

Drosophila melanogaster Antimicrobial Defense

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The Drosophila melanogaster host defense is complex but remarkably efficient. It is a multifaceted response to a variety of fungal, bacterial, and parasitic invaders. Current knowledge is discussed on recognition of infectious microorganisms and on the activation of intracellular signaling cascades that concur with the expression of numerous immune-responsive genes, among which, to date, the most prominent appear to encode potent antimicrobial peptides.

Insects are remarkably resistant to microbial infections. The role of hemocytes (blood cells) in this resistance was fully appreciated by early researchers of the late nineteenth century [1, 2]. By early in the twentieth century, it was understood that septic injury induces the appearance in the cell-free hemolymph (blood) of large-spectrum potent antimicrobial activities [3–5]. It was not until 1981, however, that these activities were ascribed to inducible cationic antimicrobial peptides with the discovery of cecropins and attacins by Steiner et al. [6]. In the 1980s, an increasing number of antimicrobial peptides were identified from insects and, later, from mammalian species, and it is now thought that all metazoans (and metaphyta) rely on various types of antimicrobial peptides as part of their host defense [7].

In the early 1990s, researchers in our laboratory and in others turned to the use of Drosophila melanogaster to investigate the control of expression of antimicrobial peptides. A hallmark of these studies was the discovery in 1996 that, in D. melanogaster, the Toll-signaling pathway, initially described for its role in dorsoventral patterning in the embryo [8], was reused later in development to combat fungal and gram-positive infections [9]. It was also found that the defense against gram-negative sepsis was dependent on another signaling pathway, termed the Imd (immune deficiency) pathway [10]. These discoveries can be linked to the observation that, during D. melanogaster host defense, a differential induction of antimicrobial peptide genes occurs after infection by various classes of microorganisms. The D. melanogaster immune response is not aspecific but can differentiate among various classes of microorganisms. A striking example is seen in D. melanogaster that are naturally infected by entomopathogenic fungi. These flies exhibit an adapted response mediated through the selective activation of the Toll pathway and produce only peptides with antifungal activities [11].

Although a rapidly increasing number of genes involved in intracellular signaling during infections was discovered, it was not until 2001 that the role of the first bona fide pattern recognition receptor was identified through a mutagenesis screen [12]. The gene that had been mutated with a resulting loss of resistance to gram-positive bacterial infections was found to encode a member of a family of circulating or transmembrane proteins initially described in lepidopteran species as peptidoglycan recognition proteins (PGRPs) [13, 14]. Mutants in a gene encoding a structurally related protein and involved in recognition of gram-negative infection were described a few months later [15–17]. Finally, the initiation of a defense reaction against fungi was found to be independent of these 2 genes but was shown to require a blood protease [18].

Our understanding of the D. melanogaster immune response has been described in recent reviews from our
THE SYSTEMIC ANTIMICROBIAL DEFENSE OF D. MELANOGASTER

The host defense of D. melanogaster is a multifaceted process. A septic injury induces proteolytic cascades in the hemolymph (phenol oxidase and coagulation cascades), defense reactions by hemocytes (i.e., phagocytosis of microorganisms or encapsulation of larger-sized invaders), and the synthesis and release of antimicrobial peptides. The epithelial surfaces of the body, which serve as first-line defenses against microorganisms, mount a so-called local response: the epidermis, cells of the digestive and genital tracts, and tracheae all produce antimicrobial peptides that inhibit microbial growth at the infection site [25–27]. The hallmark of the humoral defense reaction is the immune-induced synthesis by the fat body, an equivalent of the mammalian liver, of antimicrobial peptides that are secreted into the hemolymph, where their combined concentrations reach 300 μM in infected flies [28, 29].

Figure 1 summarizes our understanding of these peptides. They have mostly a large spectrum of activity, although they often have preferential targets. Drosomycin and metchnikowin are essentially antifungal molecules; defensin (and metchnikowin) is active against gram-positive bacteria; cecropins, drosocin, attacins, and diptericins are anti–gram-negative peptides, although cecropins variously affect gram-positive bacteria and some fungal strains [30]. None of these peptides is cytotoxic at the concentrations found in the hemolymph. The mode of action of these molecules is not fully understood and probably is not unique. They are essentially membrane active and induce perturbations of the prokaryotic or fungal cell membrane, leading to efflux of solutes and often to very rapid death of the microorganisms [31, 32].

TWO DISTINCT SIGNALING PATHWAYS REGULATE THE SYSTEMIC ANTIMICROBIAL RESPONSE

The cloning in the early 1990s of the D. melanogaster genes encoding antimicrobial peptides revealed the presence of κB-like response elements in their promoters [33, 34]. Establishment of transgenic fly lines with mutated elements indicated they were mandatory for immune inducibility of these genes [35, 36]. κB response elements, which were first described in mammals, confer inducibility to some immune-responsive genes via transactivators of the NF-κB/Rel family [37] (reviewed in [38]).

At the time that data were obtained regarding the role of κB-like response elements in D. melanogaster, a single NF-κB family member was known in the fly, Dorsal, which is involved in dorsoventral patterning in the early embryo [39, 40]. Two additional NF-κB/Rel family members have since been identified in D. melanogaster: DIF (dorsal-related immunity factor) [41] and Relish [42] (figure 2). Several independent studies in the mid-1990s found significant similarities between the activation of Dorsal by the Toll pathway during embryonic de-
Figure 2. Drosophila melanogaster homologues of the mammalian Rel/NF-κB proteins. Two representative human Rel proteins are shown: p65 (RelA) and p105/p50. The Dorsal gene was first implicated in dorsoventral patterning; DIF (dorsal-related immunity factor) and Relish were later characterized during studies on D. melanogaster immune response. Rel proteins share extensive homology within a 300-aa region (REL domain) responsible for DNA binding and protein dimerization and contain a nuclear localization signal (NLS). p105/p50 and Relish contain ankyrin (Ank) motifs in the C-terminal area. p65, Dorsal, and DIF are retained in the cytoplasm of uninduced cells by binding to an ankyrin motif-rich inhibitor (IxB or Cactus). Their nuclear translocation requires signal-dependent dissociation form this inhibitor. For p105 and Relish, signal-dependent proteolytic cleavage (arrows) is required for nuclear translocation of the REL domains. Numbers refer to amino acids in various proteins.

Development in D. melanogaster and the cytokine-induced (interleukin [IL]–1) activation of NF-κB in mammalian cells [43]. These similarities prompted our laboratory to undertake a genetic analysis of the immune induction of antimicrobial peptides in Toll pathway mutants in D. melanogaster.

In 1996, we showed that induction of the antimicrobial peptide drosomycin required Toll and several other components of the embryonic Toll-signaling pathway [9]. In contrast, induction of antibacterial peptides (e.g., diptericin) appeared to be Toll independent but required the presence of the imd gene (for immunodeficiency) [10]. Furthermore, mutants of the Toll pathway were highly susceptible to fungal infections, but behaved as wild type when challenged with gram-negative bacteria. Conversely, imd mutants were more susceptible than wild-type flies to gram-negative bacterialchallenge and resisted fungal infections, as did wild-type flies. We concluded that 2 distinct signaling pathways controlled the resistance to microbial infections and the induction of the various antimicrobial pathways.

The Toll pathway. The role of the Toll pathway in the immune response is particularly important during natural fungal and gram-positive bacterial infections (figure 3). Toll is activated via a cleaved form of the polypeptide Spaetzle, which is structurally similar to mammalian nerve growth factor [44]. Processed Spaetzle interacts with the extracellular leucine-rich domain of Toll. The intracellular domain of this receptor has a TIR (Toll-IL receptor) homology domain, which is also present in DmMyD88 [45], in all D. melanogaster Tolls, in mammalian Toll-like–receptors [23], and in many plant proteins involved in defense reactions [46, 47].

A receptor-adaptor complex is formed on the intracytoplasmic side of Toll, which comprises 3 death domain proteins: DmMyd88 (mentioned above), Tube, and the kinase Pelle. This complex signals to the ankyrin domain protein Cactus, which is phosphorylated by an undefined kinase (distinct from Pelle) and dissociates from the NF-κB/Rel protein DIF. Although Cactus becomes degraded, DIF translocates into the nucleus and directs the transcription of the drosomycin gene [48] plus that of some 350 additional genes induced by natural fungal infection (up-regulation by a factor ≈ 2), many with unknown functions [49] (see also [50, 51] for other genome-wide analyses of immune response in D. melanogaster).

In addition to Toll, the D. melanogaster genome contains 8 Toll-related receptors with similar extracellular leucine-rich repeats and intracytoplasmic TIR domains (for details see [52, 53]). As for Toll, the genes encoding these receptor proteins are expressed during embryogenesis with tissue-specific and stage-dependent patterns characteristic for each individual family member [54]. However, at present there is no firm experimental evidence that, except for Toll, any of the other 8 Toll-related receptors plays a role in the host defense of D. melanogaster, although Toll 5 and Toll 9 induce the expression of the drosomycin gene in cell culture [52, 55, 56].

Imd pathway. The Imd pathway is primarily activated by infection with gram-negative bacteria and controls resistance to these microorganisms (figure 4). The transmembrane receptor of this pathway has not been firmly identified (see PGRP-LC, below). It is also not known whether this putative receptor is activated by direct interaction with microbial patterns (as with mammalian Toll-like receptors) or by the end product of a proteolytic cascade (as for Toll). The imd gene was recently identified as a death domain protein (this death domain is most similar to that of mammalian RIP (tumor necrosis factor–α receptor-interacting protein) [57].

The Imd protein probably interacts with DmFADD [58, 59] and the caspase-8 homologue DREDD [60, 61]. Loss of function mutations in the genes encoding both DmFADD and DREDD silence the Imd pathway [58–61]. The mitogen-activated protein kinase kinase kinase (MAP3KKK) dTAK1 acts downstream of Imd/DmFADD and activates an IkB kinase (IKK) signalosome equivalent [62] consisting of D. melanogaster homologues of mammalian IKKβ and IKKγ/NEMO (NF-κB essential modifier) [63–65]. Wild-type DmIKKβ and DmIKKγ are required for normal anti–gram-negative responses [64, 65]. The NF-κB/Rel family member of the Imd pathway is the protein Relish, which is cleaved by an unknown caspase: the Rel homology domain translocates into the nucleus,
Figure 3. Toll-dependent induction of immune genes by fungal and gram-positive infections. Cysteine knot growth factor Spaetzle is activated through cleavage by blood proteases, which may be activated by fungi and gram-positive bacteria. Cleaved Spaetzle interacts with the membrane receptor Toll. The current view is that as a result of Toll activation by Spaetzle, the receptor/adaptor complex formed by the TIR (Toll-interleukin receptor) domain of Toll and MyD88 and the death domain (DD) proteins, Tube and Pelle, triggers the phosphorylation of Cactus by an unknown kinase. (Precise molecular mechanisms of this complex process have not been worked out.) Phosphorylated Cactus is degraded and DIF (dorsal-related immunity factor) translocates into the nucleus to induce the expression of drosomycin and additional genes. Drosophila melanogaster contains 8 additional genes coding for Toll-related proteins and all have an intracellular TIR domain. Their role in immunity, if any, remains to be established. ?, Unidentified Cactus kinase.

whereas the ankyrin repeat domain remains in the cytoplasm [66]. Cleaved Relish activates the transcription of the genes encoding peptides, such as diptericin, but also those of many other genes (≥220 genes are up-regulated by a factor of ≥2 by gram-negative bacterial infection), some with unknown function [49].

FROM SEPTIC INJURY TO ACTIVATION OF THE TOLL OR IMD PATHWAYS

As noted, over the last few years, we have gained a fair understanding of the intracellular signaling pathways that control transcription of the antimicrobial peptide genes and of many other immune-responsive genes, but significant gaps in our knowledge remain (e.g., identities of the Cactus kinase and the protease that cleaves Relish in response to immune challenge), but we believe that these gaps will be filled in a foreseeable future. A quintessential question that was not adequately addressed during the investigations mentioned pertains to the activation of the 2 intracellular signaling pathways during infection.

The study of the necrotic mutation in 1999 clearly showed that Toll does not directly sense microbial infection. It is activated (as during embryogenesis) by a cleaved form of the cysteine knot cytokine-like polypeptide, Spaetzle [67]. Recognition of infectious non-self thus was known to occur upstream, and the quest for the actual pattern recognition receptor of fungal and gram-positive bacterial infection (the predominant activators of the Toll pathway) remained open. The breakthrough came with the generation by Michel et al. [12] of a mutant fly line (named “semmelweis”) that failed to activate Toll in response to gram-positive bacterial challenge and had a dramatically lowered resistance to this type of infection [12].

The gene mutated by random ethyl methane sulfonate (EMS) mutagenesis in the experiments by Michel et al. [12] turned out to be PGRP-SA, a member of a large evolutionary conserved family of so-called PGRPs. Other researchers independently isolated the first PGRPs in lepidoptera through binding experiments of gram-positive bacteria to insect hemolymph [13, 14]. At about the same time, the D. melanogaster genome was found to contain 13 members of this family [68], which also has representatives in humans, mice, and cattle [14, 69, 70]. Figure 5 compares the members of the PGRP families in various species, as shown by gene databases (see also the legend to figure 5).

In D. melanogaster, the PGRPs do not appear to play redundant roles. The semmelweis mutation, which affects PGRP-SA (figure 5; 1 of the 13 members), only blocks Toll activation by some gram-positive bacteria [12]. Toll activation by fungal infection remains wild type in these mutants. How sensing of gram-positive bacteria by PGRP-SA, the semmelweis protein,
translates into cleavage of Spaetzle and activation of Toll, has yet to be worked out in molecular terms. The process certainly occurs in the circulating hemolymph, as transfer of wild-type hemolymph to mutant semmelweis flies restores the capacity of these flies to activate Toll in response to gram-positive bacterial challenge and confers a wild-type survival to the mutant flies [12].

A few months after the report on semmelweis, researchers in 3 independent studies discovered the role of another PGRP family member in resistance to gram-negative infection [15–17]. The candidate gene in this case encodes a putative transmembrane receptor, PGRP-LC, and potentially can form several splice isoforms. Overexpression of the PGRP-LC gene activates the Imd (but not the Toll pathway) in the absence of immune challenge. It is not yet clear how PGRP-LC can bring about this activation. The gene sequence does not point to any obvious signaling domain. It is probably premature to qualify PGRP-LC as either a lipopolysaccharide receptor or even the receptor of the Imd pathway; however, a wild-type copy of the PGRP-LC gene is undoubtedly required for activation of the Imd pathway by gram-negative infection.

Neither mutation discussed above (in the PGRP-SA and PGRP-LC genes) affects induction of Toll by fungal infection. How this type of infection elicits an immune response upstream of the Toll receptor remained elusive until very recently. Ligoxygakis et al. [18] showed that an EMS-induced mutation in the persephone gene, which encodes a previously unknown serine protease, blocks the induction of the Toll pathway by fungi and resistance to this type of infection [18]. Ectopic expression of the persephone protease is sufficient to induce Toll activation in the absence of an immune challenge, as is transfer of hemolymph from flies overexpressing persephone. The persephone sequence predicts an NH$_2$-terminal prodomain, which contains a cysteine-rich clip module most similar to those of Easter and Snake, which are zymogens of the embryonic cascade leading to Spaetzle cleavage. It remains unclear how fungal infection activates the persephone zymogen. Of interest, mutations in persephone do not affect responses to gram-positive and gram-negative bacterial infections.

**PERSPECTIVES**

In this short review, we attempt to draw a global picture of the *D. melanogaster* antimicrobial defense as it emerges after a short decade of investigations. We have drawn a thread from the recognition of infectious non-self by pattern-recognition receptors to the activation of intracellular signaling pathways and the expression of immune-responsive genes, among which we have highlighted the antimicrobial peptides.

We are convinced that future research will not change the general outline we present; however, we know our discussion is oversimplified in several crucial respects. First, the host de-
**Figure 5.** Schematic representation of peptidoglycan recognition proteins (PGRP) in several Drosophila species by size: short (S; <210 residues long; A–C) and long (L; D and E). A, Drosophila melanogaster (D.m.). B, Other insects: Calpodes ethlius (C.e.), Bombyx mori (B.m.), and Trichoplusia ni (T.n.). C, Mammals: Mus musculus (M.m.), Bos taurus (B.t.), Rattus norvegicus (R.n.), Camelus dromedarius (C.d.), and Homo sapiens (H.s.). D, D.m. E, H.s. and M.m. Different domains are indicated by numbering of the bordering amino acids: putative signal peptide (green), transmembrane domain (red), and PGRP domain (blue). Cysteins are indicated by vertical bar and disulfide connectivity by “⊓.” Only the disulfide connections for the bovine PGRP have been experimentally proven [70]; 2 are putative (Bommo [13] or Trini [14]); the rest are extrapolated from the bovine model. When only the last cysteine remains unbound, it is shown as a “G,” indicating a potential for dimerization or metal ion coordination. For each gene, the species abbreviation is given followed by the gene symbol (if it exists) and a accession number to a databank (FBgn00xxxxxx for Flybase, Swp:xxxxxx for Swiss-Prot, for SPTrEmbl if no indication). For PGRPs, 3 different functions have been proposed: binding to peptidoglycan, (e.g., B.m. [13]), participation in cuticular degradation during the molting process (e.g., C.e.) [71], and antimicrobial activity (e.g., B.t.) [70].
fense, as mentioned, is not limited in *D. melanogaster* (nor in other organisms) to the synthesis and release of antimicrobial peptides. We do not deny the crucial role of these molecules. Mutants that fail to produce these peptides have significantly lower survival rates after infection than wild-type flies. This finding can be partially changed by introducing functional antimicrobial peptide genes into these compromised flies [72].

As we have repeatedly mentioned, several hundred genes are markedly up-regulated by immune challenge, in addition to the antimicrobial peptides, and their contribution to fighting infection has not yet been seriously addressed. As for the control of expression of the antimicrobial peptide genes, we are fairly safe in admitting that only the Toll and the Imd pathways are involved here. Indeed, in flies carrying mutations in both pathways, none of the genes encoding these peptides remains inducible. A recurrent question pertains to a possible cross-talk of the 2 pathways, and in vitro experiments with *D. melanogaster* cell lines have led to the assumption that heterodimers of Relish and DIF or Dorsal can exert specific roles in controlling some of the peptide genes [73]. Promoter analysis of the drosomycin gene points to the existence of both DIF- and Relish-response elements, although further clarification is needed. At this stage, we should leave open the possibility of intracellular cross talk between activated Toll and Imd pathways.

The major challenge of the next few years will be to understand the mechanisms that link microbial invasion to activation of Toll/DIF and Imd/Relish signaling pathways. The data that we analyzed provide genetic evidence that the persephone and PGRP-SA (semmelweis) and PGRP-LC genes are required for the respective inductions by fungal and gram-positive and gram-negative bacterial infections. They do not, however, provide insight into the mechanisms by which the microorganisms interact with pattern recognition proteins nor how this interaction is translated into activation of the pathways. Possibilities include the conformational change of the pattern recognition proteins, leading to activation of specific zymogens, but reliable data are still missing. A further complication arises from the fact that most microorganisms carry a plethora of structural patterns, which raises the possibility that a given pathogen could activate concomitantly both pathways. There is evidence that this occurs in some instances [11, 48].

Finally, we did not substantially review the contribution of blood cells and the various proteolytic cascades (phenol oxidase and coagulation cascades) and the complement-like system in the overall resistance to infection. We are confident, however, that the recent generation of a significant number of mutants affecting these various facets of the host defense will help unravel this part of the picture over the next years.

References

transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. EMBO J 1998; 17:1217–27.


