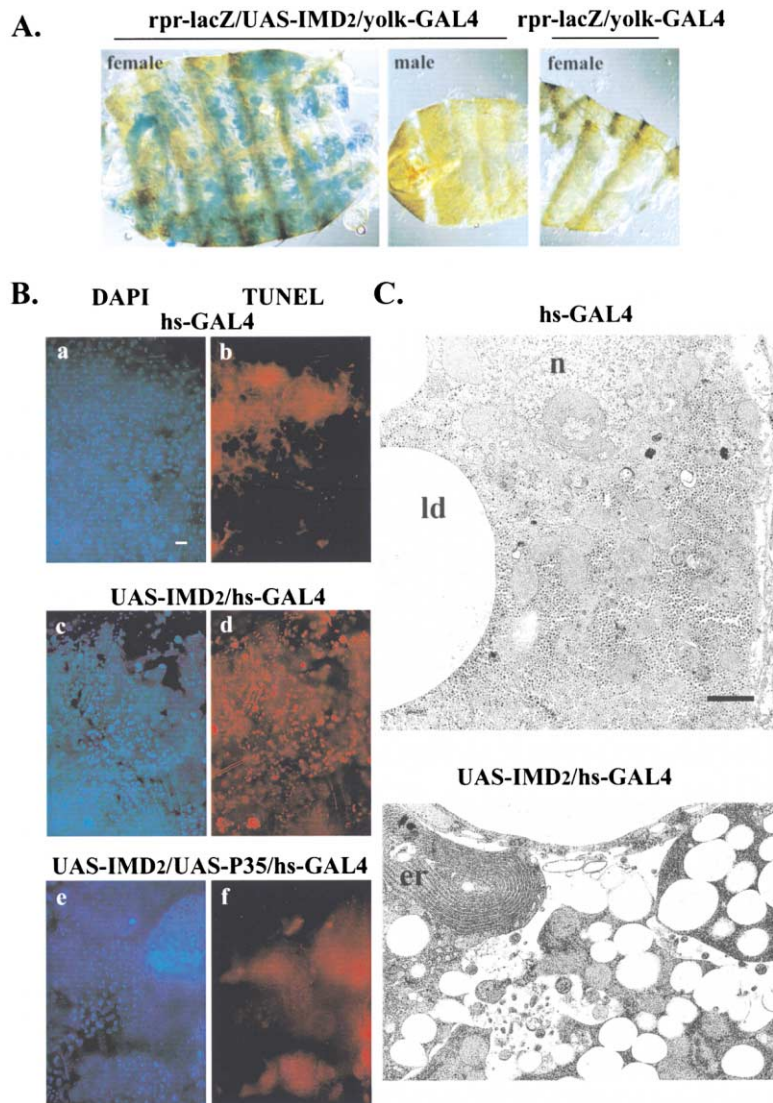


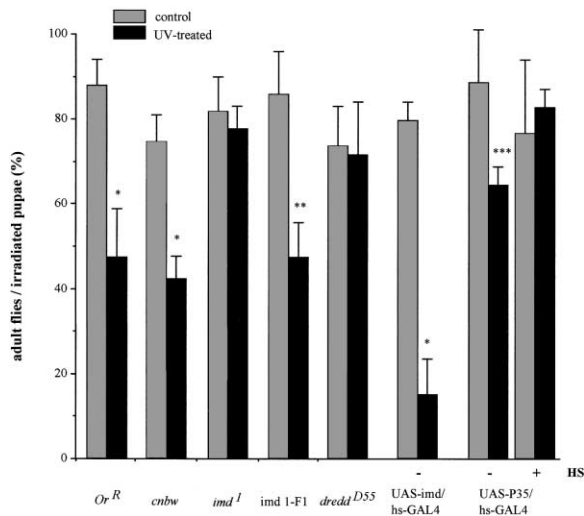
Figure 5. Overexpression of *imd* Induces *reaper-lacZ* Expression in Adult Fat Body and Promotes Apoptosis

(A)  $\beta$ -galactosidase staining (37°C for 30 min) of abdomens dissected from 4-day-old *rpr-lacZ/UAS-IMD<sub>2</sub>/yolk-GAL4* and *rpr-lacZ/yolk-GAL4* flies. Before dissection, flies were kept at 29°C overnight to enhance the activity of the GAL4 transcriptional activator. Lac-Z staining is only detected in females carrying both the active *yolk-GAL4* driver and the *UAS-IMD* transgene. Note the absence of staining in *rpr-lacZ/UAS-IMD<sub>2</sub>/yolk-GAL4* males and *rpr-lacZ/yolk-GAL4* females. *rpr*, reaper. (B) TUNEL staining of abdomens dissected from flies heat shocked at 37°C for 1 hr and subsequently placed at 29°C for 15 hr. Panels a and b: the *UAS-IMD* transgene is absent in *hs-GAL4* control flies. Panels c and d: expression of *imd* is driven by *hs-GAL4* in *UAS-IMD<sub>2</sub>/hs-GAL4* flies. Panels e and f: coexpression of *imd* and *p35* by the *hs-GAL4* driver in *UAS-IMD<sub>2</sub>/UAS-P35/hs-GAL4* flies. Nuclei were stained with DAPI. TUNEL-positive cells are labeled in red. TUNEL-positive cells were not detected in *UAS-IMD<sub>2</sub>/hs-GAL4* in the absence of heat shock (data not shown). The scale bar represents 25  $\mu$ m. (C) Transmission electron microscopy of fat body cells. Abdomens were dissected 40 hr after heat shock from control flies (*hs-GAL4*) and *UAS-IMD<sub>2</sub>/hs-GAL4* flies. Heat shock-dependent expression of *imd* induces the morphological changes of apoptosis in fat body cells, whereas *hs-GAL4* alone has no effect. The scale bar represents 1  $\mu$ m. er, endoplasmic reticulum; ld, lipid droplet; n, nucleus.

coexpression of the viral caspase inhibitor P35 (data not shown). This protein inactivates most of the executioner caspases of the death program (Hay et al., 1994). We further noted that overexpression of *imd* by the fat body-specific driver *yolk-GAL4* induced the transcription of a *reaper-lacZ* reporter (Nordstrom et al., 1996) in this tissue (see Figure 5A). Reaper is a key activator of apoptosis in *Drosophila* (White et al., 1996). A TUNEL analysis of transgenic flies overexpressing *imd* also revealed a remarkably large number of labeled nuclei in fat body cells, as compared to controls (Figure 5B). This effect was suppressed by coexpression of the antiapoptotic protein P35. TEM analysis revealed that the fat body cells exhibit the classical morphological aspects of apoptosis, that is, densification and fragmentation of the cytoplasm, membrane blebbing, and stacking of the endoplasmic reticulum (Figure 5C).

These observations raised the question as to whether the *imd*-dependent antibacterial response involves activation of an apoptotic program. However, when injecting gram-negative bacteria into flies, we never observed

appearance of TUNEL-positive cells in the fat body nor any induction of the *reaper* reporter gene, although the antibacterial peptide genes were fully induced (data not shown). We reasoned that the *imd*-dependent control of an apoptotic pathway could be triggered by stimuli different from bacterial infection. One well-established experimental system that induces apoptosis in *Drosophila* is DNA damage by UV irradiation (Brachmann et al., 2000; Manji and Friesen, 2001; Zhou et al., 1999). We subjected pupae to increasing doses of UV treatment and noted at 50,000  $\mu$ J/cm<sup>2</sup> a significant mortality in emerging flies. This lethality is, at least partially, linked to induction of apoptosis by the UV treatment, as it is significantly reduced in flies overexpressing the apoptosis inhibitor P35. Remarkably, when we subjected *imd* pupae to UV treatment, lethality was also significantly reduced. The reduction in the level of lethality was clearly related to the product of the *imd* gene, as first, in flies overexpressing *imd*, we observed an increased sensitivity to UV irradiation, and second, reintroduction of a wild-type copy of the *imd* gene into *imd*<sup>1</sup>



**Figure 6. Loss of *imd* Function Renders Flies Resistant to UV Irradiation**

Twenty-four-hour-old pupae of the indicated genotypes were untreated (light gray bars) or UV-irradiated with 50,000  $\mu\text{J}/\text{cm}^2$  (dark gray bars), and the percentage of insects surviving to adulthood was scored. For UAS-P35/hs-GAL4 flies, survival of pupae to UV irradiation was compared in the absence of and upon heat shock (HS) as indicated (+/-). Note that the higher level of survival observed in UV-irradiated UAS-P35/hs-GAL4 pupae in the absence of heat shock as compared to controls (*Or<sup>R</sup>* and *cnbw*) probably reflects the basal activity of the *hsp* promoter. The results represent the average of at least three independent experiments. The error bars represent the standard deviation. P values are calculated by comparing the survival rate of flies of each genotype with and without UV treatment (\* $P < 0.0005$ , \*\* $P < 0.001$ , \*\*\* $P < 0.005$ ).

mutant flies restores their sensitivity to UV irradiation to levels comparable to wild-type (see Figure 6). Interestingly, *dredd* mutant flies were also resistant to UV irradiation. In contrast to the clear effect of UV damage on apoptosis-related mortality, this treatment did not induce the expression of *dipterizin* in the absence of a microbial challenge (data not shown).

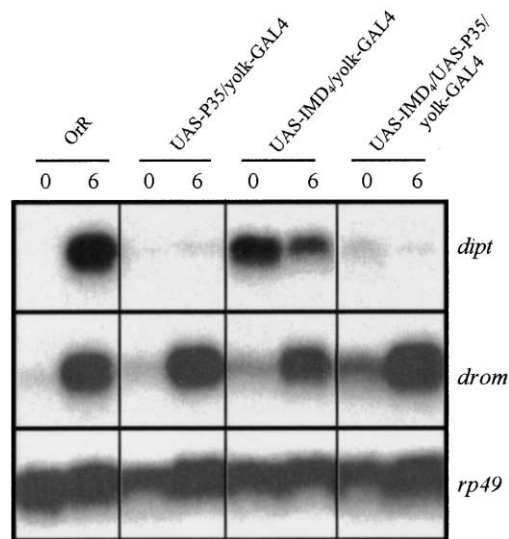
### P35 Abolishes Immune Inducibility of the Antibacterial Peptide Genes

The observations that the *imd*-induced cell death is antagonized by P35 raised the question as to whether P35 also affects the immune induction of antibacterial peptides. We indeed found that overexpressing *p35* in adult fat body cells blocked the challenge-dependent transcription of *dipterizin* (Figure 7). Furthermore, the induction of the *dipterizin* gene by overexpression of *imd* was also abolished by coexpressing *p35*. Note that induction of *drosomycin* by an immune challenge was not affected by P35. These results indicate that P35-sensitive caspases act downstream of *imd*.

### Discussion

#### IMD, a Novel *Drosophila* Death Domain-Containing Protein

Although the *imd* mutation was described in 1995 (Lemaitre et al., 1995), the identification of the corresponding gene has long remained elusive. The generation of



**Figure 7. Ectopic Expression of p35 Antagonizes *imd* and Blocks Immune-Inducible *dipterizin* Transcription**

Total RNA (20  $\mu\text{g}$ ) from unchallenged (0) and bacteria-challenged (6 hr after infection) female flies of the following genotypes: *OregonR* (*Or<sup>R</sup>*), UAS-P35/yolk-GAL4, UAS-*IMD*<sub>Δ</sub>/yolk-GAL4, and UAS-*IMD*<sub>Δ</sub>/UAS-P35/yolk-GAL4. Transcription of *dipterizin* (*dipt*), *drosomycin* (*drom*), and *rp49* was analyzed as indicated. Note that in this experiment, *drosomycin* expression levels from unchallenged flies (0) are higher than those observed in most experiments.

a large series of deficiencies has finally led to the precise mapping of the transcription unit within a 2 kb region. As argued above, the *imd* gene encodes a 30 kDa protein with a C-terminal death domain (DD). This 80-residue conserved domain is shared by a growing number of proteins (Feinstein et al., 1995). It is characterized by the presence of six sequential  $\alpha$  helices interrupted by loop regions and is usually located at the C terminus (Tartaglia et al., 1993). Death domain-containing proteins include death receptors, adaptor proteins, transcription factors, and structural proteins, which serve diverse cellular functions essentially by mediating homo- and heterotypic protein-protein interactions (Baldin et al., 1995). In addition to *imd*, four death domain-containing proteins have been described in *Drosophila*: first, the serine/threonine kinase PELLE, a homolog of the mammalian kinases IRAKs, which are associated with the IL-1 and the Toll-like receptors (Cao et al., 1996; Shelton and Wasserman, 1993). Second, the adaptor protein TUBE (Letsou et al., 1991). Third, DmFADD, the *Drosophila* homolog of FADD (Fas-associated death domain protein; Chinnaiyan et al., 1995; Hu and Yang, 2000). PELLE and TUBE play a role in the Toll signaling pathway both during embryogenesis and in the antifungal response (Grosshans et al., 1994; Lemaitre et al., 1996). The role of DmFADD is not fully elucidated. It has been reported to interact with the caspase DREDD (Hu and Yang, 2000). The fourth protein in the group associates an N-terminal death domain with a C-terminal TIR (Toll-Interleukin receptor homolog) domain and appears homologous to mammalian MyD88 (Wesche et al., 1997).

As discussed already, the sequence of the DD encoded by the *imd* gene is closest to that of mammalian



RIP, which reportedly interacts with the death receptors TNFR1 and Fas (Hsu et al., 1996; Stanger et al., 1995). It has been proposed that recruitment of RIP to the receptor complex plays an important role in mediating NF- $\kappa$ B activation (TNFR1) and/or apoptosis (Fas, TNFR1) by these receptors (Ashkenazi and Dixit, 1998; Grimm et al., 1996; Kim et al., 2000; Lin et al., 1999), and RIP has been shown to be mandatory for TNFR1-dependent NF- $\kappa$ B activation in response to TNF $\alpha$  (Kelliher et al., 1998; Ting et al., 1996). It is of relevance to note that the DD of IMD also shows significant homology to that present in TNFR1, TRADD, and FADD. In the mammalian system, TNFR1, TRADD, RIP, and FADD interact through their DDs in the context of inflammatory and apoptotic responses to TNF $\alpha$  (Nagata, 1997). Outside of the death domain, however, the IMD and RIP proteins are largely divergent. In fact, in contrast to RIP, the much shorter IMD lacks an N-terminal serine/threonine kinase domain (which, however, is dispensable for NF- $\kappa$ B activation; Hsu et al., 1996; Ting et al., 1996). Thus, structurally, IMD does not qualify as a *sensu stricto* homolog of RIP.

The sequence of *imd<sup>d</sup>* differs from wild-type *imd* by a single nucleotide substitution, changing Ala31 to Val31. Although this substitution could seem minor, the mutation fully accounts for the immune deficiency phenotype of *imd<sup>d</sup>* flies. This is indeed demonstrated by our observations that the introduction of a wild-type copy of *imd* into mutant *imd<sup>d</sup>* flies is sufficient to restore (1) immune inducibility of all the antibacterial peptides; (2) binding of protein extracts from immune-challenged flies to NF- $\kappa$ B-responsive elements; and (3) survival to bacterial infections. This inference is further supported by the observation that transfection of wild-type *imd* into S2 cells leads to expression of antibacterial peptide genes, whereas the mutated *imd<sup>d</sup>* form fails to induce this expression.

#### The *imd* Signaling Cascade

The early papers in the field had fully appreciated that wild-type *imd* was mandatory for the immune induction of Dipterin and the other antibacterial peptides, but not for that of the antifungal peptide Drosomycin (Lemaitre et al., 1995, 1996). As mentioned in the Introduction, four distinct genes have now been characterized which, when mutated, induce a roughly similar phenotype. These genes code for three well-established components within signaling cascades: (1) Relish is a member of the NF- $\kappa$ B family of inducible transactivators (Dushay et al., 1996; Hedengren et al., 1999). Like its mammalian counterpart, p105, it has a C-terminal I- $\kappa$ B-like domain, which accounts for its retention in the cytoplasm in the absence of challenge. Nuclear translocation of the Rel homology domain of Relish requires endoproteolytic cleavage by an as yet unidentified protease (Stöven et al., 2000). (2) Two genes code for the components of a mammalian signalosome equivalent, that is, for homologs of IKK $\beta$  (*ird5*) and IKK $\gamma$  (*kenny*; Lu et al., 2001; Rutschmann et al., 2000a; Silverman et al., 2000). Both genes act upstream of *Relish*, by a mechanism which has not been established, but which may involve phosphorylation of Relish by IKK $\beta$  (Silverman et al., 2000). (3) Finally, the *dredd* gene encodes a close homolog of

mammalian caspase-8 (Chen et al., 1998). As Relish is not challenge-dependently cleaved in *dredd* mutant flies, DREDD must act upstream of this transactivator in the signal transduction pathway (Stöven et al., 2000).

A major concern in this study was to clarify the relationship between *imd* and these four other known genes in the pathway. Our results clearly show that *imd* acts upstream of both *kenny* and *dredd*. Taken together with the studies mentioned above (Silverman et al., 2000; Stöven et al., 2000), they indicate that IMD functions upstream of the DmIKK signaling complex, which in turn activates Relish directly or indirectly. These data establish an *imd/kenny/ird<sup>5</sup>/Relish* gene cassette in the control of the antibacterial peptide genes. The presence in IMD of a death domain with similarity to that of the adaptor protein RIP suggests that the IMD protein may be part of a receptor-adaptor complex. By analogy with the role of RIP in TNFR1 signaling, IMD could function by recruiting the signalosome (DmIKK $\gamma$ /DmIKK $\beta$ ) to the upstream receptor-adaptor complex (Devin et al., 2000; Poyet et al., 2000; Zhang et al., 2000).

Both the receptor and the ligand(s) activating the IMD pathway remain unknown. This is in sharp contrast with the Toll pathway, which controls the antifungal response of *Drosophila* and namely the synthesis of the antifungal peptide Drosomycin. In the latter case, genetic analysis indicates that microbial ligands activate an extracellular proteolytic cascade culminating in the cleavage of the cytokine-like polypeptide Spätzle (Levashina et al., 1999). Cleaved Spätzle in turn interacts with Toll and initiates a signal transduction pathway that leads to the transcription of *drosomycin*. This does not, however, activate the synthesis of the antibacterial peptides discussed in this paper (Lemaitre et al., 1996). Conversely, as shown here, overexpression of *imd* leads to the expression of the antibacterial peptide genes but not that of *drosomycin*. These data indicate that within the predominant immune-responsive tissue of adult flies, that is, the fat body, the Toll and the IMD pathways are functionally separated and that each controls, via specific Rel transcription factors, a set of given antimicrobial peptide genes. This functional separation of the two signaling pathways within the cells is corroborated by our results that in flies subjected to infection by a bacterial mix (gram-positives and gram-negatives), expression of the antiapoptotic protein P35 abolishes inducibility of the antibacterial peptides, but not that of *drosomycin*.

#### The Link between IMD and Apoptosis

As mentioned in the Results section, we were initially surprised to note that overexpressing *imd* resulted in larval lethality, expression of the *reaper* gene in fat body cells of adult flies, and apoptosis of these cells. Most of these effects could be, at least partially, rescued by coexpressing the antiapoptotic protein P35. The presence of a death domain in *imd*, however, might bring these results in line with recent studies showing that overexpression of death domain proteins induces apoptosis in mammalian cells (Chinnaiyan et al., 1995; Hsu et al., 1995; Stanger et al., 1995). Overexpressing the death domain proteins RIP, FADD, and TRADD has been shown to lead to cell death by recruitment of apical

caspases, namely caspase-8 and -10, which in turn activate downstream effector caspases to execute the apoptotic program (Nagata, 1997; Scream and Xu, 2000). RIP, whose death domain is similar to that of IMD, has been proposed to induce cell death by recruiting FADD, which subsequently may activate caspase-8. Again, as already mentioned, *Drosophila* has a FADD homolog (DmFADD) and a caspase-8 homolog (DREDD) (Kim et al., 2000; Lin et al., 1999). Furthermore, IMD, DmFADD, and DREDD can associate into a multimeric complex in *Drosophila* S2 cells (R. Medzhitov, personal communication).

At this stage, we cannot determine whether *imd* plays a role in developmentally regulated apoptosis. We observed that *imd* is expressed at significant levels during stages of embryogenesis and pupariation, when massive apoptosis is known to occur (Abrams et al., 1993; Jiang et al., 1997). However, *imd* mutants are viable and show no obvious developmental defect, indicating that either *imd* is not involved in these processes or that redundant control mechanisms could substitute for its role. Interestingly, it has been recently reported that *reaper* loss-of-function mutants are also viable (C. Peterson and K. White, personal communication), despite the accepted role of *reaper* as a key player in *Drosophila* apoptosis through caspase activation and inactivation of inhibitor of apoptosis proteins (Goyal et al., 2000). In the context of the studies of immune defenses, which are central to our present interests, the crucial question pertains to the potential links between the control of expression of the antibacterial peptides and the induction of apoptosis. Here, two lines of evidence draw a clear distinction: for one, bacterial challenge induces antibacterial peptide genes but does not result in detectable apoptosis; further, UV irradiation leads to P35-sensitive lethality, but does not induce antibacterial peptides. In other words, the data available today do not point to a role of apoptosis in the host response to bacterial infection.

## Experimental Procedures

### Cloning and Transgene Construction

For rescue experiments, an EcoRI-StuI 5.1 kb fragment encompassing the ORF and the regulatory regions of the *imd* gene was subcloned from cosmid 119C2 (FlyBase) into the pP{caSpeR} vector to generate the P{w+, imd.5.1} transgene. The construct was introduced into *w; imd<sup>1</sup>* flies by P element-mediated transformation (Spradling and Rubin, 1982). For overexpression of *imd* in flies, an XhoI-EcoRI 1.5 kb fragment corresponding to the full-length *imd* cDNA (GH20785; Research Genetics) was subcloned into the pP{UAST} transformation vector (Brand and Perrimon, 1993) to generate the P{UAS-*imd*.SN} transgene. The construct was introduced into *w; imd<sup>1</sup>* and *w* flies. To generate the *yolk-GAL4* driver, 929 bp of the YP<sub>1</sub> promoter (Fban0002985) was cloned upstream of the GAL4 coding region in pGATB (Brand and Perrimon, 1993), using a HindIII site and a PCR-generated BamHI site. The P{GAL4-YP<sub>1</sub>.JMR} transgene specifically directs expression of GAL4 in fat body cells of 3- to 5-day-old females. For transfection of S2 cells, the coding sequences of the *imd* or the *imd<sup>1</sup>* mutated genes were cloned by PCR in pPac (Tauszig et al., 2000). c-Myc- and HA-epitope tags were introduced at the N and C termini, respectively, of the expression constructs to generate the expression plasmids pPac-Myc-*imd*-HA and pPac-Myc-*imd<sup>1</sup>*-HA. The nucleotide sequence of all the constructs was confirmed by sequencing.

### Fly Strains and Procedures

Flies were grown on a standard medium and at 25°C, except for specific experiments. For rescue of the *imd<sup>1</sup>* phenotype, the transgenic lines *w; imd<sup>1</sup>*; P{w+, imd.5.1}M1 (*imd<sup>1</sup>*-M1) and *w; imd<sup>1</sup>*; P{w+, imd.5.1}F1 (*imd<sup>1</sup>*-F1) were used. The driver lines with insertions on the third chromosome were the following: *w; P{w+, GAL4-da.G32}* (*da-GAL4*; Bloomington), *w; P{w+, GAL4-Hsp70.PB}89-2-1* (*hs-GAL4*; Bloomington), and *w; P{w+, GAL4-YP<sub>1</sub>.JMR}20* (*yolk-GAL4*; this study). Driver P elements carrying flies *da-GAL4* and *hs-GAL4* were crossed with *w; imd<sup>1</sup>* flies to obtain flies of the following genotypes: *w; imd<sup>1</sup>*; P{w+, GAL4-*da.G32}* and *w; imd<sup>1</sup>*; P{w+, GAL4-*Hsp70.PB}89-2-1*. For overexpression of *imd* by the UAS/GAL4 system, the following UAS-*IMD* insertion lines were used: *w; imd<sup>1</sup>*; P{w+, UAS-*imd*.SN}F20 (*imd<sup>1</sup>*/UAS-*IMD*<sub>1</sub>), *w; P{w+, UAS-*imd*.SN}M24* (UAS-*IMD*<sub>2</sub>), *w; P{w+, UAS-*imd*.SN}F30* (UAS-*IMD*<sub>3</sub>), *w; +; P{w+, UAS-*imd*.SN}M8* (UAS-*IMD*<sub>4</sub>), and *w; +; P{w+, UAS-*imd*.SN}F32* (UAS-*IMD*<sub>5</sub>). The *w; P{w+, UAS-p35.H}* (UAS-P35) line was employed. Driver lines were crossed to lines carrying UAS transgenes, and the effect of overexpression was analyzed in the progeny. Heat shock of larvae and adult flies was performed by incubation at 37°C for 1 hr, followed by 1 hr at 29°C. The *w; P{w+, Rpr-11-lacZ}* (*rpr-lacZ*) line was used to monitor expression of the *reaper* (*rpr*) gene. For epistasis experiments, UAS-*IMD* was overexpressed in *dredd<sup>055</sup>* (Leulier et al., 2000) and *key<sup>1</sup>* (Rutschmann et al., 2000a) flies. Survival experiments were performed as previously described (Rutschmann et al., 2000a). Immune challenge was carried out by pricking adult flies with a mixture of *E. coli* and *M. luteus*.

### Generation of Deficiencies by Male Recombination and Mapping

For male recombination, the P elements located in 55C9 (l(2)k04808; FlyBase) and in 55C5 (VG(2)A; Sun et al., 1995) were used. P element-carrying males were crossed to *w; cn bw*; P{w+, Δ(2-3) Sb} / *TM6b* to generate *w; cn bw / P; P{w+, Δ(2-3)} / +* males. These flies were backcrossed to *cn bw* and the progeny was analyzed for the presence of either *cn* or *bw* markers. 225,000 flies were screened and 460 recombinants were recovered (0.2%), among which 30% were *w+*. These lines were tested for the ability to complement the *imd<sup>1</sup>* mutation in a microwell colorimetric assay using a *Pdip1-lacZ* reporter gene (Rutschmann et al., 2000b). Sixty lines were further characterized by sequencing the breakpoints of the deficiencies using plasmid rescue or inverse PCR.

### RNA Preparation and Northern Blot Analysis

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

### Gel Shift Assay

Nuclear extracts of adult flies either unchallenged or 6 hr after bacterial challenge were prepared, and gel shift assays were performed as previously described (Kappler et al., 1993) using the following probes: wild-type (5'-ATCGGGGATTCCTTTT-3') and mutated (5'-ATCGATTATTCCTTTT-3') NF-κB-like binding sites of the *dip1* promoter.

### Transfection Assays and Western Blot Analysis

S2 cells (Invitrogen) were grown and transfected as previously described (Tauszig et al., 2000), using 0.1 μg of *attacin*- or *drosocin-luciferase* reporter plasmids, 0.1 μg of β-galactosidase-expressing control vector, and 1 μg of expression vector. LPS (*E. coli* serotype 055:B5; Sigma) was added to cells at 10 μg/ml for 16 hr. For Western blot analysis, cells were resuspended in Laemmli buffer and proteins were separated by 7.5% denaturing SDS-PAGE and electroblotted onto a nitrocellulose filter. The membrane was probed with monoclonal c-Myc antibody (Roche) and the proteins were revealed by enhanced chemiluminescence (Amersham, ECL).

### Sequencing of the *imd* Gene

DNA from homozygous flies was extracted using a single fly preparation protocol. PCR reactions were carried out as described (Rutschmann et al., 2000a), using the following primers: *imd*-sense 5'-TAC CAC CAC CAA ATT GAA AAA GAG-3' and *imd*-antisense 5'-CCG

GCT GGC TTG GAC ATC-3'. DNA was sequenced by Appligene-Onco (France). Control flies were either *OregonR* or *cn bw*.

#### TUNEL Staining and Transmission Electron Microscopy

Abdomens from adult females were dissected in PBS. After removal of the digestive and reproductive systems, the remaining cuticle preparations were fixed for 1 hr in 4% formal. After washing three times in PBS for 5 min, tissues were permeabilized for 15 min in 0.1% sodium citrate/0.1% Triton X-100, followed by incubation in methanol (15 min). After washing (see above), cuticles were stained for 3 hr at 37°C using the In Situ Cell Death Detection Kit (Roche). After two washes in PBS, the tissues were stained with DAPI for 10 min, rinsed in PBS for 10 min, and mounted in 80% glycerol. For transmission electron microscopy (TEM), adult females were analyzed 40 hr after heat shock. Cuticle preparations were fixed in 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 1 hr at 4°C and postfixed with osmium tetroxide. Samples embedded in araldite/epon were sectioned and counterstained with lead citrate and uranyl acetate.

#### UV Irradiation of Pupae

Sixty to one hundred 24-hr-old pupae were collected in Petri dishes and irradiated with 20,000, 50,000, and 100,000  $\mu\text{J}/\text{cm}^2$  on their dorsal side using a Stratelinker 1800 (Stratagene) with a 254 nm UV source. Expression of P35 in UAS-P35/hs-GAL4 pupae was induced by heat shock for 1 hr at 37°C immediately after UV irradiation. Irradiated pupae were maintained at 25°C, and the number of emerging adult flies was counted. Experiments were repeated at least three times and statistical analysis was performed by the Student's *t* test.

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