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The *Drosophila* caspase Dredd is required to resist Gram-negative bacterial infection

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The *Drosophila* innate immune system discriminates between pathogens and responds by inducing the expression of specific antimicrobial peptide-encoding genes through distinct signaling cascades. Fungal infection activates NF-κB-like transcription factors via the Toll pathway, which also regulates innate immune responses in mammals. The pathways that mediate antibacterial defenses, however, are less defined. We have isolated loss-of-function mutations in the caspase encoding gene *dredd*, which block the expression of all genes that code for peptides with antibacterial activity. These mutations also render flies highly susceptible to infection by Gram-negative bacteria. Our results demonstrate that Dredd regulates antibacterial peptide gene expression, and we propose that Dredd, Immune Deficiency and the P105-like rel protein Relish define a pathway that is required to resist Gram-negative bacterial infections.

INTRODUCTION

Innate immune systems in insects and mammals recognize distinct classes of microbes and activate effector genes through conserved signaling pathways. In mice, the Toll-like receptor 4 is primarily involved in the recognition of lipopolysaccharide, a component of Gram-negative bacteria, while Toll-like receptor 2 mediates Gram-positive bacterial recognition (Takeuchi *et al.*, 1999; Beutler, 2000). These two receptors utilize similar signaling cascades to activate NF-κB, a central transactivator of many immune and inflammatory genes. In *Drosophila*, molecules directly involved in microbial recognition remain poorly characterized (Khush and Lemaitre, 2000; Kim *et al.*, 2000); however, it is apparent that the *Drosophila* innate immune system discriminates between pathogens and responds by inducing the expression of specific antimicrobial peptide-encoding genes (reviewed in Engstrom, 1999; Anderson, 2000; Imler and Hoffmann, 2000; Khush and Lemaitre, 2000). Genetic analysis demonstrates that the Toll signaling cascade controls an antifungal response (Lemaitre *et al.*, 1996). This pathway is triggered by the proteolytic cleavage of the Toll ligand, Spätzle (Spz), and leads to activation of the rel proteins DIF and Dorsal (Manfruelli *et al.*, 1999; Meng *et al.*, 1999; Rutschmann *et al.*, 2000). Mutations that block this cascade reduce the expression of the antifungal peptide gene *drosomycin* and increase susceptibility to fungal infection (Lemaitre *et al.*, 1996). In addition, a subset of the genes encoding peptides with antibacterial activity are induced to lower levels in flies deficient for Toll signaling, indicating that this pathway also plays a role in antibacterial immune responses (Lemaitre *et al.*, 1996). Mutations in the molecularly uncharacterized *immune deficiency* (*imd*) gene, by contrast, only affect the induction of genes with antibacterial activity and increase susceptibility to bacterial infections (Lemaitre *et al.*, 1995). *imd;Toll* double mutants fail to express any antimicrobial genes, suggesting that *imd* and *Toll* define two essential pathways that regulate antimicrobial gene expression (Lemaitre *et al.*, 1996). Recently, Hedengren *et al.* (1999) determined that mutations in *relish*, a gene encoding a P105-like rel protein, reduce the expression of all antimicrobial genes after bacterial infection and concluded that *relish* may function downstream of *imd*. Here, we show that the Dredd caspase, which regulates apoptotic pathways in *Drosophila*, also mediates immune responses to infection by Gram-negative bacteria.

RESULTS

The *dredd* caspase regulates *Drosophila* immune responses

To identify genes that control *Drosophila* antibacterial immune responses, we screened for mutations on the X chromosome that

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affect the expression of the antibacterial peptide gene *diptericin* after bacterial infection (R.S. Khush and B. Lemaitre, unpublished results). Among 2500 EMS mutagenized lines, we isolated five viable, recessive mutations (named *B118*, *F64*, *L23*, *D55*, *D44*) of a gene that is required for the expression of a *diptericin–GFP* reporter gene in larvae after bacterial infection (Figure 1A). In addition, northern blot analysis shows that adults homozygous for each of the five alleles do not express the *diptericin* gene after bacterial injection (Figure 1B). We mapped the *B118* allele to cytological region 1B9–1B13 on the proximal tip of the X chromosome and identified a small deficiency, *Df(1)R194*, which does not complement *B118* (data not shown). Deficiency *Df(1)R194* spans four previously identified genes: *rpL36*, *l(1)1Bi*, *dredd* and *su(s)* (Chen *et al.*, 1998) (Figure 1C). Several results demonstrate that *B118* is a mutation in *dredd*: (i) *B118* is allelic to a viable *P* element insertion (*EP-1412*) inserted 50 bp upstream of *dredd* coding sequences (data not shown; Figure 1C); (ii) the two genes flanking *dredd*, *su(s)* and *l(1)1Bi* complement *B118* (data not shown); (iii) a small deficiency, *Df(1)dredd*^{D3}, which we generated by imprecise *P* element excision, and which removes *dredd* and affects the 5′ upstream sequences of *su(s)*, blocks *diptericin* expression after bacterial infection (Figure 1B and C); and (iv) a *P* element insertion, *P[dredd*$^{+}$*]*, containing 7.6 kb of genomic DNA, including *dredd* but not *su(s)* and *l(1)1Bi* (Chen *et al.*, 1998), fully restores *diptericin* expression in *B118* flies (Figure 1B and C). All five *dredd* EMS mutations block *diptericin* expression after infection to the same degree as *Df(1)dredd*^{D3}, indicating that they are probably null alleles (Figure 1B; see Methods). The *P* element insertion in line *EP-1412* generates a strong hypomorphic *dredd* mutation since a small amount of *diptericin* expression is detectable after infection (Figure 1B).

dredd encodes an apical caspase and is an effector of the apoptosis activators *reaper*, *grim* and *hid* (Chen *et al.*, 1998). One or more *dredd* transcripts are specifically enriched in cells programmed to die and *dredd* overexpression induces apoptosis in SL2 cells (Chen *et al.*, 1998; Rodriguez *et al.*, 1999). In mammals, the closest *dredd* homologs are caspases 8 and 10, which mediate apoptosis induced by members of the tumor necrosis factor receptor family (Reed, 1999). Caspases are produced as inactive zymogens termed pro-caspases; when activated, mature caspases catalyze the proteolytic cleavage of death substrates that are associated with apoptosis (Reed, 1999). The isolation of mutations in *dredd* that block *diptericin* expression after infection demonstrates that Dredd also regulates immune responses. In addition, a *dredd–lacZ* reporter gene is constitutively expressed in all adult and larval tissues including the fat body, the major immuno-responsive tissue in insects (Figure 1D). Infection does not, however, appear to increase *dredd* expression levels (data not shown).

The five *dredd* alleles all contain point mutations that affect different regions of the *dredd* protein (Figure 1E). Alleles *B118*, *D55* and *F64* generate either premature stop codons or frameshift changes in the Dredd prodomain. D44 has a missense mutation in sequences encoding the first death effector domain (DED), a region thought to mediate protein–protein interactions (Reed, 1999). In the protein encoded by allele *L23*, a tryptophan (W) in the caspase domain is replaced by an arginine (R) residue. The strong phenotype of alleles *D44* and *L23* indicates that

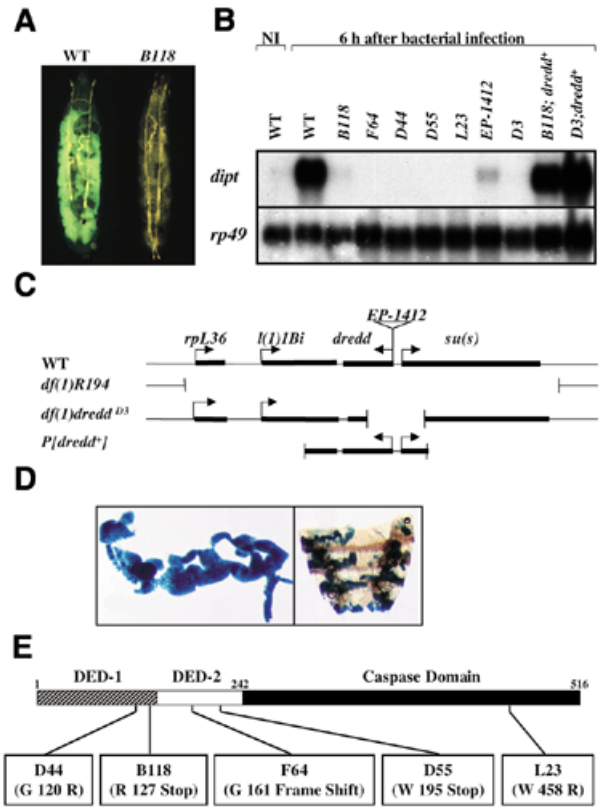

Fig. 1. *dredd* is required for *diptericin* expression in larvae and adults. (**A**) Bacterial infection induces the expression of the *diptericin–GFP* reporter gene in the fat bodies of wild-type (WT) larvae. This induction is blocked by the *B118* mutation. (**B**) Northern blot analysis of total RNA extracted from adult flies infected with a mixture of Gram-positive (*Micrococcus luteus*) and Gram-negative (*Escherichia coli*) bacteria shows that all five alleles of *dredd* (*B118*, *F64*, *D44*, *D55*, *L23*) and the *Df(1)dredd*^{D3} (*D3*) deficiency completely block *diptericin* expression. *diptericin* is weakly expressed in the *EP-1412* line that carries a *P*-element insertion in the *dredd* gene. *diptericin* expression is restored in *B118* and *D3* flies carrying the *P[dredd*$^{+}$*]* transgene. *rp49* expression was monitored as a loading control. NI: non-infected. (**C**) A genomic map of the *dredd* locus (Chen *et al.*, 1998) showing the *P*-element insertion site (*EP-1412*), the sequences deleted in deficiencies *Df(1)R194* and *Df(1)dredd*^{D3}, and the genomic DNA contained in the *P[dredd*$^{+}$*]* transgene. (**D**) Histochemical staining for *lacZ* activity shows that a *P[dredd–lacZ]* reporter gene is constitutively expressed in the fat bodies of uninfected larvae (left) and adults (right). (**E**) The five EMS-induced alleles of *dredd* each contain a single point mutation that generates the indicated changes in the Dredd protein.

Dredd domains affected in these alleles are essential for Dredd function in immunity.

dredd mediates antimicrobial peptide gene expression in response to Gram-negative bacterial infections

The isolation of *dredd* mutations that block *diptericin* expression enabled us to characterize *dredd*'s role in mediating *Drosophila* antimicrobial host defense as well as *dredd*'s relationship to other genes that function in this response. Pricking adult flies with a mixture of Gram-positive and Gram-negative bacteria

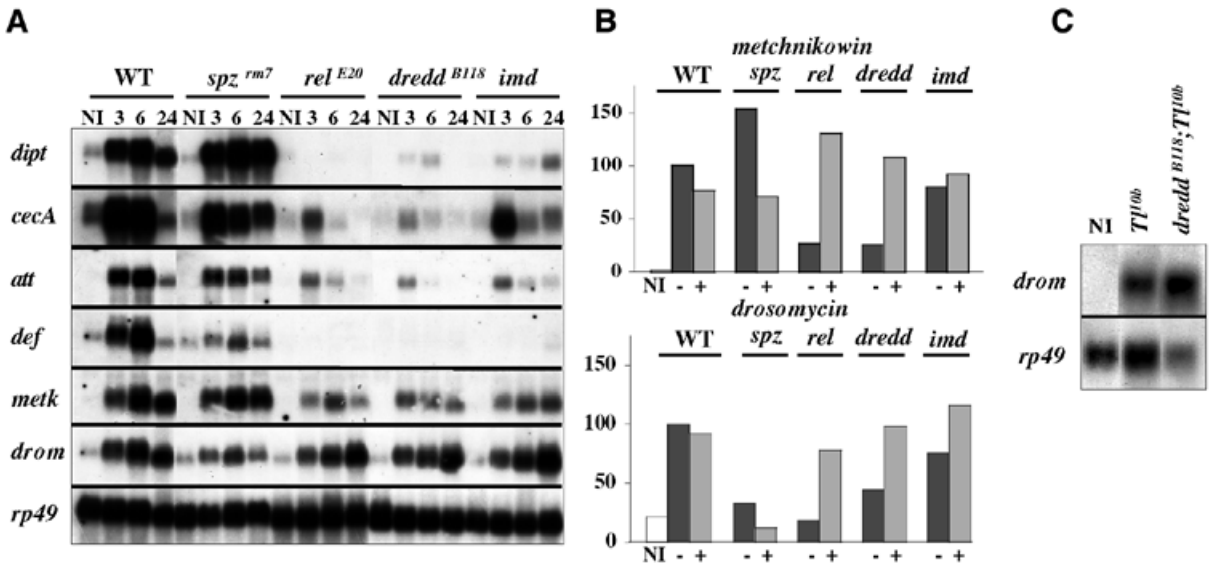

Fig. 2. Dredd regulates the expression of antibacterial genes. (**A**) A time course of antimicrobial gene expression in different mutant adults infected with a mixture of *E. coli* and *M. luteus* shows that *dredd*, *relish* and *imd* predominantly control the expression of the antibacterial genes (*diptericin*, *cecropin A*, *attacin*, *defensin*). The northern blot was performed with total RNA extracted from wild type, and *dredd*, *imd*, *rel* and *spz* mutant adults at different time intervals after challenge (as indicated in hours). Flies were incubated at 25°C. The blot was successively hybridized with the following cDNA probes: *diptericin* (*dipt*), *cecropin A* (*cecA*), *attacin* (*att*), *defensin* (*def*), *metchnikowin* (*metk*), *drosomycin* (*drom*) and *rp49*. (**B**) The quantification of *metchnikowin* and *drosomycin* expression in different mutant adults collected 6 h after infection by either Gram-negative (–, *E. coli*) or Gram-positive (+, *M. luteus*) bacteria indicates that *dredd* and *relish* are required for the expression of these genes after Gram-negative, but not Gram-positive bacterial infection. As observed for the other antimicrobial genes the effect of the *imd* mutation on the expression of these genes is weaker than the *dredd* and *relish* mutations (Figure 2A). The signals from a northern blot were quantified with a Bio-Imager system and the levels of *metchnikowin* and *drosomycin* expression were normalized with the corresponding value of the *rp49* signal. (**C**) The high levels of *drosomycin* gene expression in uninfected Tl^{10b} and $dredd^{B118};Tl^{10b}$ adults demonstrate that *dredd* is not required for the Tl^{10b}-driven constitutive expression of *drosomycin*.

activates the expression of all the genes that encode antimicrobial peptides in *Drosophila* (Figure 2A). In the $dredd^{B118}$ mutant, however, mixed Gram-positive/Gram-negative infections only induce the expression of the antifungal gene *drosomycin* and the gene coding for Metchnikowin, which has both antifungal and antibacterial activity (Figure 2A); *diptericin*, *cecropin A*, *attacin A* and *defensin* are expressed at <5% of wild-type levels and *metchnikowin* is expressed at 50% of the wild-type level (Figure 2A; quantification data not shown). Antimicrobial gene expression is similarly affected in flies homozygous for rel^{E20}, a strong or null mutant allele of *relish* (Hedengren *et al.*, 1999) and *imd*, although most of the antibacterial genes are expressed at slightly higher levels in *imd* flies (Figure 2A). By contrast, a mutation in the *spz* gene, which blocks Toll activation, reduces *drosomycin* induction by mixed Gram-negative/Gram-positive bacterial infection and reduces the induction of some of the antibacterial genes (*defensin*, *attacin*, *cecropin A*) (Figure 2A) (Lemaitre *et al.*, 1996). These data demonstrate that mutations in *dredd* are phenotypically similar to mutations in *imd* and *relish*, and that these three genes regulate all *Drosophila* antibacterial peptide gene expression.

Hedengren et al. (1999), however, previously showed that *drosomycin* and *metchnikowin* are expressed to only 10–20% of the wild-type level in rel^{E20} flies after infection by the Gram-negative bacteria *Enterobacter cloacae*. The difference between their results and our observation that *drosomycin* and *metchnikowin* are significantly induced in *relish* mutants after mixed Gram-negative/Gram-positive infections could be explained by the type of infection. To define further the roles of *imd*, *dredd* and *relish* in activating *metchnikowin* and *drosomycin* after different types of bacterial infection, we quantified *metchnikowin* and *drosomycin* expression in different mutant backgrounds 6 h after infection with either Gram-negative *Escherichia coli* or Gram-positive *Micrococcus luteus* bacteria. The $dredd^{B118}$ and rel^{E20} mutations strongly reduce *metchnikowin* and *drosomycin* induction by Gram-negative bacterial infections (~20–50% of wild-type levels), while the *imd* mutation has a weak effect; by contrast, *metchnikowin* and *drosomycin* are expressed at close to wild-type levels in the *imd*, $dredd^{B118}$ and rel^{E20} mutants after Gram-positive bacterial infection (Figure 2B). We conclude, therefore, that *dredd* and *relish* play a greater role in inducing *metchnikowin* and *drosomycin* after Gram-negative bacterial infection than after Gram-positive bacterial infection.

The observation that *drosomycin* and *metchnikowin* expression is almost completely abolished in *imd;Toll* double mutants (Lemaitre *et al.*, 1996; Levashina *et al.*, 1998) suggests that Gram-positive bacterial infection triggers the expression of *metchnikowin* and *drosomycin* via the Toll pathway. In agreement, our analysis shows that mutations in *spz* affect *drosomycin* gene expression more strongly after Gram-positive than after Gram-negative bacterial infection, and that the constitutive activation of the Toll pathway in the Tl^{10b} mutant (Lemaitre *et al.*, 1996) leads to *drosomycin* expression in the absence of

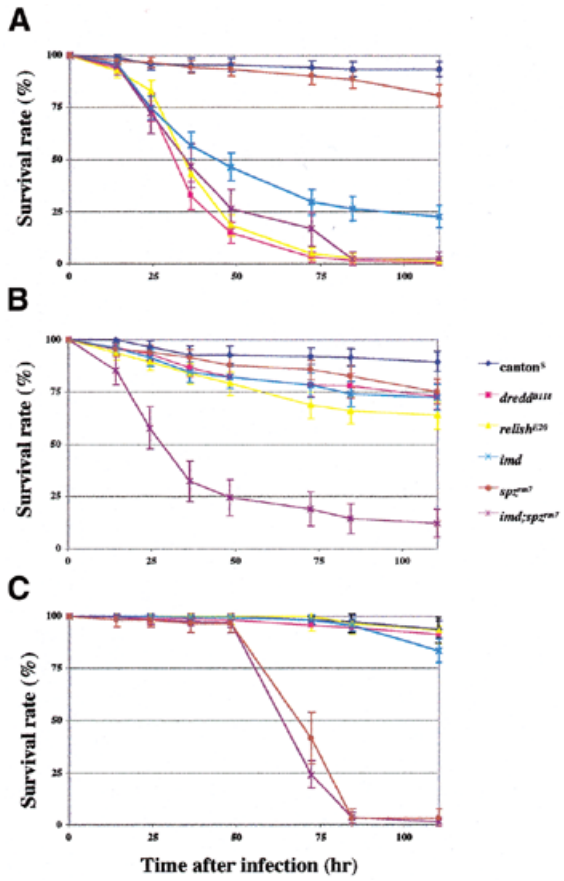

Fig. 3. *dredd* is required for resistance to Gram-negative bacterial infection. (**A**) Mutations in *dredd*, *relish* and *imd* render adult flies highly susceptible to *E. coli* infection. (**B**) Only flies carrying both the *imd* and *spz* mutation are sensitive to *M. luteus* infection. (**C**) The *spz* gene is required for resistance to natural infection by *Beauveria bassiana*. The survival rate of wild-type (Canton^S, diamonds), *imd* (×), *spz^{rm7}* (circles), *dredd^{B118}* (squares), *rel^{E20}* (triangles) and *imd;spz^{rm7}* (asterisks) infected flies are presented with confidence intervals (p <5%). One hundred to 200 adults, aged 2–4 days, were pricked and transferred at 29°C to a fresh vial every 3 days. Adults were pricked with a needle previously dipped into either *E. coli* (A), *M. luteus* (B) or naturally infected by *B. bassiana* (C). The mutated flies tested here exhibited >80% survival 100 h after challenge by a clean injury (data not shown).

dredd activity (Figure 2C). *metchnikowin*, however, is still expressed to a high level in *spz* mutants after Gram-positive bacterial infection, indicating that *metchnikowin* induction by Gram-positive bacterial infection may also be mediated in part by the Imd pathway.

dredd mediates resistance to Gram-negative bacterial infections

The susceptibility to microbial infection observed in *dredd, imd, relish, spz* and *imd;spz* mutants is correlated with the expression pattern of antimicrobial genes in these mutants. *dredd^{B118}*, *rel^{E20}* and *imd;spz^{rm7}* adults are highly susceptible to bacterial infection by Gram-negative bacteria, and *imd* adults are slightly less susceptible (Figure 3A for *E. coli*; data not shown for *Erwinia carotovora carotovora*). These survival results confirm that the activation of defense responses to Gram-negative bacterial infection require *imd, dredd* and *relish*. Only the *imd;spz^{rm7}* double mutants, however, are highly susceptible to bacterial infection by Gram-positive bacteria (Figure 3B for *M. luteus*; data not shown for *Aerococcus viridans*), indicating that resistance to Gram-positive bacteria is regulated by both the Toll and Imd pathways. Finally, only *spz^{rm7}* and *imd;spz^{rm7}* mutants are highly sensitive to natural infection by the entomopathogenic fungus *Beauveria bassiana* (Figure 3C) or injection of *Aspergillus fumigatus* spores (data not shown), confirming that responses to fungi are largely activated by the Toll pathway (Lemaitre *et al.*, 1996, 1997).

DISCUSSION

In this study we have identified Dredd, a *Drosophila* apical caspase previously shown to function in apoptosis (Chen *et al.*, 1998), as a regulator of the antibacterial response. *dredd* mutants display very low inducibility of all the genes that encode peptides with antibacterial activity and a high susceptibility to Gram-negative bacterial infection. This *dredd* immune phenotype is similar to the *relish* and *imd* phenotypes; we predict that the Imd, Dredd and Relish proteins function in a common signaling pathway that regulates antibacterial peptide gene expression. Based on the respective activites of Dredd as a caspase and Relish as a transcriptional transactivator (Dushay *et al.*, 1996), we also hypothesize that Dredd functions upstream of Relish in the control of antimicrobial gene expression. Our hypothesis is supported by the observation that Dredd is required for Relish activation via endoproteolytic cleavage (Stöven *et al.*, 2000). We believe that the weaker effects of the *imd* mutation on antibacterial gene expression place the *imd* gene product at an early stage of the antibacterial cascade where multiple responses, some of which bypass *imd*, trigger the activation of the pathway. Alternatively, the *imd* mutation may represent a hypomorphic allele.

Caspases were originally identified as effectors of apoptosis, but there is increasing evidence that caspases also function in other physiological processes. Recent studies suggest that the recruitment of the caspase-8 precursor to the TNF-R1 signaling complex either activates NF-κB through a Traf2-, RIP-, NIK- and IKK-dependent pathway or, after proteolytic processing of caspase-8, induces apoptosis (Hu *et al.*, 2000). Our data indicate that Dredd, a close homolog of caspase-8, may also have dual functions in NF-κB signaling and apoptosis in *Drosophila*. Further biochemical analysis is necessary to determine whether Dredd participates directly in Relish activation or functions further upstream.

Deciphering the mechanisms that enable *Drosophila* to differentiate between pathogens and mount specific immune responses is essential for understanding innate immunity. Recent studies indicate that the Toll pathway is mainly activated in response to fungal and Gram-positive bacterial infection (Lemaitre *et al.*, 1997; Rutschmann *et al.*, 2000). We now present several observations that suggest that *imd, dredd* and *relish* mediate most of the responses to Gram-negative bacterial infection: (i) these genes regulate the antimicrobial peptide genes that are most highly induced by Gram-negative bacterial infection (Lemaitre *et al.*, 1997); (ii) *dredd* and *relish* control the

induction of *metchnikowin* and *drosomycin* after Gram-negative bacterial infection; and (iii) these three genes are required for resistance to Gram-negative bacterial infection. We propose a model whereby antimicrobial gene expression in *Drosophila* adults is regulated by a balance of inputs from the Toll pathway and the Imd pathway, which includes Imd, Dredd and Relish, and that these two pathways are differentially activated by different classes of microorganisms. Identifying the receptors that discriminate between invading microbes and stimulate these pathways presents an exciting challenge in the study of innate immunity.

METHODS

***Drosophila* strains.** Canton^S flies were used as a wild-type standard. *imd, spz, Tl* alleles are described elsewhere (Lemaitre *et al.*, 1996). *rel^{E20}* is a strong or null allele of *relish* (Hedengren *et al.*, 1999). *B118, F64, D44, D55* and *L23* are five EMS mutations of *dredd* that were generated in a *y,w* chromosome. Flies homozygous or hemizygous (*in trans* to *Df(1)dredd^{D3}*) for these five mutations are phenotypically identical to flies homozygous for *Df(1)dredd^{D3}* with respect to both *diptericin* and *attacin* induction after challenge and susceptibility to *E. carotovora carotovora* infection, thereby indicating that they are genetically null mutations of *dredd* (data not shown). *Df(1)dredd^{D3}* was generated by imprecise *P* element excision of *EP-1412*. Other mutant lines are described in the text. *diptericin–GFP* is *P* transgene containing a fusion between 2.2 kb of upstream sequence of *diptericin* and the *Green Fluorescent Protein* gene. The precise fly genotypes are: *imd*: *br, pr, imd*; *spz^{rm7}*: *spz^{rm7}/spz^{rm7}*; *rel*: *rel^{E20}, e*; *dredd^{B118}*: *y,w, dredd^{B118}*. For survival studies, we have used a *rel^{E20}* stock from which the *ebony* mutation was removed since *ebony* affects survival levels (Lemaitre *et al.*, 1996). Similar survival rates were observed with *y,w, dredd^{B118}* and y+ *Df(1)dredd^{D3}/y,w, dredd^{B118}* flies, indicating that our results do not reflect deleterious effects due to the *y* marker. *Drosophila* stocks were maintained at 25°C. Infected animals were incubated either at 25°C (northern blot) or at 29°C (survival).

Infection experiments. Bacterial infections were performed by pricking third instar larva or adults with a thin needle previously dipped into a concentrated culture (OD ~200) of *E. coli, M. luteus* or a mixture of the two bacteria. Natural infections with *B. bassiana* were performed by shaking anesthetized flies for 30 s in a Petri dish containing a sporulating fungal culture (Lemaitre *et al.*, 1997). Bacterial and fungal strains were previously described (Lemaitre *et al.*, 1997).

Northern blot analysis. Total RNA extraction and northern blotting experiments were performed as described in Lemaitre *et al.* (1997).

Sequencing of *dredd* alleles. DNA was extracted from adult flies and the *dredd* ORF was amplified by PCR using the Expand Long Template PCR kit (Boehringer Mannheim) for each of the mutant alleles and then sequenced using an ABI 373 automated sequencer.

Cloning and transformation of *P[dredd–lacZ].* To determine if 5′ regulatory sequences regulate *dredd* expression after bacterial infection in the fat body, transgenic lines carrying an ~3.3 kb promoter fragment fused to lacZ were tested for β-galactosidase activity in third instar larvae. The *dredd* 3.3 kb fragment was generated by PCR from genomic DNA obtained from *yw* flies as described above. The following modified oligonucleotides containing an *Eco*RI and a *Bam*HI site, respectively, were used: 5′-GGCACTCG-CGAATTCAATGCTGCTAGCATGGTC-3′ and 5′-ATTGGATCCG-GCCATGGCGATCGGGAGTATACA-3′. The PCR-amplified fragment was then digested with *Eco*RI and *Bam*HI, gel purified and ligated into *Eco*RI–*Bam*HI-cut pCaSpeR-AUG-β-gal vector. Flies bearing this construct were generated by *P* element-mediated germline transformation. X-gal staining was described previously (Lemaitre *et al.*, 1995).

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