Antimicrobial peptide defense in *Drosophila*

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Summary

Drosophila responds to a septic injury by the rapid synthesis of antimicrobial peptides. These molecules are predominantly produced by the fat body, a functional equivalent of mammalian liver, and are secreted into the hemolymph where their concentrations can reach up to 100 μM. Six distinct antibacterial peptides (plus isoforms) and one antifungal peptide have been characterized in *Drosophila* and their genes cloned. The induction of the gene encoding the antifungal peptide relies on the *spätzle/Toll/cactus* gene cassette, which is involved in the control of dorsoventral patterning in the embryo, and shows interesting structural and functional similarities with cytokine-induced activation of NF-κB in mammalian cells. An additional pathway, dependent on the as yet unidentified *imd* (for *immune-deficiency*) gene, is required for the full induction of the antibacterial peptide genes. Mutants deficient for the *Toll* and *imd* pathways exhibit a severely reduced survival to fungal and bacterial infections, respectively. Recent data on the molecular mechanisms underlying recognition of non-self are also discussed in this review.

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Introduction

Animals have developed two basic host defense reactions against invading microorganisms, which are classically referred to as innate and adaptive immunity. Innate, non-adaptive immunity represents a first line defense and involves both cellular reactions by specialized blood cells and the rapid synthesis of proteins with a wide range of activities, such as opsonisation of microorganisms, inhibition of proteases, intercellular signaling or direct antimicrobial action. In mammals, the systemic aspect of innate immunity is referred to as acute phase response[1] and is triggered by cytokines released by macrophages upon encounter of microorganisms. Whereas innate immune reactions are present in all classes of the animal kingdom, adaptive immunity is restricted to gnathostome vertebrates. Its two hallmarks are the existence of a large repertoire of recognition molecules, generated by somatic rearrangement of gene fragments in lymphocytes (immunoglobulins, T-cell receptors), and memory, which results from the clonal expansion of selected lymphocytes.

Insects are particularly resistant to infections by microorganisms. Their defense reactions rely on both cellular and humoral mechanisms (for recent reviews, see refs 2-5). The cellular aspects include phagocytosis and encapsulation of invading microorganisms and are particularly well developed in the ancient insect orders[6]. The humoral facet involves the activation of proteolytic cascades leading to melanization and coagulation. In the recent insect orders, a striking aspect of the humoral host defense is the rapid synthesis by the fat body of antimicrobial peptides, which are released into the hemolymph to counter the development of microorganisms[7-9]. Antimicrobial peptides are detected in insect hemolymph as early as 2 to 4 hours after a septic injury. Their concentrations vary greatly and range from 1 to 100 μM, which corresponds to the levels at which they are active against their respective microbial targets. They exhibit large and complementary spectra of activity against various microorganisms. Their mode of action is still poorly understood. We will summarize our present information on the structures of the inducible antimicrobial peptides of insects and then focus on what we have recently learned about the control of their gene expression in *Drosophila*.

Inducible antimicrobial peptides in insects

The first inducible antimicrobial insect peptides to be fully characterized were the cecropins, isolated from pupae of *Hyalophora cecropia* by Boman and associates[10]. Since the discovery of cecropins, more than 100 inducible antimicrobial peptides have been described from various insect

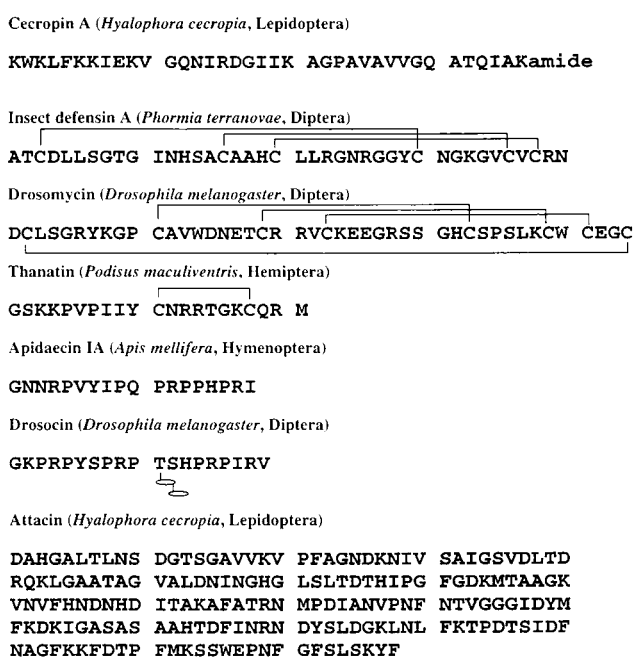

Fig. 1. Amino-acid sequences of representative members of each of the antimicrobial peptide families in insects. *Hyalophora* cecropin A[10], insect defensin from *Phormia*[25], drosomycin[28], thanatin[31], two proline-rich peptides (apidaecin IA[33] and drosocin[35]), and a glycine-rich polypeptide (*Hyalophora* attacin[43]) are shown. The intramolecular connections of the disulfide bridges are indicated, as well as the position of the O-linked disaccharidic motif of drosocin.

sources[11]. For convenience, these molecules can be grouped into four major families, which we shall briefly discuss hereafter.

(1) Cecropins

Cecropins are 31- to 39-residue cationic peptides devoid of cysteine residues[12] (Fig. 1). They consist of an amphipathic N-terminal helix and a hydrophobic C-terminal helix separated by a short flexible hinge. All known cecropins are C-terminally amidated. More than 20 isoforms of cecropins have been described from Lepidoptera and Diptera and they are mostly active against Gram-negative bacteria[4,11-14]. Surprisingly, these molecules have not been reported to date from other insect orders. A cecropin homologue has been described in extracts of the pig intestine[15], but studies aimed at cloning the corresponding gene in pigs have remained so far inconclusive. Cecropins were shown to permeabilize the bacterial membranes through their amphipathic helix structure[12].

(2) Cysteine-containing antimicrobial peptides

These peptides are 2-5 kDa molecules with two to eight cysteine residues, which form intramolecular disulfide bridges (Fig. 1). Three types of cysteine-containing peptides have been analysed in some detail:

(a) Insect defensins

Insect defensins are 4-5 kDa peptides which consist of a central amphipathic α-helix linked *via* two disulfide bridges to a C-terminal antiparallel β-sheet[16,17]. The N-terminal residues of these molecules form a flexible loop linked *via* a disulfide bridge to the β-sheet[17]. Some 30 defensins have been described from a variety of insect orders[11]. Insect defensin homologues are also present in scorpions[18,19] and molluscs[20,21]. Insect defensins (also referred to as sapecins[22,23]) were initially given their name on the basis of partial sequence similarities with mammalian defensins[24,25]. 3-D structures of insect and mammalian defensins have since been worked out and their analysis has shown a major difference in that the mammalian defensins consist solely of β-sheets[26] and lack the characteristic central amphipathic α-helix of invertebrate defensins[17]. The latter are essentially active against Gram-positive bacteria[11]. Studies with *Phormia* defensin showed that this molecule disrupts the permeability barrier of the cytoplasmic membrane of Gram-positive bacteria by a voltage-dependent process[27].

(b) Drosomycin

Drosomycin is a 44-residue peptide containing eight cysteine residues engaged in four intramolecular disulfide bridges[28]. It consists of a central α-helix linked to an antiparallel β-sheet *via* two disulfide bridges, as in insect defensins. In comparison with the latter molecules, drosomycin shows an extended N-terminal sequence forming an additional β-sheet (M. Ptak, personal communication). This structure is reminiscent of that of plant defensins recently described from various plant families, namely from *Brassicaceae*[29,30]. As is the case for plant defensins, drosomycin is predominantly active against filamentous fungi, by inhibiting spore germination or delaying the growth of hyphae, which therefore exhibit abnormal morphology[28].

(c) Thanatin

Thanatin is a 21-residue cationic peptide with sequence homology to frog skin antimicrobial peptides of the brevinin family[31,32]. It has a single disulfide bridge and exhibits a remarkably large spectrum of activity against both Gram-positive and Gram-negative bacteria and against filamentous fungi. Thanatin has been isolated so far only from the bug *Podisus maculiventris* (Hemiptera).

(3) Proline-rich peptides

Hymenoptera, Diptera, Hemiptera and Lepidoptera synthesize a variety of mostly small-sized (2-3 kDa) proline-rich peptides predominantly active against Gram-negative bacteria (Fig. 1)[33-38]. Their mode of action is unknown at present; in the case of apidaecin and of drosocin, which are proline-rich peptides isolated from honey-bees and from *Drosophila*, respectively, all-D isoforms are inactive[39,40], suggesting that the native peptides act *via* chiral receptors.

Some of the proline-rich peptides carry an O-glycosylated substitution (e.g. drosocin[35], pyrrhocoricin[36], lebocin[38]) which is necessary for their full activity. Proline-rich antibacterial peptides are also present in bovine neutrophiles[41] and in pig intestine[42], although sequence homology with the insect peptides does not appear to exist.

(4) Glycine-rich polypeptides

These form a heterogeneous family of immune-inducible insect polypeptides, with sizes ranging from 8 to 30 kDa[43,44], which have in common a higher-than-average proportion of glycine residues (10-21%)[11]. They are mainly active against Gram-negative germs. It has been shown in *Hyalophora* that the glycine-rich polypeptide attacin inhibits the synthesis of outer membrane proteins in *E. coli* by interfering with *omp* gene transcription[45]. The prototype of this family is attacin (Fig. 1), initially isolated from the moth *Hyalophora cecropia*[43]. Glycine-rich antibacterial polypeptides are present in many insect orders. To date, no structural homologues have been reported from Vertebrates.

Lysozymes

Lysozymes also participate in the insect host defense[46,47]. These ubiquitously distributed enzymes of the animal kingdom are large (ca. 14 kDa) cysteine-rich polypeptides with anti-Gram positive activity, and cleave the peptidoglycan bonds of the bacterial cell wall. They are present in many insect species and were shown to be induced by immune challenge in Lepidoptera[48-50]. Surprisingly however, this challenge represses their expression in *Drosophila*[51].

In *Drosophila*, on which this review focuses, an immune challenge induces the synthesis of the following antimicrobial peptides: (1) several cecropin isoforms[52,53]; (2) the cysteine-containing peptides defensin[54] and drosomycin[28]; (3) the proline-rich peptides drosocin[35,55] and metchnikowin[56]; and (4) the glycine-rich polypeptides attacin[57] and diptericin[58]. Work in progress in this laboratory points to the existence of several additional inducible antimicrobial peptides in this species.

The expression of the genes encoding antimicrobial peptides

The genes and/or cDNAs encoding the antimicrobial peptides of *Drosophila* have now been cloned and gene sequences for some of the inducible antibacterial peptides are also available from Lepidoptera, Hymenoptera and other Diptera. The organisation of some relevant genes is presented in Fig. 2. In *Drosophila*, the genes encoding antimicrobial peptides are mostly unique and intronless, with the remarkable exception of cecropins (grouped in a cluster of four closely related genes, each containing a short intron[52,53]) and attacins (several genes detected[57]). They code for prepropeptides, containing a signal sequence, a prosequence, which can be short but the limits of which are not always clearly defined, and the mature peptide sequence. Occasionally, as in drosocin, the prosequence is located C-terminally to the mature peptide sequence[35].

The promoter regions of these genes were found to contain sequence motifs with a high degree of similarity to established *cis*-regulatory elements of some mammalian acute-phase response genes. In particular this is the case for motifs similar to mammalian NF-κB (Nuclear Factor-kappa B) response elements (RE), which appear to be present in single or multiple copies in all the promoters of immune-inducible antimicrobial peptide genes of insects[14,54,55,58-60].

In the case of the diptericin and the cecropin genes, experiments with transgenic fly lines carrying reporter genes fused to wild-type, mutated or truncated promoter sequences, clearly showed that these κB-related sequences are mandatory for immune-inducibility of both genes[60,61]. Associated motifs (e.g. motifs homologous to the mammalian Interleukin-6 response elements and half-

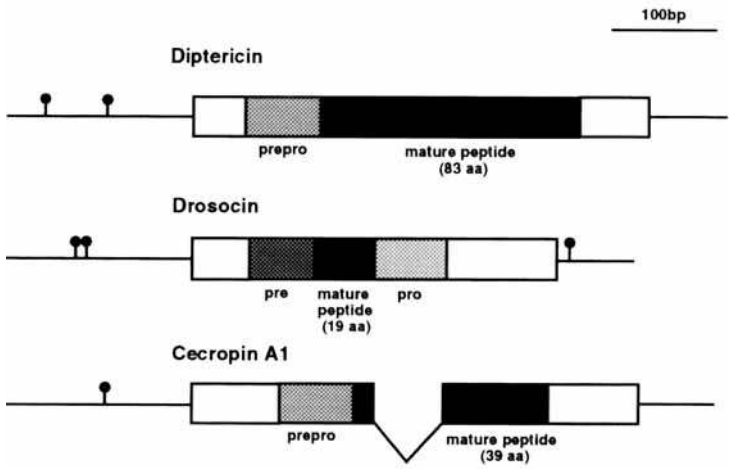

Fig. 2. Structure of the genes encoding three antibacterial peptides of *Drosophila*: diptericin[58], drosocin[55] and cecropin A1[52]. The mature peptides are shown in black and the prepro regions in dashed boxes. The white boxes represent the 5′ and 3′ untranslated regions. The position of the κB-related decameric sequences in the upstream or downstream genomic regions are indicated (●).

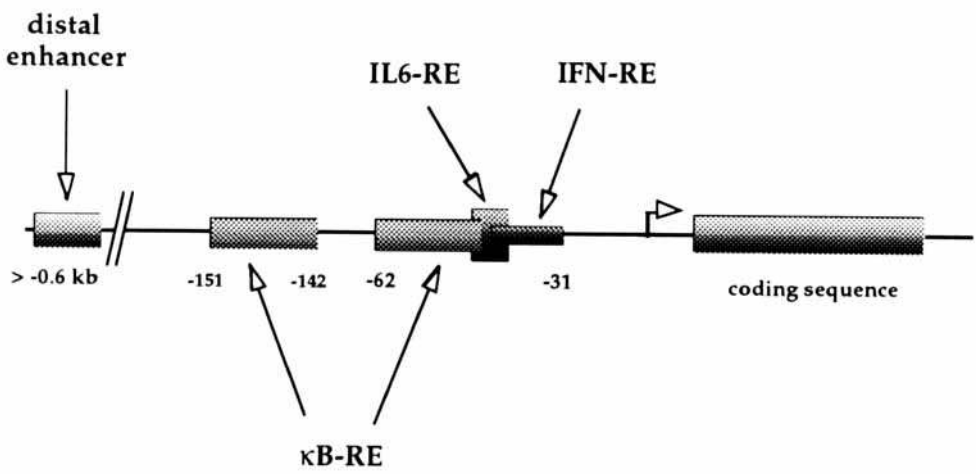

Fig. 3. Regulatory elements in the promoter of the diptericin gene[61-64]. The two κB response elements (κB-RE) confer immune inducibility to the gene. Other regulatory sequences such as IL6 response elements (IL6-RE) and interferon response elements (IFN-RE), together with a distal enhancer located between –0.6 and –2.2 kb, are responsible for the upregulation of the induced expression.

sites for Interferon Regulatory Factor binding) appear to upregulate the level of transcription[62,63] and additional enhancer elements are located more upstream (see Fig. 3)[64]. The demonstration that the nucleotide sequence motifs related to NF-κB response elements control the immune-inducibility of the diptericin and cecropin genes raised the question of whether the transactivating proteins binding to these motifs are homologous to mammalian NF-κB, which acts as a rapidly inducible transactivator on a large number of immune-responsive genes in this class[65,66]. NF-κB is composed of two subunits, both containing a so-called Rel-homology domain (the name is derived from the v-Rel oncogene, which causes a severe form of avian reticuloendotheliosis). NF-κB was initially described as a p50/p65 heterodimer, but several other Rel proteins can heterodimerize to constitute functional NF-κB complexes with different DNA binding affinities and functions. In unstimulated mammalian cells, NF-κB is present in the cytoplasm complexed to an inhibitory protein, I-κB. The activation of cytoplasmic NF-κB by various stimuli results in the dissociation from I-κB and the concomitant translocation into the nucleus where it triggers the expression of target genes (Fig. 4). Interestingly, a Rel homologue functions in early embryonic development of *Drosophila* to direct dorso-ventral patterning[67-69]. This homologue, the morphogen dorsal, is complexed in the cytoplasm of syncytial blastoderm stage embryos to an inhibitory protein, the product of the *cactus* gene, which is a homologue of mammalian I-κB. In the ventralmost region of the embryo, a processed form of the protein spätzle serves as an external ligand and, upon binding to Toll, activates a signaling cascade which leads to the dissociation of dorsal from cactus. As a consequence, the dorsal protein translocates into the nucleus and regulates the restricted expression of zygotic genes involved in the formation of the dorso-ventral pattern (Fig. 4). Strikingly, the *Drosophila* transmembrane protein Toll, which serves as a receptor for the spätzle ligand, shares sequence homology in its intracellular domain with that of the interleukin-1

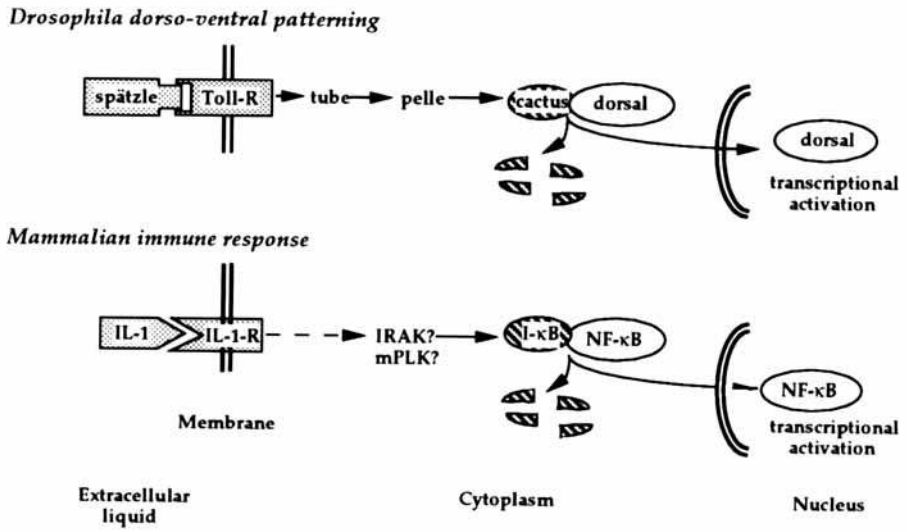

Fig. 4. Model for the conserved pathway leading to the nuclear translocation of dorsal and NF-κB[69]. On the ventral side of the *Drosophila* embryo, binding of the ligand spätzle to the receptor Toll triggers the signal transduction through tube and pelle, which ultimately leads to the dissociation of the dorsal/cactus complex and to dorsal nuclear import. In the mammalian immune responsive cell, binding of IL-1 to its receptor activates an as yet incompletely described pathway, which possibly includes the pelle homologue IRAK[72] (IL1 receptor-associated kinase) or mPLK[73] (mouse pelle-like protein kinase). This activation leads to the dissociation of the NF-κB /I-κB complex.

receptor[70,71]. In addition, pelle, a serine-protein kinase of the Toll-mediated signaling cascade, shows sequence homology with kinases associated with cytokine signal transduction (IRAK, for IL-1 receptor-associated kinase[72] and mPLK, for mouse pelle-like protein kinase[73]).

The structural and functional similarities between gene cassettes involved in *Drosophila* dorsoventral patterning and in the mammalian immune response, invited the question of whether antimicrobial gene expression in *Drosophila* is subordinated to a similar control mechanism. A molecular genetic analysis has recently provided some answers to this question[74,75]. It was first shown, and in some cases confirmed, that all the genes of the dorsoventral regulatory cascade extending from *spätzle* to *dorsal* are expressed in larvae and adults; these genes had originally been described primarily as maternally expressed genes. The analysis in *Drosophila* adults of the immune-inducibility of the antimicrobial peptides in mutant backgrounds revealed that in mutants deficient for any of the genes of the regulatory cascade, the induction of the drosomycin gene is dramatically affected, except for *dorsal*-deficient mutants, in which drosomycin gene induction is not decreased. The effect on the inducibility of the antibacterial peptide genes varies from gene to gene: diptericin and drosocin induction is not affected, but that of cecropins, attacin and defensin is significantly reduced in the mutants. In *Toll* gain-of-function mutants, in which the transmembrane receptor is constitutively active, the drosomycin gene is signal-independently expressed at levels similar to those normally observed in immune-challenged wild-type adults. Similarly, in *cactus*-deficient mutants, in which the transactivating Rel protein(s) is constitutively nuclear, the drosomycin gene is constitutively expressed. Significantly, however, in both the *Toll* gain-of-function and the *cactus*-deficient mutants, the antibacterial peptide genes are not constitutively transcribed, although they remain inducible by immune challenge.

These results indicate that in *Drosophila* adults, the *Toll* signaling pathway is necessary and sufficient for the induction of the gene encoding the antifungal peptide drosomycin, but that the genes encoding antibacterial peptides are dependent on an additional regulatory cascade. The serendipitous discovery of a recessive mutant, *imd* (for *immune deficiency*)[76], is a first indication for the existence of a second regulatory pathway: in homozygous *imd* mutants, the expression of the antibacterial peptide genes upon immune challenge is dramatically affected, whereas the drosomycin gene remains inducible. These data are summarized in Fig. 5, in which two clearcut pathways are presented: (1) the Toll signaling cascade, which controls drosomycin gene expression, and (2) the *imd* pathway, which appears to control antibacterial gene expression. Evidence has been obtained that only the diptericin and the drosocin genes are primarily dependent on the *imd* pathway, whereas cecropin, insect defensin and attacin gene inductions require a contribution from the *Toll* signaling

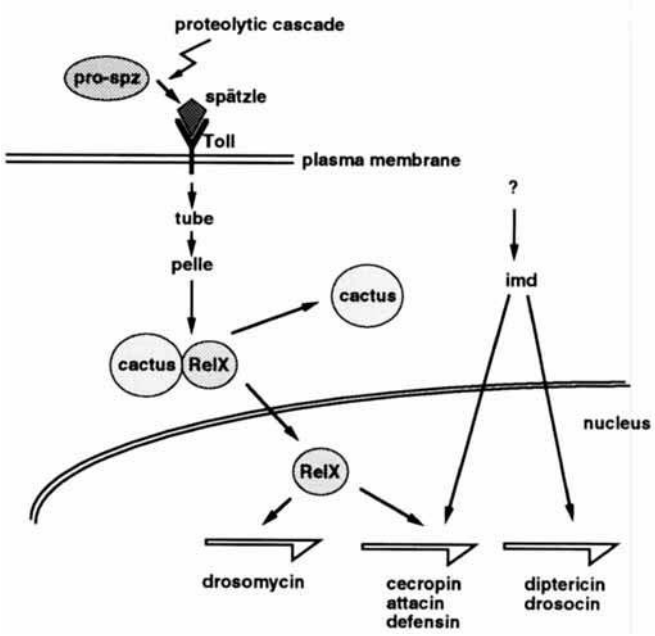

Fig. 5. Model for the control of expression of genes encoding antimicrobial peptides in the *Drosophila* adult fat body (reprinted from ref. 75, with permission). Two distinct pathways activate the expression of antimicrobial peptides. Drosomycin is induced *via* a Rel protein, which is retained in the cytoplasm of the fat body by cactus. The dissociation of the cactus/RelX complex is mediated by the *Toll*, *tube* and *pelle* gene products. It is proposed, by analogy with the embryonic system, that spätzle is present in the hemolymph and is processed by a protease of a proteolytic cascade induced upon septic injury, and acts as a ligand to activate the Toll receptor. All the genes encoding antibacterial peptides require the *imd* gene product for their induction. The full induction of the cecropin A, defensin and attacin genes also depends on the Toll pathway.

pathway (although none of these genes is constitutively expressed when the *Toll* pathway is signal-independently activated, e.g. in *Toll* gain-of-function mutants). The *imd* gene has not yet been cloned.

In Fig. 5, the identity of the transactivator bound to the cactus protein, and presumably released upon immune challenge, is indicated as Rel X. Three Rel proteins are at present known from *Drosophila*: dorsal[67,77], Dif (for dorsal-related immune factor)[78] and Relish (an analogue of mammalian p105, the precursor of p50)[79]. Transfection experiments indicate that these Rel proteins can activate antimicrobial peptide gene expression *via* the κB-related sites mentioned above. As stated above, however, in *dorsal*-deficient mutants, antimicrobial peptide genes remain fully inducible[74,75], suggesting that either dorsal is not involved in this process, or that other Rel proteins can substitute for its function. *Dif*-deficient mutants are not yet available; a series of studies performed on cecropin gene expression suggests nevertheless that this protein is a good candidate for Rel X[80]. An involvement of Relish is also an open possibility.

As illustrated in Fig. 5, the Toll ligand initiating the signaling cascade which leads to drosomycin gene induction is presumably derived from the *spätzle* gene product. This

assumption is based on the fact that in *spätzle*-deficient mutants, drosomycin fails to be induced by immune challenge. In the embryonic system, spätzle is cleaved within the vitelline fluid by a serine-protease to a shorter polypeptide, which is considered to be the active ligand for the Toll receptor[81,82]. Serine-proteases which act to process spätzle have been identified in the embryo (the *snake* and *easter* gene products)[82]. In adult *Drosophila* carrying loss-of-function mutations for the genes which encode these proteases, the induction of the drosomycin gene by immune challenge is not compromised[75], indicating either that easter and snake are not required to process the spätzle protein in the immune response, or that other proteases can substitute for their function. The peptide sequence of the spätzle protein points to possible structural analogies with the 'cysteine-knot' family of proteins[83], which comprise growth factors such as PDGF, TGFβ and NGF, as well as coagulogen, the clotting protein from horseshoe crab[84], which are also processed to their active form by serine-proteases. An attractive working hypothesis is that proteolytic cascades, triggered by injury (namely the coagulation cascade), lead to the processing of the spätzle protein in the hemolymph of *Drosophila*, thus generating the active ligand form which binds to the Toll receptor on the fat body cells and induces the signaling cascade leading to transcription of the drosomycin gene.

The role of antimicrobial peptides in the host defense of *Drosophila*

The fact that insects produce significant amounts of antimicrobial peptides in response to a septic injury has led most authors to consider that these molecules play a major role against invading microbes. Similar assumptions are made with regard to mammalian and plant antimicrobial peptides, although experimental evidence has been lacking so far in any of these systems. The observation in *Drosophila* that in *Toll*-deficient and in *imd* mutants the synthesis of the antimicrobial peptides is severely compromised, has provided a welcome model with which to investigate the role of these molecules in the host defense under *in vivo* conditions. In a series of experiments performed in wild-type and mutant adults of *Drosophila* challenged with either Gram-negative *E. coli* or the filamentous fungus *Aspergillus fumigatus*, Lemaitre and co-workers[75] have now reported that: (1) *imd* mutants, in which the challenge-induced synthesis of antibacterial peptides is dramatically lowered, exhibit a severely reduced survival rate when injected with *E. coli* as compared to *Toll* deficient or wild-type flies; however their resistance to infection with the fungus *A. fumigatus* is similar to that of wild-type flies; (2) conversely, *Toll*-deficient flies, in which drosomycin induction is severely compromised, but not that of the antibacterial peptides, are poorly resistant to infection by *A. fumigatus* but show a survival rate to *E. coli* which is similar to that of wild-type adults. Significantly in *imd* mutants, the number of *E. coli* per fly increases by three magnitudes within 24 hours following an infection, whereas no bacterial growth is observed in wild-type or *Toll*-deficient adults. These results demonstrate that the *imd* and *Toll* pathways are both essential for full antimicrobial resistance. They establish a correlation between the impairment of antifungal gene induction and reduced resistance to fungal infection and, conversely, between the impairment of antibacterial gene induction and reduced resistance to bacterial infection. Evidently, however, these results do not rule out the possibility that the *Toll*-deficient and *imd* mutations also affect immune mechanisms other than the antimicrobial peptide synthesis (such as cellular reactions[85]), which could contribute to survival from microbial infections.

Perspectives

This short overview has focused on the antimicrobial peptides in *Drosophila* and the control of their expression following immune challenge. Although significant progress has been made in recent years in this field, many essential questions remain to be answered. Among these are: (1) the characterization of the proteolytic enzymes leading to the formation of active Toll ligands and their induction by injury; (2) the precise roles of the Rel proteins in the immune response; (3) the characterization of the *imd* pathway and the identity of the *imd* gene.

Two areas were not covered in this review: the first pertains to the recognition of microorganisms. Receptors capable of binding a broad range of polyanionic ligands, oxidized lipoproteins, LPS, apoptotic cells, etc. have been characterized on embryonic hemocytes, cultured cell lines and fat body cells. These are a class C scavenger receptor[86] and croquemort[87], which shows sequence similarities with mammalian CD36. It is unclear whether ligand binding to these receptors induces the transcription of the antibacterial peptide genes. The second area, mentioned in the Introduction, concerns cellular immunity. *Drosophila* hemocytes are responsible for phagocytosis of microorganisms and encapsulation of larger intruders[88], but the molecular mechanisms underlying this facet of immunity have yet to be understood.

The most unexpected recent developments in the field of *Drosophila* immunity obviously relate to (1) the use of a same gene regulatory cassette in dorsoventral patterning and host defense and (2) the functional and structural similarities between some aspects of innate immunity in insects and mammals. It is hoped that use of these powerful tools of *Drosophila* genetics will help to gain a better insight on the evolution of the innate response in the animal kingdom.

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