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

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Received June 26, 2000; revised August 11, 2000; accepted August 15, 2000

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; Kutschmann 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...
affect the expression of the antibacterial peptide gene diptericin after bacterial infection (R.S. Khush and B. Lemaître, 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)11B, 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)11B, complement B118 (data not shown); (iii) a small deficiency, Df(1)dreddP2, 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)11B (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)dreddP2, 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).

**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)dreddP2 (D3) deficiency completely block diptericin expression. dredd 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)dreddP2, 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...
activates the expression of all the genes that encode antimicrobial peptides in Drosophila (Figure 2A). In the dreddB118 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). Antibacterial gene expression is similarly affected in flies homozygous for relE20, 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 relE20 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 dreddB118 and relE20 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, dreddB118 and relE20 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 Tl10b 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 (CantonS, diamonds), *imd* (circles), *spz* (squares), *dredd* (triangles) and *imd;spz* (asterisks) infected flies are presented with confidence intervals (p < 0.05). One hundred to 200 adults, aged 2-4 days, were pricked and transferred at 29°C to a fresh vial every day. 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*, *ref* and *imd;spz* 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* 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* and *imd;spz* mutants are highly susceptible 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 activities 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. CantonS flies were used as a wild-type standard. *imd*, *spz*, *Tl* alleles are described elsewhere (Lemaire et al., 1996). *ref*20 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 (*trans* to *Di(1) dredd*P13) for these five mutations are phenotypically identical to flies homozygous for *Di(1) dredd*P21 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). *Di(1) dredd*P21 was generated by imprecise P element excision of EP-1412. Other mutant lines are described in the text. *dip*tericin–*GFP* is a 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*936/spz*937/spz*938; *rel: ref*20, *v; dredd*B118; *y,w, dredd*B118. For survival studies, we have used a *ref*20 stock from which the *ebony* mutation was removed since *ebony* affects survival levels (Lemaire et al., 1996). Similar survival rates were observed with *y,w, dredd*B118 and *y* *Di(1; dredd)*P21/*y,w, dredd*P21 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 prickling 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 sporingul fungal culture (Lemaire et al., 1997). Bacterial and fungal strains were previously described (Lemaire et al., 1997).

**Northern blot analysis.** Total RNA extraction and northern blotting experiments were performed as described in Lemaire 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 *EcoRI* and a BamHI site, respectively, were used: 5′-GGCCACTCG-GCAATTCATCGTCGAGCATGTC-3′ and 5′-ATGGATCC-GCCATGGCCGATGATATAA-3′. The PCR-amplified fragment was then digested with EcoRI and BamHI gel purified and ligated into EcoRI–BamHI-cut pCaSpeR-AUG–β-gal vector. Flies bearing this construct were generated by P element-mediated germline transformation. X-gal staining was described previously (Lemaire et al., 1995).

**ACKNOWLEDGEMENTS**

We thank Dan Hultmark for providing *relish* mutants and our colleagues Isaline Rowe, Stéphanie Gobin, Thomas Ranson, Sheila Vidal, Ennio de Gregorio and Pedro Santamaria for assistance and stimulating discussions.

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DOI: 10.1093/embo-reports/kvd073