Relish and the Regulation of Antimicrobial Peptides in *Drosophila melanogaster*

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“You must always search for truth.

*Truth does not depend on the point of view.*”

- Linus Pauling (1901-1994)

Till min familj...

*Tack för att ni finns!*
This thesis is based on the following articles. They will be referred to in the text by their Roman numerals.


ABSTRACT

The fruit fly *Drosophila melanogaster* has been a powerful model system in which to study the immune response. When microorganisms breach the mechanical barrier of the insect, phagocytosing cells and a battery of induced antimicrobial molecules rapidly attack them. These antimicrobial peptides can reach micromolar concentrations within a few hours. This immediate response is reminiscent of the mammalian innate immune response and utilizes transcription factors of the NF-κB family.

We have generated loss-of-function mutants of the NF-κB-like transcription factor Relish in order to investigate Relish’s role in the *Drosophila* immune response to microbes. Relish mutant flies have a severely impaired immune response to Gram-negative (G-) bacteria and some Gram-positive (G+) bacteria and fungi and succumb to an otherwise harmless infection. The main reason for the high susceptibility to infection is that these mutant flies fail to induce the antimicrobial peptide genes. The cellular responses appear to be normal.

Relish is retained in the cytoplasm in an inactive state. We designed a set of expression plasmids to investigate the requirements for activation of Relish in a hemocyte cell line after stimulation with bacterial lipopolysaccharide. Signal-induced phosphorylation of Relish followed by endoproteolytic processing at the caspase-like target motif in the linker region released the inhibitory ankyrin-repeat (ANK) domain from the DNA binding Rel homology domain (RHD). Separation from the ANK domain allowed the RHD to move into the nucleus and initiate transcription of target genes like those that encode the inducible antimicrobial peptides, likely by binding to κB-like sites in the promoter region.

By studying the immune response of the Relish mutant flies in combination with mutants for another NF-κB-like protein, Dorsal-related immunity factor (Dif), we found that the *Drosophila* immune system can distinguish between various microbes and generate a differential response by activating the Toll/Dif and Imd/Relish pathways. The recognition of foreign microorganisms is believed to occur through pattern recognition receptors (PRRs) that have affinity for selective pathogen-associated molecular patterns (PAMPs). We found that the *Drosophila* PRRs can recognize G- bacteria as a group. Interestingly, the PRRs are specific enough to distinguish between peptidoglycans from G+ bacteria such as *Micrococcus luteus* and *Bacillus megaterium* and fungal PAMPs from *Beauveria bassiana* and *Geotrichum candidum*.

This thesis also investigates the expression of the antimicrobial peptide genes, *Diptericin B* and *Attacin C*, and the putative intracellular antimicrobial peptide gene *Attacin D*, and explores a potential evolutionary link between them.
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ABBREVIATIONS

The abbreviations that are used in this thesis are listed in alphabetical order. All genes and gene products are written in *italics*. Protein names begin with a capitalized letter and are written in a regular font.

<table>
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<tr>
<td>18w</td>
<td>18 wheeler</td>
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<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
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<td>ANK</td>
<td>ankyrin-repeat</td>
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<td>Anp</td>
<td>Andropin</td>
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<td>aPKC</td>
<td>atypical Protein kinase C</td>
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<td>Att</td>
<td>Attacin</td>
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<td>Cec</td>
<td>Cecropin</td>
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<td>CIF</td>
<td><em>Cecropia</em> immunoresponsive factor</td>
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<td>DAP</td>
<td>diaminopimelic acid</td>
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<td>Def</td>
<td>Defensin</td>
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<td>Dif</td>
<td>Dorsal-related immunity factor</td>
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<td>DIF</td>
<td><em>Drosophila</em> immunoresponsive factor</td>
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<td>Dpt</td>
<td>Dipterycin</td>
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<td>Dro</td>
<td>Drosocin</td>
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<td>Drosophila</td>
<td><em>Drosophila melanogaster</em></td>
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<td>Drs</td>
<td>Drosomycin</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FRH</td>
<td>FLAG-Relish-RGSHis</td>
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<tr>
<td>G</td>
<td>glycine-rich</td>
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<td>G−</td>
<td>Gram-negative</td>
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<td>G+</td>
<td>Gram-positive</td>
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<td>GNBP</td>
<td>Gram-negative bacteria binding protein</td>
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<td>ICE</td>
<td>Interleukin-1 converting enzyme</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IKK</td>
<td>IκB kinase</td>
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<td>IL-1R</td>
<td>IL-1 receptor</td>
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<td>imd</td>
<td>immune deficiency</td>
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<td>IRAK</td>
<td>IL-1R-associated kinase</td>
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<td>Inhibitor kappa B</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>lipopolysaccaride</td>
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<td>Lys</td>
<td>Lysozyme</td>
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<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
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<tr>
<td>MASP</td>
<td>MBL-associated serine protease</td>
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MBL  Mannan-binding lectin
mbn-2  *lethal (2) malignant blood neoplasm*
MPAC  mature Pro-domain of Attacin C
*Mtk*  *Metchnikowin*
NF-κB  Nuclear factor kappa B
*Nmdmc*  *NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase*
NO  nitric oxide
P  proline-rich
PAMP  pathogen-associated molecular pattern
PG  peptidoglycan
PGRP  Peptidoglycan recognition protein
PO  Phenoloxidase
Pro-PO  Prophenoloxidase
Pro-ppA  Prophenoloxidase-activating enzyme
PRR  pattern recognition receptor
R1  Region 1
*Rel*  *Relish*
*RHD*  Rel homology domain
RIP  TNF-receptor-interacting protein
RNAi  RNA interference
S2  Schneider’s line 2
SR-CI  Scavenger receptor CI
TAK  Transforming growth factor beta-activated kinase
TCR  T-cell receptor
TEP  thiolester-containing peptides
TF  transcription factor
TIR  Toll/IL-1R
*Tl*  *Toll*
TNF  Tumor necrosis factor
TRAf  TNF receptor associated factor
TUA  teichuronic acid
Turandot  Tot
INTRODUCTION

Species as diverse as slime moulds, insects and humans defend themselves from intruders who see their beings as a superb environment to nourish and propagate their species. Agents known to cause disease include viruses, bacteria, fungi, protozoa and helminths (parasitic worms). Throughout evolution, organisms have developed several ways to defend themselves against these agents, from unspecific mechanical barricades to highly specific weapons such as antibodies.

Although the study of mammals such as mice is of great interest since they might reveal the secrets to our own survival in a microbial environment, research in primitive organisms such as amoeba often provide a more convenient way to investigate the architecture of our complex immune system. For my Ph.D. thesis, I have used the widely studied insect, *Drosophila melanogaster*, as a model system to develop a deeper understanding of the biology of immune defense. Before I describe the particular experiments and results that have been the focus of my graduate work, I will briefly review the differences between the vertebrate immune system and the immune system of *Drosophila* in the next two sections of the introduction. The following sections of the introduction will present some recognition and effector molecules of *Drosophila*, and the final part of the introduction describes Relish and the other Rel factors that regulate the expression of antimicrobial peptides in *Drosophila*.

**Immune defense in vertebrates**

Vertebrates have two branches of immune responses, the innate immune system of ancient origin and the adaptive immune system that is specific for vertebrates. The cornerstone of vertebrate immunity is the possibility to “remember” previous infections and thereby mount a faster and stronger immune reaction the next time the individual encounters the same pathogen. This protection is possible because of the adaptive immune system. The adaptive immune system is generated when immune cells bind to a specific antigen through a specific cell surface receptor, such as the immunoglobulin (Ig) molecule on B-lymphocytes or the T-cell receptor (TCR) on T-lymphocytes, in the context of a second stimulating signal. This activated lymphocyte starts to divide rapidly into a clone of genetically identical effector cells. A few of these effector lymphocytes are set aside and develop into memory cells and migrate to a lymph gland where they will survive for an unknown length of time. These memory cells can be rapidly activated when the individual once again encounters the same pathogen [described in (1)].

The specificity of an Ig or a TCR is determined by rearrangements and hypermutations of the gene segments encoding the variable part of this receptor in each new lymphocyte that is developed from the stem cells. Each lymphocyte derived from the stem cells has a unique homogeneous set of Igs or TCRs with a unique affinity for different antigens. However, these highly specific Ig or TCR
antigen receptors can sometimes recognize self-antigens. This could be very dangerous and lead to an attack by the immune system against the host’s own cells and tissues, a condition known as autoimmunity. Although there is a negative selection process to remove the self-reactive receptor-bearing cells, a tight control of the initiation of the adaptive immune response is still very important. This control is managed by the innate immune system.

In contrast to the adaptive immune system, the innate immune system is functional at birth and includes the first line of defense against foreign agents. Epithelial surfaces provide a mechanical and chemical barrier by secretion of antimicrobial proteins and peptides, which prevent intruders from colonizing the host. For example, the tight junctions that join epithelial cells prevent entry of microorganisms into deeper tissues. Furthermore, antimicrobial peptides display a broad spectrum of activity against microbes. Antimicrobial peptides and polypeptides are present in both prokaryotes and eukaryotes as key elements of the innate immune system in animals and plants [reviewed in (2-4)]. The rapid killing and mode of action of these peptides are believed to restrict selection of resistant microbes. These properties, in combination with their low toxicity on mammalian cells, make antimicrobial peptides suitable for drug development.

Microbes that have succeeded in breaching the mechanical and chemical barrier are immediately attacked by complement proteins and phagocytosing macrophages. The complement system involves a group of serine proteases and carbohydrate-binding proteins that can be activated through interactions with microorganisms and is a major part of the innate immune system (1). Binding of the complement proteins C1q or C3b+B to microorganisms activates associated serine proteases and triggers the classical or alternative pathway for complement activation, respectively (Fig. 1). The activation of complement consists of a serine protease cascade that releases activated downstream serine proteases along with protein fragments that act as chemoattractants for inflammatory cells. Some of these protein fragments also bind to microorganisms and act as opsonins for phagocytosing cells. Other protein fragments ultimately produce a membrane attack complex with potential to kill some microbes by damaging their cell envelope.

Activated macrophages trigger an inflammatory response by secreting cytokines and chemokines that induce acute phase proteins and attract other immune cells. Cells of the innate immune system (for example neutrophils, macrophages and dendritic cells) express invariant receptors encoded in the germline. Thus, all receptors of the same type have the same specificity and affinity for antigens. It has been suggested that these receptors recognize pathogen-associated molecular patterns (PAMPs) instead of specific epitopes as do the Igs and TCRs and thus are referred to as pattern recognition receptors (PRRs) (5). One of the classical PRRs is the mannann-binding lectin (MBL) that is present at low concentrations in plasma and is induced by the liver during the acute-phase response (6). This receptor binds to sugar residues on bacteria and
initiates the MBL pathway of complement activation together with two MBL-associated serine proteases (MASP-1 and -2) (Fig. 1). Only bacteria that contain the sugars mannose and fucose in the correct order and spacing in their cell wall will bind to and activate this complex. The cell-bound C-type lectin, macrophage mannose receptor, binds to microorganisms in a similar fashion as the MBL and is an example of a cell-surface PRR that functions directly as a phagocytic receptor (7).
**Drosophila melanogaster**

*Drosophila melanogaster*, also known as the fruit fly, belongs to the family *Drosophilidae* within the insect group *Dipteria*. It is a small, easy to breed, fly that undergoes complete metamorphosis from egg to adult fly within 14 days. *Drosophila* has been used in research since the beginning of the twentieth century and has been a valuable tool in the study of genetics and developmental biology. Genes have been mapped to all three autosomes and the two sex chromosomes with the help of visual marker mutations or hybridization to the polysome chromosomes of the larvae salivary glands. It has been estimated that the 180 Mb *Drosophila* genome (of which 120 Mb is euchromatin) contains approximately 13,600 genes (8). About 700 of the genes have been classified as transcription factors and 8,884 of the genes encode proteins with unknown or unclassified function. It has been determined that 47 of the annotated genes encode defense or immune proteins whereas 149 genes are believed to encode proteins involved in the immune response. Several genomic or proteomic expression analysis experiments have revealed hundreds of genes, depending on the stringency of the analyses, that are up or down regulated in *Drosophila* in response to immune-challenge (9-12). These genes, many of which are uncharacterized, are thought to be involved in the immune response. A subfraction of these immune genes can be divided into three groups; immediate early, early, and repressed genes relative to their induction kinetics.

Insects are complex creatures with properties that might astonish even the wise. Researchers have demonstrated that *Drosophila* need their sleep-wake cycle (13), their physiology and behavior are affected by their circadian rhythm (14), they have a memory for odors and physical punishment (15), and males impress females by “singing” and tapping their feet (16). In this context, it is maybe not surprising that a fundamental biological process like an immune defense system is present in flies.

**Fruit flies like a banana…**

Why would an insect like *Drosophila* need an immune system? Spoiled fruit is a heaven for microorganisms as well as flies. *Drosophila* feed on microorganisms within decaying fruit. One can imagine that being an insect larvae or adult in a crowded environment puts you in a likely position to get an infection. Although the life span of *Drosophila* is short and the number of offspring is large it is nothing compared to some harmful microorganism’s propagation rate. *Drosophila* is equipped with an extracellular layer of chitin (the cuticle), which functions both as a structural support and as a protecting barrier (Fig. 2). However, a wound causing a crack in the chitin or the openings of the gut and trachea could be perfect entry points for microorganisms.
Defense – the fruit fly style

*Drosophila* has a rapid and efficient immune system that is reminiscent of the vertebrate innate immune system [reviewed in (18-22)]. Various tissue (including epithelia and fat body) and blood cells (hemocytes) respond swiftly by secreting a battery of effector proteins and peptides into the hemolymph, intestine, and gonads following infection by bacteria or fungi (Fig. 2). Some of these secreted proteins are members of the complement-like proteolytic cascade that leads to the activation of phenoloxidase (Fig. 1). Phenoloxidase catalyzes a reaction of phenol and oxygen that generates reactive quinones. The quinones react with neighboring molecules and cause the hemolymph to clot (melanization) around intruders and wounds, and are also involved in the encapsulation of parasites (23). Some of the proteins are pattern recognition receptors (PRRs) that bind to pathogen-associated molecular patterns (PAMPs) from microorganisms. The PRRs are either enzymatic proteins or signaling molecules that trigger an immune response. Some of the PRRs might also act as opsonins. Some of the other secreted proteins and peptides have antimicrobial properties that either kill or inhibit the growth of microorganisms. All of these effector proteins and peptides, in addition to the reactive oxygen species are part of the humoral immune response in the fly.

*Drosophila* has a variety of specialized blood cells, the hemocytes, which, in response to an infection, collectively phagocytose and encapsulate foreign objects and secrete antimicrobial factors (Fig. 2). These cells comprise the cellular immune response. It is questionable as to whether the humoral and cellular reactions should be considered as separate systems but rather interacting components of the overall immune system. For example, *Drosophila* larvae fail to induce an antimicrobial response in the fat body after bacterial infection in the absence of hemocytes or injury, suggesting that various cells contribute to the stimulation of the fat body response (24).

*The term fruit fly is a very broad definition and is maybe not the best name for *Drosophila melanogaster*, a fly that lives on microorganisms in spoiled fruit. The true fruit fly might instead be flies belonging to the family *Tephritidae*, which live on fresh fruit and are a feared pest in fruit producing regions of the Mediterranean and Orient. The term fruit fly as a description for *Drosophila* occurred as early as 1923 and has been used exclusively in textbooks since 1945. Previous common names for *Drosophila melanogaster* are pommac or vinegar fly (17).*
Figure 2. **Immune responses of Drosophila.** The cellular part of the immune response consists of blood cells (hemocytes) such as phagocytosing plasmatocytes, large flattened lamellocytes that are involved in encapsulation of foreign objects and parasites, and crystal cells that contribute to the phenoloxidase cascade. The epithelial cells and the fat body cells respond to infection by secreting humoral factors. The humoral part of the immune response contains antimicrobial peptides (AMP), peptidoglycan recognition receptors (PGRP), gram-negative bacteria binding proteins (GNBP), reactive oxygen species such as nitric oxide, likely cytokines, thiolester-containing peptides (TEP), potential stress response Turandot peptides (Tot), and proteins that are involved in wound healing, clotting, and encapsulation reactions such as the phenoloxidase cascade proteins. Reviewed in [(20-22)].
A dynamic immune system

The *Drosophila* immune reactions are dynamic and vary depending on several factors such as the location of the infection, the developmental stage of the animal in which the infection occurs, the amount of time in which the infection has proceeded, the type of immune challenge that is encountered, and even the timing in respect to the circadian rhythm. For example, the local immune response that occurs in epithelial cells is distinct from the systemic immune response that occurs in the fat body cells in that it involves activation of different pathways for inducing antimicrobial peptides (25-27). In addition, transcription modulators that are used for the fat body-mediated systemic immune response in larva are not used in the adult fat body response. This difference reflects the different origin of these two individually developed tissues (28). Tissue-specific modulators interact with specific clustered motifs in the promoter regions of immune genes (29). Interestingly, switching one tissue-specific modulator-binding site for another can change the tissue-specific expression.

In addition to tissue specificity, the time following infection is an important aspect to consider. For example, it is possible that activation of hemocytes trigger the expression of a cytokine that signals the fat body to secrete peptides such as the Turandot (Tot) peptides (30-32). Such a situation reflects a secondary response, but could easily be thought of as a primary response. Furthermore, as we described in publication III, the *Drosophila* immune system generates a selective response by inducing different pathways depending on the type of infecting microorganism (11, 33-36). In addition, some immune genes fluctuate during the circadian rhythm of the fly and might explain why some immune mutants of certain alleles are more susceptible to infection at night than in the morning (37, 38). Although these dynamic variations complicate the research, it makes the *Drosophila* immunity field all the more interesting. However, my thesis is focused on the section of the *Drosophila* immune system that involves the systemic immune response in adult flies.

Pattern recognition receptors

The *Drosophila* Toll receptor was discovered in a screen for mutations affecting *Drosophila* development (39). Since then, toll-like receptors (TLRs) have been found in a wide variety of organisms including humans, nematodes and plants (40-42). A decade after the discovery of *Drosophila* Toll, it became apparent that Toll and TLRs are important components of the immune response (33, 40, 43). Several mammalian TLRs and/or their co-receptors might bind directly to various microbial PAMPs such as peptidoglycan (PG), lipopolysaccharide (LPS), dsRNA and CpGDNA and thus represent mammalian examples of pattern recognition receptors [reviewed in (44)]. These interactions induce the adaptive immune system by triggering the production of pro-inflammatory cytokines, chemokines, and co-stimulatory molecules. The *Drosophila* Toll receptor is involved in the induction of
antimicrobial peptides, especially the antifungal peptide Drosomycin. However, none of the nine TLRs in Drosophila have shown to bind directly to a PAMP (19). Instead, the family of 13 peptidoglycan recognition proteins (PGRPs) (45, 46), the Gram-negative bacteria binding proteins (GNBPs) (47, 48), and Scavenger receptor CI (dSR-CI) (49) are thought to be part of the Drosophila PRR collection.

**Antimicrobial peptides and polypeptides**

In June of 1918, R. W. Glaser published his observations that “extracellular antagonistic substances” in blood from previously immunized grasshoppers had potency to kill bacteria (50). The first antimicrobial peptide isolated and fully characterized from an insect was Cecropin from the moth Hyalophora cecropia (51). Since then, more than 170 antimicrobial peptides and polypeptides have been identified in insects (3). The importance of these peptides to fight a microbial infection was recently demonstrated by the ability to partially restore resistance to infection by overexpression of a single antimicrobial peptide in fly mutants that lacked the ability to induce antimicrobial peptide genes (52).

Most of the peptides or polypeptides can be grouped into three different classes based on structural differences; 1) peptides with intra-molecular disulfide bonds forming hairpin-like $\beta$-sheets or mixed $\alpha$-helical/$\beta$-sheet structures, 2) peptides forming amphipathic $\alpha$-helices, or 3) peptides with a high number of proline and/or glycine residues (3, 4). The rest of this thesis will focus on the regulation of different antimicrobial peptides by the Drosophila immune system. Therefore, I have grouped the Drosophila antimicrobial peptides into three groups according to their activity rather than their structure; 1) antibacterial, 2) antifungal and 3) both antibacterial and antifungal. Since only a few of the Drosophila antimicrobial peptides have been isolated and biochemically characterized, I have extrapolated the antimicrobial properties of the peptides isolated from other insects to the Drosophila peptides. It should be noted that this extrapolation might not reflect the true properties of the Drosophila peptides.

**Peptides with antibacterial activity**

Antimicrobial peptides that exhibit only antibacterial activity include Diptericin, Attacin, Drosocin, and Andropin (Fig. 3). I have also included the peptidoglycan recognition protein, PGRP-SC1BI, in this group. PGRP-SC1B has enzymatic activity against PG from G$^+$ bacteria such as Staphylococcus aureus, Bacillus megaterium, and Micrococcus luteus (53). Although PGRP-SC1B digests purified PG, it does not show antibacterial activity on live bacteria. However, it is unknown if it possesses antimicrobial activity against bacteria in synergy with other immune proteins.

Diptericin is a glycine-rich peptide with one glycine-rich domain at the C-terminus, but also has a short proline-rich (P) region in the N-terminus. This O-glycosylated peptide (54) was first isolated from bacteria-challenged third instar Phormia terranovae larvae (55). Diptericins isolated from P. terranovae and Sarcophaga peregrina exhibit activity against the Gram-negative (G) bacteria
Escherichia coli and Shigella sonnei, but not against the G- bacteria Enterobacter cloacae, Pseudomonas aeruginosa, Proteus vulgaris, and Proteus rettgeri, or the Gram-positive (G+) bacteria M. luteus, Staphylococcus aureus, Bacillus subtilis, Bacillus thuringiensis, and Corynebacterium bovis (55, 56). When growing cultures of E. coli were treated with purified Diptericin from S. peregrina, long chains of cells were observed in transmission electron microscopy (55, 56). Two Diptericin genes (DptA and DptB) have been described in Drosophila (II) (57, 58) but neither of their protein products has been biochemically characterized.

Attacin was first isolated from immune-challenged H. cecropia (59) and is the largest of the antimicrobial peptides. The peptide contains two C-terminal glycine-rich (G) domains that have to be released from the N-terminal prodomains by proteolytic processing of a furin-like RVRR cleavage site in order to achieve full antimicrobial activity (60). Attacins are effective against the G- bacteria E. coli, Acinetobacter calcoaceticus, and Pseudomonas maltophilia, but not against E. cloacae or the G+ bacterium B. megaterium (59). Attacin interferes with bacterial cell division, which ultimately leads to death of the bacteria. Four Attacin genes (AttA, AttB, AttC, and AttD) have been described in Drosophila (II) (61-63). Parts of the Attacin C peptide have been isolated from Drosophila by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (64, 65). A truncated form of the P domain of Attacin C [mature Pro-domain of Attacin C (MPAC)] contains two post-translational modifications (65). The concentration of MPAC in adult Drosophila was estimated to 5 μM 15 hours after injection with E. cloacae, which was approximately half of the maximum concentration that was reached after 24 hours. Synthetic MPACs (with or without glycosylation) had limited effect on growing E. coli. However, synthetic MPAC had a synergistic effect with Cecropin A on the G- bacteria Agrobacterium tumefaciens, E. cloacae, E. coli, Erwinia carotovora carotovora, P. aeruginosa, Salmonella typhimurium, the G+ bacteria B. megaterium and M. luteus, and the fungus Neurospora crassa, but not against the G- bacterium Serratia marcescens, the G+ bacteria S. aureus and Streptococcus pyogenes, or the yeast strain Candida albicans (65).

Drosocin is a short (19 amino acids), proline-rich peptide that contains an O-glycosidic substitution on threonine-11. The substitution is a disaccharide consisting of N-acetylgalactosamine and galactose (66). Synthetic Drosophila Drosocin was active against the G- bacteria E. coli, S. typhimurium, Klebsiella pneumoniae, E. cloacae, Xanthomonas campestris, A. tumefaciens, and the G+ bacterium M. luteus but not against the G- bacteria P. aeruginosa, Pseudomonas cepatia, E. carotovora, S. marcescens, P. vulgaris, Alcaligenes faecalis, or the G+ bacteria Listeria monocytogenes, S. aureus, S. pyogenes, and B. megaterium (67). Killing of G+ bacteria by Drosocin takes several hours and all D-enantiomers are inactive suggesting that its activity is stereospecific. One Drosocin gene (Dro) has been described in Drosophila (68).

Andropin is a male-specific antibacterial peptide that is active against the G+ bacteria B. megaterium, B. subtilis, and M. luteus and against the G- bacterium
E. coli in ionic-buffered conditions. Its expression is restricted to the ejaculatory duct and is induced after mating. One Andropin gene (Anp) has been described in Drosophila (69).

**Peptides with antifungal activity**

Drosomycin was the first antifungal peptide isolated from insects (70) and is the only member of this group. This 44 amino acid-peptide contains eight cysteines comprising four intra-chain disulfide bonds and is resistant to heat treatment, proteases, and pH alterations *in vitro*. It was estimated that Drosomycin can reach concentrations as high as 100 μM in the hemolymph yet is effective against the fungi N. crassa, Botrytis cinerea, Fusarium culmorum, Alternaria brassicola, Alternaria longipes, Nectria haematococca, Fusarium oxysporum, and Ascochyta pisi but not against Trichoderma viride or Trichoderma hamatum at concentrations of 5 μM or less. The bacteria E. coli, E. cloacae, P. cepacia, Streptococcus agalate, Streptococcus sanguis, S. aureus, M. luteus, and Bacillus megaterium were insensitive to treatment with Drosomycin. Lower concentrations of Drosomycin delay growth of hyphae. Higher concentrations of the peptide inhibit spore germination (70). Seven Drosomycin genes (Drs, Drs-1, dro2, dro3, dro4, dro5, and dro6) are present in the Drosophila genome, but only the expression of Drs and dro-5 have been detected (9, 70).

**Peptides with antibacterial and antifungal activity**

The antimicrobial peptides Cecropin, Metchnikowin, and Defensin belong to this group. I have also included Lysozyme in this group.

Cecropin from H. cecropia was the first antimicrobial peptide to be isolated and biochemically characterized (51). The Cecrins are processed from larger precursors and form amphipathic α-helices. They are active against bacteria, fungi, and have some effect on Plamodium and Leishmania parasites (71-73). Cecropin A was detected at concentrations of 25-50 μM in Drosophila hemolymph. Both natural and synthetic Drosophila Cecropin A has activity against the G- bacteria E. coli, E. cloacae A. tumefaciens, E. carotovora, P. aeruginosa, and S. typhimurium, the G+ bacteria B. megaterium, M. luteus, and B. subtilis, the fungi Metarhizium anisopliae and N. crassa, and the yeasts Saccharomyces cerevisiae, Geotrichum candidum, and Dipodascopsis uninucleata. It is not active against the G- bacterium S. marcescens, the G+ bacteria S. aureus and S. pyogenes or the fungus Beauveria bassiana (65, 74, 75). Four Cecropin genes and two pseudo genes (CecA1, CecA2, CecB, CecC, Cec-Ψ1, and Cec-Ψ2) have been described in Drosophila (76, 77).

Metchnikowin is a strongly cationic (pI of 11.6), proline-rich (>25%), three-kDa peptide that was isolated from immune-challenged adult Drosophila (78). Two isoforms, differing only in amino acid residue number three, have been identified. Like Attacin, Metchnikowin is processed into an active form but displays no antimicrobial effect on the G- bacterium, E. coli. Metchnikowin inhibits the growth of the G+ bacterium M. luteus and the filamentous fungus N.
Figure 3. The antimicrobial peptides and proteins of *Drosophila.* A) The antimicrobial peptides Diptericin, Attacin, and Drosocin are active against G⁻ bacteria, Andropin is active against G⁺ bacteria, Drosomycin is active against fungi, Metchnikowin is active against fungi and G⁺ bacteria, and Cecropin and Defensin are active against fungi and G⁻ and G⁺ bacteria. Lysozyme and some PGRPs are lytic enzymes that digest PG from G⁺ bacteria. Although PGRP-SC1B can digest PG it is not known if this has an inhibitory effect on the bacteria. See the text for references. B) An example of three promoter elements in the *CecA1* and *CecA2* genes [adopted from (88)]. The κb-like site binds Rel factors and is essential for induction. The GATA site binds the dGATAb factor Serpent and is necessary for tissue specificity and full induction (28). The region 1 (R1) site is necessary for full induction and binds an unknown factor that is not any of the Rel factors (91).

*crassa* by an unknown mechanism (78). Although two isoforms of Metchnikowin have been isolated, only one gene (*Mtk*) has been described in *Drosophila.*

The first defensins from insects were isolated from a cell line derived from the flesh fly *S. peregrina* and black blow fly larvae of *P. terranovae* challenged with bacteria (79, 80). They are widely distributed in insects and also found in mollusks. However, they should not be confused with mammalian defensins (3). Insect defensins are cysteine-rich, antimicrobial peptides that are believed to form channels in the cytoplasmic membrane of bacteria such as *M. luteus* (71). As little as a one-minute exposure to the peptide is sufficient to kill susceptible bacteria by lysis at concentrations as low as 0.1-10 μM under low salt conditions. Insect
defensins are active against a wide range of G+ bacteria, and some G- bacteria, fungi, yeast and Plasmodium oocysts. Drosophila Defensin has been isolated and successfully tested for activity against G+ M. luteus (81). One Defensin gene (Def) has been described. It is believed that, like other Defensins, Drosophila Defensin is synthesized as a preprodefensin. In addition to the signal peptide, Defensin is thought to be endoproteolytically processed after the RQKR amino acid motif resulting in a 30 residue prosegment and the mature peptide (81).

Discovered in 1922, lysozyme is found in a wide range of species such as bacteriophages, insects and humans (71). In contrast to the inducible antimicrobial peptides, lysozyme is constitutively expressed in the digestive tract and acts as a bactericidal enzyme that hydrolyzes PG from G+ bacteria. Lysozyme from Drosophila has activity against the G+ bacterium M. luteus (82). Furthermore, lysozyme D displays chitinase activity (83). Chitin is a component of insect cuticles, Octosporea spores, fungal cell walls, and some algae. Interestingly, it was recently shown that several lysozyme genes are upregulated in response to infection with Octosporea muscaedomesticae parasites (84). Eight lysozyme genes (LysA, LysB, LysC, LysD, LysE, LysP, LysS, and LysX) have been described in Drosophila (82, 85). In addition, a lysozyme-like gene (CG16756) that is induced by a parasite infection has been identified in the Drosophila genome (84).

**Regulation of antimicrobial peptide induction**

In response to the injection of microbes and microbial components, the transcription of antimicrobial peptide genes is rapidly increased in Drosophila and other insects. In 1991, Sun et al. observed a conserved sequence in the promoter region of the Attacin genes from the moth, H. cecropia, that was similar to the binding site for the mammalian transcription factor, nuclear factor kappa-B (NF-κB) (86). In addition, they found similar κB-like sequences upstream of all immune genes that had been isolated from H. cecropia and Drosophila. The authors noticed that the induction of immune genes from H. cecropia shared some characteristics to the induction of NF-κB-regulated immune, inflammatory, and acute phase genes in mammals and correctly predicted that immune genes in insects could be regulated by a NF-κB-like transcription factor binding to this site. One year later, they isolated the Rel factor, Cecropia immunoresponsive factor (CIF) that had affinity for the conserved NF-κB-like sequence (87).

In Drosophila, promoter studies of the antimicrobial peptide genes CecA1 and DptA in mbn-2 cells and larvae revealed a protein-DNA complex as early as 15 min after immune stimulation (88, 89). Analogous to the Rel factor from H. cecropia, this κB-motif-binding activity was named Drosophila immunoresponsive Factor (DIF). The observations that the binding of H. cecropia CIF to the κB-like sequence was inhibited by antibodies directed against mammalian Rel proteins and that pure, recombinant mammalian protein p50 (part of NF-κB) could bind to the Drosophila DptA κb-related binding motif in vitro suggested that immune activation was evolutionarily conserved between insects and mammals. It has
since been demonstrated that many antimicrobial peptide genes contain at least one of two evolutionarily conserved motives, the GATA and Region 1 (R1) sites, in addition to the κB-like site (29, 90). These sites are clustered together within the κB-like site of the promoter region (Fig. 3). However, except for the κB-like site, none of the tested additional sites are bound by any of the Drosophila Rel proteins (90, 91). While the κB-motif is essential for induction of CecA1, both the GATA and the R1 sites are necessary for full induction of the CecA1 gene. The GATA site has been found in the promoter region upstream of a large number of antimicrobial peptide genes (29, 90) and is necessary for full induction of the CecA1 gene in the larval fat body after immune-challenge (28). The dGATAb factor Serpent binds to this GATA site. The R1 site has been found in the promoter region upstream of the antimicrobial peptide genes encoding Cecropin, Defensin, Metchnikowin, and Drosomycin, but not upstream of DptA or Dro (91). However, the transcription factor that binds to the R1 site is still unknown.

In 1996, Lemaitre et al. (33), showed that part of the pathway that regulates the formation of the dorso-ventral axis in the Drosophila embryo through the NF-κB-like transcription factor Dorsal, was involved in the induction of some of the antimicrobial peptide genes. They proposed that two different pathways control the regulation of antimicrobial peptides. The Toll pathway, activated by fungi, would regulate the induction of the antifungal peptide gene Drs. The Imd pathway, activated by bacteria, would regulate the induction of the antibacterial peptide gene DptA. However, the transcription factor Dorsal did not seem to be essential for either pathway in the adult fly (33).

**NF-κB-like transcription factors**

In mammals, various combinations of proteins bearing homology to v-Rel [p105 (p50), p100 (p52), RelA (p65), RelB, and c-Rel] form homo- or heterodimers that are collectively referred to as NF-κB [reviewed in (94, 95)]. NF-κB controls the expression of acute phase proteins in the liver, participates in hematopoeisis and in the inducible expression of cytokines and cell surface receptors in lymphoid cells. The N-terminal part of the protein contains the Rel homology domain (RHD) that is involved in dimerization, DNA-binding, and interaction with the inhibitor, inhibitor kappa-B (IκB). NF-κB is present in the cytoplasm of unchallenged mammalian cells as an inactive complex with IκB (Fig. 4). Dissociation of IκB from NF-κB is required for nuclear translocation of NF-κB and the transcription of target genes.

b*The mbn-2 cell line is composed of differentially heterogeneous malignant phagocytotic blood cells, including plasmatocytes, derived from third instar larvae of the recessive-lethal Drosophila mutant lethal (2) malignant blood neoplasm (92). These cells can induce an immune response of antimicrobial peptides after stimulation with microorganisms or microbial components (93).
NF-κB-like transcription factors in Drosophila

In Drosophila, there are three known NF-κB-like transcription factors; Dorsal, Dorsal-related immunity factor (Dif), and Relish (Fig. 4) (96-98). Like their mammalian counterparts, all three Drosophila Rel factors are present in the cytoplasm of naive cells but translocate to the nucleus following immune challenge (97, 99, 100).

Dorsal was identified as a maternally expressed gene that, when disrupted by mutagenesis, caused dorsalized embryos (39). In addition to its maternal expression, Dorsal is expressed in the fat body of larvae and adult flies (99, 101-103). Dorsal is retained in the cytoplasm by the IκB-like protein, Cactus, and is activated and translocated to the nucleus on the ventral side of the embryo during the dorso-ventral axis formation or in fat body cells after immune-challenge through activation of Spätzle and Toll (104). In addition to embryonic defects, overactivation of the Toll/Dorsal pathway, including overexpression of Dorsal itself, induced lamellocyte differentiation and caused the formation of melanotic capsules (105) suggesting a role for Dorsal in hemocyte differentiation and activation.

Dorsal-related immunity factor (Dif) was cloned by a low stringency hybridization screen using conserved regions from the Dorsal gene that encoded the Rel domain (97). In contrast to Dorsal, Dif peaks during larval, pupal and adult stages. However, it was shown that Dif could partially rescue the dorsalized phenotype seen in Dorsal mutants (106) suggesting that some redundancy between the two Rel proteins exists. Dif is expressed in the fat body and translocates to the nucleus after immune stimulation (97, 102, 103). In mutants expressing a constitutively active form of Toll (Toll^{108}), Dif is localized primarily in the nucleus. In addition, Drs induction was repressed in Dif mutants (102, 107, 108). Transactivation assays in mbn-2 cells revealed that Dif preferably activates promoters containing κB-like sequence from the CecA1 promoter when compared to the κB-like sequence from the DptA promoter (109).

Relish was found in a PCR-based differential display screen for genes that were induced in flies in response to bacteria challenge (98). In contrast to Dorsal or Dif, Relish is a Rel protein that carries its own inhibitor in the C-terminal part of the protein, like the mammalian NF-κB precursor proteins p105 and p100 that give rise to the p50 and p52 proteins (95). Signal-dependent processing of Relish separates the Rel homology (RHD) domain from the ankyrin repeat (ANK) domain (100). This processing facilitates the translocation of the RHD-containing N-terminal part of Relish to the nucleus where it stimulates the transcription of target genes (IV).

When I started as a graduate student, it was suspected that at least one of these Rel factors was responsible for the induction of antimicrobial peptide genes after immune challenge in Drosophila. It was unclear as to which one was involved. When overexpressed individually in mbn-2 cells, it was observed that all three Drosophila Rel factors could activate transcription of CecA1 and/or DptA
However, Dif alone demonstrated the strongest activation of the CecA1 promoter when compared to co-expression with Dorsal or Relish (109). Of the three Drosophila Rel factors, only Dorsal mutant flies were available at the time. In 1996, using these mutant flies, Lemaitre et al. demonstrated that Dorsal was not essential for antimicrobial peptide induction after bacterial challenge (33). Consequently, it became of interest to investigate the role of the other two Rel factors, Dif and Relish. However, no mutants were yet available.

Figure 4. NF-κB-like factors of mammals and Drosophila. A) Simplified model of NF-κB activation [reviewed in (94, 95)]. Before activation, Rel factors are held in the cytoplasm in a complex with the inhibitor molecule IκB. Upon activation, IκB is phosphorylated, dissociates from the NF-κB dimer and gets targeted for degradation by the proteasome. The released NF-κB dimer translocates to the nucleus and activates transcription of target genes. B) Rel proteins have a Rel homology domain (RHD) in the N-terminal part of the protein that is involved in dimerization, DNA binding, and interaction with IκB. The Drosophila Rel proteins, Dorsal and Dif, has similarities to the mammalian proteins RelA (p65), RelB, and c-Rel. Relish has similarities to mammalian p100 (p52) and p105 (p50). The C-terminal part of Relish and the similar mammalian proteins p100 (p52) and p105 (p50) has ankyrin repeats like the inhibitory molecule IκB. The figure is adopted from (98).
AIMS OF THIS THESIS

To generate *Relish* deficient mutants and investigate the role of Relish as a transcription factor in the immune system of *Drosophila melanogaster*. 
RESULTS AND DISCUSSION

Relish, a central factor in the control of humoral but not cellular immunity in Drosophila (Publication I)

The Relish gene is complex and located in the intron of the Nmdmc gene. Four different promoters regulate the expression of four transcripts that vary in length at the 5’ end (2.7, 3.1, 3.4, and 3.5 kb) (I) (98). The 2.7 kb transcript was observed only in early embryos and in unchallenged female adults. This observation indicated that a maternal transcript was expressed from the nurse cells in the ovary and then deposited into the embryo (98). The putative Relish protein encoded by this 2.7 kb transcript differs from the other Relish proteins in that it is truncated just N-terminal of the first Rel-homology domain. The 3.5 kb transcript presumably has a weak promoter since it is rarely detected by Northern blot analysis. The 3.4 kb transcript was observed at all stages of development and is thought to contribute to the basal expression of Relish. This transcript was induced about 15-fold after injection of adult flies with bacteria when compared to unchallenged flies (98). The 3.1 kb transcript was undetected in unchallenged adults but increased greater than 50-fold after injection with bacteria when compared to the basal level without injection (98). Previously, it had been shown that the induction of the 3.1 and 3.4 kb transcripts was much greater than the approximate three-fold induction of the Rel proteins Dorsal and Dif (98). This observation strongly suggested an involvement of Relish in the defense against bacterial infection.

In order to understand Relish’s role in the Drosophila immune system, we generated deletion mutants in the Relish gene using imprecise excision of a P-element. We screened for deletion mutants that had lost the P-element by using genetic markers, Southern blot hybridization and PCR. We found five fly strains ([Rel]E14, [Rel]E20, [Rel]E21, [Rel]E26, [Rel]E38) that contained deletions in the Relish gene and one strain ([Rel]E23) that contained a perfect excision that reverted the Relish gene back to wild-type. Of these Relish alleles, only [Rel]E20 and [Rel]E38 had a deletion that spanned all four transcription start sites. These two Relish mutant strains had an obvious immune phenotype. When injected with the G- bacterium, E. cloacae, these Relish mutant flies succumbed within 17 and 27 hours, respectively. Canton-S and [Rel]E23 control flies survived the treatment. We observed prolonged survival by injecting lower doses of bacteria. Injections with estimated doses of fewer E. cloacae bacteria per fly killed the corresponding fractions of the Relish mutants, indicating that single E. cloacae bacteria were sufficient to kill these flies. Transgenic expression of Relish in flies with [Rel]E20 background rescued the resistance to E. cloacae and highlighted the importance of Relish in the defense against E. cloacae. Additionally, the [Rel]E20 and [Rel]E38 flies were also more sensitive to injection with the fungi G. candidum, D. uninucleata or M. anisopliae when compared to control flies. However, the killing took days rather than hours as was the case for injection with the bacterium E. cloacae. This observation suggested that Relish was also involved in the antifungal response.
The reduced survival to bacterial and fungal injections was likely due to a lower induction level of antimicrobial peptides. Dushay et al. had recently shown that LPS-stimulation of mbn-2 cells previously transfected with a CecA1-lacZ reporter construct along with either full-length Relish or Rel-only expression constructs, generated a three- and ten-fold increased expression from the reporter, respectively, as compared to the empty vector control. These observations suggested that Relish was involved in CecA1 induction (98). By studying the expression of antimicrobial peptide mRNA by Northern blot, we found that RelE20 and RelE38 flies failed to induce DptA and CecA after injection with E. cloacae. The induction of the other antimicrobial peptides was also reduced substantially compared to the controls. Only 10% of the induction of AttA and Mtk, and 20% of the induction of Drs remained in the RelE20 flies. Approximately twice as high levels were observed in RelE38 flies when compared to RelE20 flies. The difference between the RelE20 and RelE38 flies was likely due to some residual Relish in the RelE38 flies but not in the RelE20 flies (100). The inducibility of CecA1 and DptA was rescued by overexpression of full-length Relish in transgenic RelE20 flies. These results indicated that Relish was a central factor in the control of the activation of these antimicrobial peptide genes after infection with E. cloacae.

Although there was deficient antimicrobial peptide gene induction, we could still observe melanization around the injection wound in these Relish mutants. The hemocyte population in Relish mutant third instar larvae and adults displayed quantities, morphologies, and properties that were similar to the hemocyte population in wild-type Relish+ larvae and adults. Phagocytosis of the G- bacteria E. cloacae, Escherichia coli and the G+ bacteria Staphylococcus saprophyticus by plasmatocytes from Relish mutants were normal. Encapsulation of the parasitoid wasp Leptopilina boulardi in Relish mutant larvae were comparable to that observed in wild-type controls. These results indicated that the cellular part of the Drosophila immune system was functional in the Relish mutants and that the function of Relish was to induce antimicrobial peptides in the humoral part of the immune system.

Different pathways for induction of antimicrobial peptides

Using Drosophila mutants, two distinct pathways for the induction of antimicrobial peptides had been characterized (33). The Toll and Imd pathways were named after a key protein in each pathway. Toll is a receptor involved in the initiation of one pathway, the Toll pathway. A loss-of-function mutation in the Imd protein is responsible for the immune deficiency phenotype found in imd (immune deficiency) flies and defines the Imd pathway (20). The two pathways selectively regulate different antimicrobial peptides. For example, activation of the Toll pathway is sufficient for induction of the antifungal peptide Drosomycin as was observed by a constitutively increased level of Drs mRNA in the absence of bacteria challenge in flies with a gain-of-function form of Toll (Toll10B) (33). imd-deficient flies failed to induce DptA after injection with bacteria (110). The imd mutant phenotype was
similar to the phenotype that we observed in the Relish loss-of-function mutants RenI and RenII (I). Furthermore, overexpression of Relish in adult flies induced DptA without injection of bacteria (I). These results suggested that Relish was part of an Imd/Relish pathway leading to induction of DptA. Recently, it was shown that expression of the antibacterial peptide gene DptA was constitutively elevated in flies in the absence of bacteria challenge after overexpression of Imd, dTAK, or Dredd (111, 112). These reports, together with results discussed in publications I-IV strengthens the argument that the Imd/Relish pathway is responsible for the induction of DptA.

It has not been as straightforward to describe the induction of antimicrobial peptides other than Drs and DptA. For example, experiments have shown that CecA and AttA are partially induced in flies that contain mutations in the genes for proteins involved in either the Toll or Imd/Relish pathways (I) (33). In addition, larvae that contained a mutation in the gene for the TLR protein 18 wheeler (18w-35) failed to induce AttA to the levels of induction that were observed in control larvae following bacterial challenge (113). This observation suggested that 18 wheeler was involved in AttA induction. One complication has been that different experiments in the Drosophila immunity field used different methods and microbes to challenge their flies. To eliminate these variables and possibly gain more understanding of which pathways regulate the induction of which peptides, we decided to examine the expression of some antimicrobial peptide genes, including our novel antimicrobial candidates DptB, AttC and AttD, in various known immunity mutants using one method and one type of bacterium for the challenge.

Expression and evolution of the Drosophila Attacin/Diptericin gene family
(Publication II)
The sequencing of the Drosophila genome revealed additional copies of putative antimicrobial peptide genes. By searching databases with the AttA gene, we came across a putative Diptericin gene (DptB) which showed 43% overall identity with DptA. The more conserved C-terminal glycin-rich (G) domain showed 52% and 37% identity with the G domains of DptA and AttA, respectively. Interestingly, unlike DptA, the proline-rich (P) and G domains encoded by the DptB gene were separated by a RVRR furin-like cleavage site that was identical to the cleavage site found in Attacins, including Attacin A. This observation suggested that proteolytic processing of Diptericin B could release the P and G domains from each other as has been reported for Attacins and might display a putative evolutionary link between DptB and the Attacin genes. Furthermore, like AttA/B, DptB had an intron upstream of the G domain, whereas DptA did not. However, this intron was not in the same context as the intron in AttA/B.

Two putative Attacin genes (AttC and AttD) were found by searching databases with the AttA gene. AttC and AttD were 75% and 33% identical to AttA, respectively. Both genes showed the same structure as the previously
described \textit{AttA/B} including the intron separating the N-terminal prodomain from the two C-terminal G domains. However, the \textit{AttD} gene was truncated in the 5′ end revealing an open reading frame that would encode an N-terminally truncated peptide that would lack the amino acids down past the corresponding RVRR cleavage site in the Attacins A and B. The peptide encoded from \textit{AttC} also contained a putative cleavage site, however the motif was RARR instead of RVRR as in Attacins A and B. A truncated form of the proline-rich prodomain from Attacin C (MPAC) that lacks the N-terminal leucine and proline dipeptide as well as the two C-terminal arginines was recently isolated from immune-challenged \textit{Drosophila}, suggesting that Attacin C is processed at the furin-like motif in a similar fashion as Drosocin (65). The MPAC peptide has two post-translational modifications, one pyroglutamic acid residue that blocks the N-terminus of the peptide and one O-glycosylation on threonine 16.

\textit{DptB}, \textit{AttC}, and \textit{AttD} were induced after injection with the G- bacterium \textit{E. cloacae} as were their relatives \textit{DptA} and \textit{AttA}. This observation indicated that these genes might encode inducible antimicrobial peptides. Although the two \textit{Diptericin} and four \textit{Attacin} genes were related, they had different promoters and hence were regulated individually. In order to further understand the relationship between these peptides and the regulation of antimicrobial peptides by different pathways, we examined the expression of the \textit{DptB}, \textit{AttC}, and \textit{AttD} genes in various adult immune mutants and compared their expression to the expression of \textit{DptA}, \textit{AttA}, and other antimicrobial peptides 6 h after injection with the G- bacterium \textit{E. cloacae}. For simplicity and consistency, we used only one strain of bacteria, one method of infection, one time point, and one developmental stage. This experiment revealed that \textit{DptB} was regulated as \textit{DptA} after challenge with \textit{E. cloacae}. The inducibility of the \textit{DptA/B} genes was abolished in the \textit{Rel}$^{E20}$ and \textit{imd} mutants. Surprisingly, males that contained loss-of-function mutations of Toll (\textit{Tl}) did not induce \textit{DptA/B}. The \textit{DptA/B} induction in \textit{Tl} females and 18\textit{w}$^{7-35}$ adults was suppressed but not to the same extent as in the \textit{Tl} males. We could not explain the discrepancy between the \textit{Tl} males and females. However, McKean \textit{et al.} proposed that increased sexual activity could reduce male immune function in \textit{Drosophila} (114). Whether there was a connection between the antimicrobial peptide gene expression in the \textit{Tl} males in our experiment and their sexual activity remains a mystery.

Inductions similar to the ones described for the \textit{DptA/B} gene were also observed for the \textit{AttA/B} genes after immune challenge of the different immune mutants. However, \textit{AttC} was clearly more affected in the 18\textit{w}$^{7-35}$ mutants than \textit{AttA/B} (>90% reduction of induction for \textit{AttC} relative to wild-type flies compared to 65% reduction of induction for \textit{AttA/B} relative to wild-type flies). But it should be noted that the expression that was seen after Northern blot hybridization using the \textit{AttA} probe represented the combined expression of the \textit{AttA} and \textit{B} genes. Therefore, one cannot exclude the possibility that the mutation in the 18\textit{w}$^{7-35}$ mutants was as important for the separate induction of either \textit{AttA} or \textit{AttB}
as for the induction of \textit{AttC} after challenge with \textit{E. cloaca}. The requirement for a functional 18 wheeler protein in adult flies for the induction of antimicrobial peptide genes in general, and \textit{AttC} in particular, have recently been questioned (115). Ligoxygakis et al. used 18\textit{w}^7-35/\textit{Df(2R)017} heterozygote adult flies, whereas our studies were based on 18\textit{w}^7-35 homozygotes (II). It is possible that the \textit{AttC} deficient phenotype that was observed in adult 18\textit{w}^7-35 homozygote flies was due to a mutation that was not uncovered by \textit{Df(2R)017}.

Curiously, the 18\textit{w}^7-35 mutation appeared to have opposite effects on the expression of \textit{AttD} and the other \textit{Attacin} genes. The expression of \textit{AttD} increased in the 18\textit{w}^7-35 mutants when compared to wild-type flies. This difference might reflect a separate mode of induction for a putative intracellularly located Attacin D peptide compared to the other extracellularly located Attacins.

\textbf{Selectivity of pathways for specific PAMPs}

Although the model for activation of the immune pathways had evolved, a direct interaction of a \textit{Drosophila} protein (PRR) with a microbial molecule (PAMP) was not proven until recently (45, 47). Nor was it known which PAMPs activate which pathways. By injecting \textit{Relish} mutants, we found that although the induction of \textit{CecA} was abolished in these mutants when challenged with the G\textsuperscript{-} bacterium \textit{E. cloaca}, some induction was seen after challenge with the G\textsuperscript{+} bacterium \textit{M. luteus} (III). Similar results [for example (112, 116)] lead to the model that fungi and G\textsuperscript{+} bacteria activate the Toll pathway and that G\textsuperscript{-} bacteria activate the Imd/Relish pathway. The differential expression of \textit{CecA} in \textit{Relish} mutants gave us an opportunity to search for the PAMP from \textit{M. luteus} that was responsible for inducing \textit{CecA} in \textit{Relish} mutants through a Relish-independent pathway. Furthermore, since Dif had been named as a transcription factor in the Toll pathway (107, 108), we thought that by using flies that were mutant in both \textit{Relish} and \textit{Dif} we could investigate if the proposed model would hold up and possibly place the \textit{M. luteus} specific PAMP in one of the pathways.

\textit{Differential activation of the NF-\kappa B-like factors Relish and Dif in Drosophila melanogaster by fungi and gram-positive bacteria} (Publication III)

In previous bacteria challenge experiments, only a few bacteria including \textit{E. cloaca} and \textit{M. luteus} were used to compare different immune mutant phenotypes. We decided to screen various G\textsuperscript{+} and G\textsuperscript{-} bacteria for \textit{CecA} induction in \textit{Relish} mutants in order to see if the proposed model would hold up. Consistent with the model, we found that none of the G\textsuperscript{-} bacteria tested could induce \textit{CecA} in the \textit{Relish} mutant. Of the G\textsuperscript{+} bacteria tested, only \textit{M. luteus} and \textit{S. aureus} could induce \textit{CecA} in the \textit{Relish} mutant. \textit{B. megaterium}, \textit{B. subtilis}, \textit{B. thuringiensis}, \textit{B. cereus}, \textit{Lactococcus lactis}, \textit{Lactobacillus acidophilus}, and \textit{Lactobacillus reuteri} did not induce \textit{CecA} in the \textit{Relish} mutant. These results suggested that the \textit{Drosophila} immune system could distinguish between different G\textsuperscript{+} bacteria and selectively activate the Toll/Dif and Imd/Relish pathways (Fig. 5). Furthermore, the Relish-independent induction of
CecA by M. luteus was abolished in Dif/Relish double-mutants suggesting that CecA could be induced by M. luteus through the Toll/Dif pathway.

The Cecropin A peptides are encoded by two separately regulated genes, CecA1 and CecA2. Because of cross-reactivity of the CecA1-derived probe that we used in our Northern blot analyses with CecA2, we investigated if the same pathway induced the two genes. First, we used transgenic flies containing the lacZ gene regulated by the CecA1 promoter in Relish+ or Relish mutant background. We could detect β-galactosidase activity above background levels in Relish+ flies after injection with M. luteus, but not in Relish mutant flies. This suggested that CecA1 was regulated through the Imd/Relish pathway and that CecA2 was regulated through the Toll/Dif pathway in the Relish mutant background. Second, we performed RT-PCR of mRNA extracted from Relish+ or Relish mutant flies injected with E. cloacaec or M. luteus using CecA1 and CecA2 specific primers. The RT-PCR experiment confirmed our previous data and suggested that the CecA1 and CecA2 genes were differentially regulated through the Imd/Relish and Toll/Dif pathways after injection with E. cloacaec and M. luteus, respectively (Fig. 5).

To find out which PAMP on M. luteus stimulated the Relish-independent CecA induction through the Toll/Dif pathway, we tested various purified compounds in the Relish mutants. Of all the compounds we tested, only the cell wall extracted with boiling SDS and purified insoluble PG from M. luteus could clearly induce CecA in the Relish mutant. PG was therefore a good candidate for

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Figure 5. Selectivity of different pathways for specific microorganisms. The figures show simplified models for differential activation of the Toll/Dif and Imd/Relish pathways after injection with different microorganisms. Some arrows do not represent a direct interaction. The question marks represent uncertain pathways. TF represents any transcription factor, including Dif and Relish. Contributions from other pathways are not excluded. A) Peptidoglycan is a macromolecule that consists of a glycan backbone [usually repeating disaccharides consisting of N-acetylmuramic acid (M) and N-acetylglucosamine (G)], a linear muramyl tetrapeptide (usually well conserved), and a peptide cross-link [the most variable part (CL)] (150). The exact architecture of the peptidoglycan may vary between bacteria strains, growth conditions, or during differentiation processes. The amino acid (AA) in position three of the tetrapeptide is usually species specific. Most commonly, the third amino acid is L-lysine (LYS) as in M. luteus. It could also be diaminopimelic acid (DAP) as in many G- bacteria strains like E. cloacaec or in the G+ bacteria like B. megaterium. However, several other variations exist. The peptide cross-link varies from a single type of amino acid to mixed amino acid peptides. The figure is adopted from Sleytr et al. (150). B) Selectivity to G- bacteria such as E. cloacaec and E. coli. C) Selectivity to G+ bacteria such as S. aureus, M. luteus, B. megaterium, and B. subtilis. D) Selectivity to fungi such as B. bassiana, S. cerevisiae, M. anisopliae, and G. candidum.
being the PAMP of interest. PG from \textit{B. subtilis} and \textit{B. megaterium} could induce \textit{CecA} in the control flies, but not in the \textit{Relish} mutants. This observation suggested that \textit{Drosophila} PRRs could distinguish between the PG preparations from these different \(G^+\) bacteria strains (Fig. 5). A recent report by Leulier \textit{et al.} (117) describing the selective activation of the Toll and Imd pathways by PG from \textit{M. luteus} and \textit{B. megaterium} confirms our finding.

Since we found that various \(G^+\) bacteria could selectively induce either the Toll/Dif or the Imd/Relish pathways, we were interested to examine if the \textit{Drosophila} immune system could also distinguish between various fungi. Our experiment showed that the Toll/Dif and the Imd/Relish pathways could differentially regulate the antimicrobial induction after injection with various fungi (Fig. 5). From these results, we concluded that \textit{Drosophila} PRRs must be able to distinguish between PAMPs from at least the fungi \textit{G. candidum} and \textit{B. bassiana}. The requirement of Relish for induction of the antifungal genes \textit{Drs} and \textit{CecA} after injection with some fungi might explain the higher susceptibility of the \textit{Relish} mutants to certain fungi that we observed in publication I.
Selective activation by G+ bacteria

Our injection experiments of the Relish and Dif mutants in publication III suggested that the Drosophila PRRs could distinguish between various microbes and differentially activate Relish and/or Dif. All of the G+ bacteria that we tested activated the Imd/Relish pathway. One potential PRR candidate for PAMPs on G+ bacteria, and thereby an activator of the Imd/Relish pathway, is the group of the Gram-negative bacteria binding proteins (GNBPs). The first GNBPs was isolated from the silkworm Bombyx mori based on its interaction with the G+ bacterium E. cloacae (118). Three GNBPs genes (GNBP1-3) and two GNBPs-like genes (CG12780 and CG13422) have been found in the Drosophila genome (47, 118). Two were found to be induced after immune-challenge in two separate genome-wide expression studies (9, 10). Two forms of GNBP-1 are known to be expressed in cell lines. One is secreted and one is membrane-bound (47). In an in vitro study, GNBP-1 was found to bind with high affinity to LPS and β-1,3-glucan, but not to PG, cellulose or chitin. Overexpression of GNBP-1 in inducible Drosophila cell lines enhanced the induction of the antimicrobial peptide genes AttA, CecA, and Drs after stimulation with LPS and β-1,3-glucan. Likewise, treatment of the cells with GNBP-1 monospecific antibodies reduced the induction of AttA, CecA, and Drs after addition of LPS. These results suggested that the GNBP-1 was a good PRR candidate for binding to LPS on G+ bacteria upstream of the Imd/Relish pathway. However, a recent RNA interference (RNAi) study of GNBP-1 in flies revealed an increased susceptibility of these mutant flies to the G+ bacteria, Enterococcus faecalis and S. aureus, but not to the G+ bacterium E. carotovora or the fungi A. fumigatus and B. bassiana (48). Furthermore, RNAi of GNBP-1 reduced the induction of Drs after infection with G+ bacteria, but not by fungi. In contrast to the in vitro data, these recent results suggested that GNBP-1 binds to G+ bacteria such as E. Faecalis and S. aureus upstream of the Toll/Dif pathway (Fig. 5). However, it is possible that binding of LPS and β-1,3-glucan by GNBP-1 in vivo is redundant. Recent RNAi experiments in mbn-2 cells showed that different splice forms of the PGRP-LC gene could encode different PGRP-LC protein variants that selectively regulated the induction of the antibacterial peptides genes DptA, AttA, and CecA by either LPS isolated from G+ bacterium E. coli (previously enzymatically treated with PGRP-SC1B to remove PG contaminants) and PG isolated from the G+ bacterium B. megaterium (46). These results suggested that the PGRP-LC gene could regulate LPS induction and might therefore rescue the GNBP-1 RNAi mutant from succumbing to infection by a G+ bacterium, E. carotovora. In addition, the PGRP-LC gene was found in an RNAi screen of Schneider 2+ (S2) cells as one of 34 gene products involved in phagocytosis. PGRP-LC was involved in phagocytosis of the G+ bacterium E. coli but not the G+ bacterium S. aureus (119).

*Schneider’s “line” 2 (S2) is an embryonic cell line from Drosophila (120). The cells are immunocompetent in that they are able to induce antimicrobial peptides after stimulation with microbial components (74).

dSee figure 5 for explanation.
Another receptor that is thought to bind G- bacteria is the *Drosophila* Scavenger receptor C1 (dSR-C1). An RNAi experiment showed that dSR-C1 is responsible for 1/5 of the binding to *E. coli* by S2 cells (49).

In addition to LPS, another PAMP candidate from G- bacteria is PG. PG from G- bacteria and some G+ bacteria species such as *Bacillus* are similar in its structure (DAP-type)\(^d\). It has been proposed that PG from G- bacteria (DAP-type), and not LPS, is responsible for inducing the Imd/Relish pathway (117). However, RNAi of *PGRP-LCa* in *mbn-2* cells reduced the induction of antimicrobial peptides *DptA*, *AttA*, and *CecA* after infection with the G- bacteria *E. cloacae* and *E. coli*, but not with the G+ bacterium *B. megaterium* (46). Unless structural differences between the PGs of these bacteria accounted for the induction discrepancy, these results suggest that LPS, and not PG, induced the antimicrobial peptide genes through PGRP-LCa (Fig. 5). However, it is possible that the structural combination of PG (DAP-type) and LPS, or another G- bacteria specific molecule altogether, induced the Imd/Relish pathway through PGRP-LCa. In the absence of biochemical binding experiments with LPS, LPS-free PG or other PAMPs to any of the putative PRRs, the interaction of G- bacteria with the *Drosophila* immune system remains an open question (Fig. 5).

**Selective activation by G+ bacteria**

The G+ bacteria *B. megaterium* and *B. subtilis*, and their PG component (DAP-type), activated the Imd/Relish pathway (III). Some of the PGRPs have been proven to bind PG. For example, PGRP-SA binds insoluble PG from *M. luteus* (LYS-type)\(^d\) with high affinity (45) whereas PGRP-LE binds to *Lactobacillus plantarum* PG (DAP-type) and *Nocardia calcaro* cell wall (DAP-type PG) but does not bind *Staphylococcus epidermis* (LYS-type) or *S. aureus* (LYS-type) (121). The first PGRP was isolated from *Bombyx mori* (122) and was later found in *Trichoplusia ni* (123), rats (124) and humans (125, 126). At present, the role of the 13 different PGRPs in immunity is an object of intense research in the *Drosophila* immunity field. PGRP-LC and PGRP-LE from *Drosophila* have recently been shown to specifically regulate the induction of antimicrobial peptides through the Imd/Relish pathway (Fig. 5) (119, 121, 127, 128) and are therefore good PRR candidates for recognition of the G+ bacteria *B. megaterium* and *B. subtilis*. In addition, RNAi of PGRP-LC reduced the expression of the antimicrobial peptides *AttA*, *DptA*, and *CecA* after infection of *mbn-2* cells with the G+ bacterium *B. megaterium* (46). The collective results from publication III, along with studies of PGRP, Toll/Dif and Imd/Relish pathway mutants [reviewed in (20-22)], suggests that *B. megaterium* and *B. subtilis* PG can activate the Imd/Relish pathway by binding to PGRP-LE (Fig. 5). Whether these PG species also bind directly to PGRP-LC, or if PGRP-LC receives a signal through binding to another protein, is unclear. Investigation of PGRP-LE loss-of-function mutant flies along with studies of direct interactions between the PGs of *B. megaterium* and *B. subtilis* with any of the putative *Drosophila* PRRs would be a useful test of this hypothesis.
The G+ bacterium *M. luteus* appears to activate both the Toll/Dif and Imd/Relish pathways (Fig. 5). Werner *et al.* showed that PGRP-SA could bind PG isolated from *M. luteus* (45). Furthermore, overexpression of PGRP-SA led to increased expression of *Drs*. These results strongly suggest that PGRP-SA, like GNBP-1, is a PRR that can activate the Toll/Dif pathway in response to some G+ bacteria (Fig. 5). However, the mechanism of stimulation of the *Drosophila* immune system by *M. luteus* is unclear. Induction of *DptA* was abolished in a PGRP-LC deficient mutant after injection with *M. luteus* suggesting that PGRP-LC could activate the Imd/Relish pathway after injection with *M. luteus* (Fig. 5). However, the PAMP associated with *M. luteus* does not necessarily have to be teichuronic acid (TUA) or a PG, but could be another immunogenic *M. luteus* PAMP.

**Selective activation by fungi**

The fungus *G. candidum* selectively activated the Imd/Relish pathway whereas *B. bassiana* primarily activated the Toll/Dif pathway (Fig. 5) (III). This result suggests that *Drosophila* has distinct PRRs that can distinguish between PAMPs on these two fungi. One fungal PAMP that has been identified is β-1,3 glucan from yeast. Like the *Bombyx* GNBP, the *Drosophila* GNBP-1 has a domain similar to the putative catalytic region of β-1,3 glucanase A1 from *Bacillus circulans* (47). This bacterium can lyse fungi, including yeast. However, the *Bombyx* GNBP protein does not possess glucanase activity but is thought to act only as a PRR. Biochemical binding experiments between GNBP-1 and other *Drosophila* PRRs to *G. candidum* and *B. bassiana* or their subcellular components might reveal the detection mechanism of fungi.

**Activation of the transcription factor Relish**

Transcription factors like NF-κB are retained in the cytoplasm by inhibitors like IκB (Fig. 4) [reviewed in (95)]. In contrast to the other Rel factors, Relish harbors its own inhibitor within the C-terminal part of the protein. Endoproteolytic processing of Relish was observed within a minute following the addition of LPS to *mbn-2* cells and was believed to be necessary for activation and nuclear translocation of Relish (100). In order to further understand the activation of Relish, we expressed a set of mutant Relish proteins in the *mbn-2* cell line and examined the processing, translocation, and phosphorylation of Relish and the transcription activity of the antimicrobial peptide genes *CecA* and *DptA* before and after addition of LPS.

**Caspase-mediated processing of the *Drosophila* NF-κB factor Relish (Publication IV)**

A set of plasmids was designed for expression of various mutant Relish proteins in transfected *mbn-2* cells. The Relish proteins were tagged with an N-terminal FLAG and a C-terminal RGS-His epitope (FRH). The transfected cells were induced with LPS and harvested and the processing of FRH was examined using Western blot analysis. Two mutations in the linker region and two C-terminal
mutations affected FRH processing.

The deletion mutant ΔC535-V552 and the point mutant D545A disrupted a putative group III caspase consensus target site suggesting that a caspase might be the Relish endoprotease. It was previously shown that Relish was not processed in Dredd mutants following infection (100). Dredd has homology with the mammalian initiator of apoptosis caspases, caspase-8 and -10. Therefore, immunoprecipitations were performed of mbn-2 cell extracts with various combinations of transiently expressed wild-type and mutant forms of the FRH and Dredd proteins. FRH proteins could immunoprecipitate Dredd proteins and vice versa, suggesting an interaction of Relish and Dredd also in the fly.

The 107 C-terminal amino acids of Relish were essential for processing of FRH suggesting that a signal-dependent modification of Relish relied on this part prior to endoproteolytic cleavage of the linker region. Since processing of Relish failed in mutants for IKKβ (kenny), the phosphorylation of Relish was investigated. Treatment of Relish with a caspase inhibitor generated a protein band of higher molecular weight after addition of LPS. This higher molecular weight band was not visible in the C-terminal deletion mutants after LPS induction. This suggested that the C-terminal region of Relish was essential for phosphorylation of Relish after immune challenge prior to endoproteolytic processing.

To analyze the importance of Relish processing for its effective translocation to the nucleus, immunohistochemistry was performed on mbn-2 cells transfected with the various FRH mutants before or after addition of LPS. All FRH mutants except for the deletion mutant ΔS29-S45 were retained in the cytoplasm prior to LPS stimulation. Only the two mutants of the putative caspase recognition site, the deletion mutant ΔC535-V552 and the point mutant D545A did not translocate to the nucleus after addition of LPS. This observation suggested that processing at the putative caspase site in the linker region of Relish was essential for the translocation of FRH.

All FRH mutants that were processed and translocated to the nucleus bound to the κB site from the CecA1 promoter in EMSA experiments. The expression from the CecA1 promoter by the various FRH mutants was examined by co-transfecting a plasmid containing a CecA1-lacZ reporter. Tranfection with wild-type FRH increased the expression from the CecA1-lacZ reporter two-fold as compared to transfection with the original vector (no FRH). The non-cleavable FRH mutants, as well as ΔH497-I515, were not able to increase expression from the CecA1 promoter above background levels. The expression increased ten- and five-fold in the constitutively translocated ΔS29-S45 deletion mutant and the ΔPEST mutant, respectively. A parallel Northern blot experiment studying the DptA expression in mbn-2 cells or ΔS29-S45 FRH mutant transgenic flies gave similar results.

Tingvall et al. showed that a CecA1-lacZ reporter was induced in the yolk and epidermis in embryos in response to LPS challenge (27). Although Relish could be a potential transcription factor for this embryonic immune response, it is possible that the 2.7 kb Relish transcript seen in 0-2 hr embryos encodes a
N-terminally truncated Relish protein with a function that is distinct from the Relish proteins that are encoded by the longer transcripts (I) (98). Similar to the ΔS29-S45 deletion FRH mutant construct and transgenic flies, this N-terminally truncated protein would not contain the N-terminal serine-rich region that is present in the larger Relish. It is possible that the N-terminally truncated form of Relish that is translated from the maternally expressed 2.7 kb transcript could be signal-independently translocated to the nucleus and potentially differentially positively or negatively regulate gene transcription in the early embryo depending on interactions with other transcription factors. Although the RelE20 mutation is viable, it is possible that the delayed development observed in the RelE20 mutant strain was due to the absence of maternally expressed N-terminally truncated Relish (Hedengren-Olcott, unpublished data).

**Evolutionary conserved Rel activation pathways**

There are striking similarities between the activation of Rel proteins in *Drosophila* and mammals (Fig. 6) [reviewed in (18, 20, 22). The intracellular components of the *Drosophila* Toll/Dif pathway are highly reminiscent of the interleukin-1 (IL-1)-induced pathway in mammals leading to induction of human β-defensin-2 in keratinocytes (20). The Toll/Interleukin-1 receptor (TIR) domain of the cytoplasmic part of Toll and IL-1R interacts with the TIR domain of a MyD88 protein in the respective organism. In addition, *Drosophila* MyD88 interacts with Tube and Pelle through a death domain. Pelle is a serine-threonine kinase and is homologous to mammalian interleukin-1 receptor-associated kinase (IRAK) and Tube has been suggested to be the functional equivalent of Mad (22). Similar to its mammalian counterparts, the Toll/MyD88/Tube/Pelle complex relays a signal that leads to phosphorylation and degradation of the IkB-like protein, Cactus. However, the kinase that phosphorylates Cactus has yet to be identified. The dissociation of Cactus from the NF-κB-like homo- or heterodimer containing Dif (Dif and / or Dorsal in larvae) allows translocation of the Rel protein dimer into the nucleus and transcription of target genes. Phosphorylation of Dorsal is necessary for its activation and it is possible that phosphorylation of Dif is also required for its activation (129, 130). However, the mechanism for signal dependent phosphorylation of Dorsal and Dif is unclear. Three TRAF homologues (dTRAF1-3) have been identified in *Drosophila*. Although the involvement of these proteins in Toll-mediated activation of Drs is uncertain (131). Interestingly, RNAi of two *Drosophila* homologues of mammalian p62 and atypical protein kinase C (aPKC), Ref(2)P and aPKC, lowers the induction of Drs after infection with G+ bacteria and are important for Dif activity, but not nuclear translocation of Dif or the degradation of Cactus (130). This suggests that translocation and activation of Dif are two distinctly regulated processes.

Extracellularly, Toll and IL-1R each bind a cellularly secreted molecule, Spätzle and IL-1, respectively. However, in contrast to IL-1, Spätzle is constitutively secreted in an inactive form and must be processed before binding to and activating
Figure 6. Activation of NF-κB-like pathways in Drosophila. Summary of the A) Toll/Dif/Dorsal and B) Imd/Relish/JNK pathways in the Drosophila immune system [reviewed in (18, 22)]. Antimicrobial peptides (AMP), transiently expressed genes (TRG), nitric oxide (NO).

Toll. Pro-IL-1 is induced by NF-κB in immune-stimulated macrophages, processed intracellularly by Interleukin-1 converting enzyme (ICE), and secreted as an active IL-1 cytokine molecule. One trypsine-like protease activates Spätzle in response to fungi (132). The Spätzle protease that is activated by bacteria has not been identified. Protease inhibitors, such as the serpin Necrotic, are likely to regulate the upstream events leading to the processing of Spätzle (133). Overexpression of the pattern recognition receptors PGRP-SA or the protease Persephone led to induction of the Toll pathway (132, 134). Furthermore, depletion of GNBP-1 by RNAi reduced the induction of Drs after infection with M. luteus, suggesting that GNBP-1 is upstream of the Toll pathway (48). However, the link between the PGRP-SA and GNBP-1 and the protease cascade and the links between the Persephone protease and fungal PAMPs are still unknown.

The Drosophila Imd/Relish pathway has similarities to the TNF-induced pathway in mammals [reviewed in (18, 20, 22)]. Imd has a death domain and similarities to mammalian TNF-receptor-interacting protein (RIP), but lacks a kinase domain and has been placed upstream of IKK, Dredd and dFADD (111, 135). dTAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) that
is homologous to mammalian TGF-β-activated kinase 1 (TAK1) and acts upstream of the IKK complex that consists of Ird5 (IKK-β) and Kenny (IKK-γ/NEMO) (112, 136, 137). The IKK complex phosphorylates Relish and is required for Relish processing (IV) (138). The caspase-8 homologue Dredd interacts with Relish and is also required for processing of Relish (IV) (116). We speculate that activated Dredd cleaves Relish at the caspase-like target site located in the linker region after Relish has been phosphorylated (IV). Interaction between the death-domain containing proteins dFADD and Dredd activates Dredd by autoprocessing (139). After phosphorylation and processing, Relish translocates to the nucleus and activates sustained response genes like the antimicrobial peptides (IV) (140).

In contrast to the sustained expression of antimicrobial peptide genes that is mediated by Relish, the Imd pathway can branch downstream of dTAK1 and regulate the transient expression of the c-Jun N-terminal kinase (JNK) cascade target genes (140). Park et al. identified 27 JNK-dependent transient response genes, many of which were unknown, and peaks within one hour after addition of LPS to S2 cells. The JNK-dependent response is rapidly suppressed by increased proteosomal degradation of dTAK1 due to expression of an unknown gene product that is regulated by Relish. It is possible that activation of the JNK part of the Imd pathway is responsible for induced apoptosis after overexpression of Imd (111). The crosstalk between the Imd/Relish pathway and the Imd/JNK pathway is similar to the crosstalk that is seen between NF-κB and JNK in mammals in response to TNFα (140).

Because of some similarities between the TNFα pathway in mammals and the Imd/Relish pathway in Drosophila it is interesting to note that two homologues for the mammalian TNFα receptor and its ligand TNF, Wengen and Eiger, have been found in Drosophila (141-143). However, it is unknown if they are involved in Drosophila immunity. Two putative receptors for the Imd/Relish pathway are PGRP-LC and PGRP-LE. Although these two putative PRRs might be the direct link to some invading microorganisms, it is not clear if they are the only receptors for the Imd/Relish pathway or if they interact directly with Imd. One possible ligand for the Imd/Relish pathway could be reactive oxygen species. It was shown that nitric oxide (NO) induced DptA in Drosophila larvae through the Imd/Relish pathway (144) suggesting that a receptor for an NO-mediated or an NO-activated signal must be present in the Imd/Relish pathway.
**The missing link**

The induction pattern of *CecA*, which is the collective expression of the genes *CecA1* and *A2*, is interesting. As described in publication III, *M. luteus* induced *CecA1* and *CecA2* through the Imd/Relish and Toll/Dif pathways, respectively. Although overexpression of Relish in transgenic flies was enough to increase transcription of *DptA*, it did not increase transcription of *CecA1* (I and III). Similarly, activation of Toll in the *Toll^10B* mutant was enough to increase transcription of *Drs*, but not to increase the transcription of *CecA2* (I) (33). Therefore, a second event must happen in order to induce the *Ceca* genes compared to the induction of the *Drs* and *DptA* genes. This missing link might reflect the effects of signal-dependent modifications of Rel factors, dynamic combinations with various Rel factors and/or unidentified transcription factors that are stimulated by an additional (could be the reciprocal) pathway. It might be possible to dissect the missing second signal event by studying *CecA1* and *CecA2* expression after introducing or deleting various factors on the protein or promoter levels in the context of the two gain-of-function mutants in cell lines or flies and look for constitutive expression of the two antimicrobial peptide genes, *DptA* and *Drs*. 
FUTURE PERSPECTIVES

The field of insect immunity dates back to the year 1865 when the microsporidian protozoan pathogen *Nosema bombysis* infected the silk producing worm in France and caused silk production to plummet from 26,000 tons to 4,000 tons (145). Louis Pasteur was asked to investigate the reason for the mysterious cause of silkworm deaths. By identifying the difference between melanized spots caused by the microorganism and spots caused by wounds that were inflicted because of an overcrowded environment, he was able to select for healthy individuals and thereby help restore silk production. Much has changed since then in the ways in which we study insect immunity, but a better understanding of insect immunity could still have both economical and physiological potential to impact our society today.

In the last few years, there has been a rapid accumulation of knowledge of insect immunity, particularly in *Drosophila* [reviewed in (18, 20-22)]. However, there are still a lot of questions remaining. For instance, which molecules recognize fungal pathogens? What are the signals for the cellular response such as wound healing, encapsulation, and phagocytosis? Do cytokine signals amplify the immune response in *Drosophila*? What are the roles of the JAK/STAT-regulated thiolester-containing proteins? Are the newly discovered Turandot proteins a link between the immune system and the stress response? How does *Drosophila* defend itself against viruses? What is the timing of the *Drosophila* immune response? Do opsonins enhance the *Drosophila* immune response? What are the interactions between microbial symbionts that have evolved together with *Drosophila* and their host? How do virulence factors interfere with the *Drosophila* immune system? What are the signals for timing and tissue specificity? Is the immune response regulated on a chromosomal structural level? And why does not an infection carry over from a pupa to an adult fly? I hope that these and many more questions will be answered in the future.
CONCLUDING REMARKS

Drosophila is an amazing organism with great potential to unravel biological processes. My Ph.D. thesis has described some of the knowledge of the immune defense mechanisms that we have obtained by studying the Drosophila immune system. For example, by disrupting the Relish gene, we found that Relish was essential for the antimicrobial defense against G− bacteria, some G+ bacteria and some fungi (I and III). These mutants have now been used in a variety of labs to study immune functions. By injecting Relish and Dif mutant flies with various microbes and microbial compounds we found that the fungus B. bassiana and PG from the G+ bacterium M. luteus activated the Toll/Dif pathway and that the fungus G. candidum and the G+ bacterium B. subtilis or B. megaterium PG activated the Imd/Relish pathway (III). The differential activation of the Drosophila immune system that we observed suggests that Drosophila has a collection of selective PRRs that can distinguish between various types of microorganisms probably by recognizing distinct microbial PAMPs. These findings might initiate a search for finding these specific PRRs and their partners. The selectivity of the Drosophila immune system for various classes of bacteria has been observed in other studies [for example (34, 48, 117, 128, 146)]. However, the observation that Drosophila can also distinguish between fungi such as B. bassiana and G. candidum and thereby have distinct PRR combinations for these fungi still stands alone. These findings might also have implications outside the Drosophila immunity field. Mammals can distinguish between various PAMPs through TLRs and their co-receptors. However, I am eagerly waiting for researchers to determine if PRRs other than the TLRs/co-receptors and the collectins in the mammalian innate immune system can also selectively distinguish between various fungi and PGs from various G+ bacteria and trigger selective immune responses accordingly.

From the site-directed mutagenesis study, we learned that Relish was processed at a caspase-like target motif in order to release the N-terminal Rel-homology and the C-terminal Ankyrin-repeat domains from each other (IV). Relish was phosphorylated in a signal-dependent fashion before the processing occurred and the endoproteolytic processing of Relish was prerequisite to nuclear translocation of the Rel-homology domain that enhanced the transcription of antimicrobial peptide genes. We also learned that the serine-rich region in the N-terminal part of Relish was important for the cytoplasmic localization of Relish suggesting that N-terminally truncated almost full-length Relish could translocate to the nucleus in the absence of an immune-stimulated signal. It is possible that the proteolytically released Rel-homology domain and the almost full-length Relish have different target specificity and activity and might therefore be involved in distinct biological processes such as embryogenesis and immunity.

The similarity of the predicted amino acid structure of Attacins A, B, C, and D with Diptericins A and B, suggests that Diptericin B might be an evolutionary
link between the Attacins and Diptericins (II). It is possible that Diptericin B is processed at the furin-like site in a similar way as the Attacins. However, Attacin D does not have this furin-like site or a signal peptide since it is truncated C-terminally of the corresponding site in the other Attacins. This suggests that Attacin D might be the first potentially intracellular antimicrobial peptide found in Drosophila.

The Diptericin and Attacin genes were regulated in a similar fashion after injection with the G bacterium E. cloacae, with a few exceptions. AttC was more affected in the 18w7-35 mutants than the combined expression of AttA and could be a useful tool for researchers that want to dissect a potential 18 wheeler-dependent immune pathway. In contrast to the other Attacin genes, expression of AttD was increased in the 18w7-35 mutants. It is possible that the regulation of AttD is completely different from the regulation of any of the antimicrobial peptides that we have studied so far and might reveal additional immune regulation pathways.

It is possible that the knowledge that I have gained from my Ph.D. studies could have implications outside the Drosophila field. We are very eager to extrapolate the immune response reactions in Drosophila to the immune response reactions in humans. But should we really do that? It is obvious that various factors alter the dynamic of the Drosophila immune response such as the mode of infection, the infecting pathogen, the time after infection, the developmental stage, the location of the infection and even the behavior of the fly. But how much does evolutionary pressure between species change the dynamic of the immune response? Insects have niches ranging from extreme to calm conditions and have evolved into a very diverse bunch. So should we even extrapolate the immune responses from Drosophila to other insects? In the context of the enormous diversity between insects, it amazes me that there are so many similarities between a tiny six-legged flying insect like Drosophila and us.

Finally, I would like to conclude my Ph.D. experience with a quote from an unknown source

“Time flies like an arrow
Fruit flies like a banana”
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