EXPLORATION OF DROSOPHILA’S IMD PATHWAY THROUGH PHIC31-MEDIATED TRANSGENESIS

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ABSTRACT

Microbial infections of gram-negative bacteria in Drosophila are recognized through the immune deficiency (IMD) pathway by a molecular mechanism not completely understood. This project initiated an analysis of the potential IMD role of PGRP-LE, a peptidoglycan recognition protein that binds bacterial cell wall fragments to activate intracellular signaling. Four PGRP-Le mutants were created (E231L, S232E, R254T, and a S232E/R254T double mutant) using genomic rescue transgenes cloned into a pattB vector. A MDP transporter, Yin, was tested as a potential intracellular transporter for TCT molecules, but found as improbable. This research will allow further study of the molecular mechanism of the IKK-mediated Relish activation in IMD pathways.
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BACKGROUND

Innate Immunity

Innate immunity was evolution’s first developed defense against disease, neutralizing the majority of daily encountered pathogens by initiating action on contact prior to the agent’s ability to cause disease (Murphy et al., 2008). Selective pressures from infectious microorganisms lead to the evolution of this system, found in all organisms (Medzhitov and Janeway, 1997). Innate immunity exists from the moment of birth, and is the first response of the body against infection (Murphy et al., 2008).

The effects of innate immunity begin immediately within the first four hours in a primary response, and lasts up to 96 hours post-infection in an induced innate response (Murphy et al., 2008). The typical innate response is inflammation, which is responsible for delivering effector molecules to infection sites, speeding blood clotting as a means of physical protection, and helping repair of damaged cells. Though lacking the specificity of the adaptive immune system, innate immunity can recognize a wide range of pathogens and trigger an immediate response while differentiating between host and foreign material. Receptors found on macrophages and other effector molecules are responsible for this identification.

Instead of recognizing individual antigens, the innate immune system recognizes pathogen-associated molecular patterns (PAMPS). Preformed and nonspecific effectors, known as pattern-recognition receptors (PRRs), recognize PAMP-containing pathogens and remove them through mechanical, chemical, and microbiological means. Unlike the adaptive immune system, whose receptors are clonally selected and bind different
antigens, the innate immune system uses receptors that are found dispersed on every cell of their specific cell type, and that recognize a wide selection of pathogens. Recognition by these PRR receptors results in either ingestion of the pathogen, chemotactic direction to the site, or stimulates effector cell production for the later induced innate response (Murphy et al., 2008).

**Drosophila’s Innate Immune System**

*Drosophila melanogaster* is a common model for genetic study, and useful for studying innate immunity. This fly has 4 stages of its life cycle, and generates a new adult generation in 10 days, useful for creating strains of desired genotypes for study. The *Drosophila* sexes are easily distinguished under microscope by looking for the dark posterior abdomen found only on males. Newly born females less than 12 hours old are generally immature and guaranteed virgin for setting up crosses between phenotypes (Kim and Kim, 2005). The similarities between *Drosophila’s* immune system and that of mammals are strong enough that the discovery of *Drosophila’s* Toll receptor and its function initiated the exploration for mammalian Toll-like receptors (Hoffmann, 2003).

*Drosophila* has no adaptive immune system, leaving the innate immune system genes strongly conserved and more easily studied. Through research, two innate immunity signaling pathways were identified, the Toll and IMD pathways, that lead to stimulation of effector molecule production (Kim and Kim, 2005), and a recent study shows that a third pathway, JAK-STAT, contributes a small part (Charroux and Royet, 2010). Innate immunity starts in *Drosophila* with recognition of pathogen-associated
molecule patterns (PAMPs). The recognizing receptors initiate signaling cascades that cause immune effector production in three different responses.

The *Drosophila* innate humoral response uses several antimicrobial peptides originating from the fat body which travel via the blood. The antimicrobial peptides used are Drosomycin, Metchnikowin, Cecropin, Defensin, Attacin, Diptericin, and Drosoci, which work cooperatively to damage the membranes of infectious cells. The promoter regions of each antimicrobial peptide gene contain a consensus sequence element similar to mammalian NF-κB, a protein complex responsible for controlling DNA transcription during mammalian innate immunity. These κB-like sequences regulate the antimicrobial peptide synthesis, stimulating a response to bacterial cell wall fragments.

The second response is a phagocytosis driven by plasmatocytes, phagocytic macrophage-like cells that allow rapid engulfing of bacteria. Lamellocytes are responsible for encapsulation of larger pathogens, and crystal cells carry enzymes used in the third response: melanization. The last response clots and repairs wounds by using the enzyme melanin which in high levels leads to increased resistance to infection.

**The Toll-like Receptor Pathway and IkB Kinases**

In mammals, the most comparable response system, in function and mechanism, to the innate immune pathways used in *Drosophila* is the LPS signaling pathway. This pathway recognizes many microbial components, notably lipopolysaccharide (LPS), peptidylglycans, lipoproteins, and bacterial DNA or RNA. Toll-like receptors are crucial to recognizing these pathogen associated molecules and leading to a response (Silverman and Maniatis, 2001).
The toll-like receptors (TLRs) have been conserved in evolution, and are involved with immunity against bacteria in a variety of ways. These TLRs recognize the pathogenic patterns of microorganisms and are present at the location of infection. Co-stimulatory molecules are induced by TLR activation and release cytokines to initiate an adaptive immune response. Importantly for this report, TLR activation starts an antimicrobial effector pathway to remove foreign material (Krutzik et al., 2001).

Innate immunity between mammals and flies is highly similar, revolving around the role of NF-κB transcriptional activator proteins. NF-κB usually exists as homo- or hetero-dimers sequestered in the cytoplasm bound to an inhibitor protein, inhibitor kappa-B (IκB). The IκB kinase (IKK) is activated by signaling pathways and phosphorylates serine residues in the N-terminal end of IκB proteins. The proteasome then degrades phosphorylated IκB, uncovering a nuclear localization signal which triggers nuclear localization of the NF-κB dimer and its binding to promoter genes to initiate transcription of the antimicrobial peptides (Silverman and Maniatis, 2001).

The Toll Pathway

The Toll pathway was discovered during a genetic investigation of embryonic patterning, and is responsible for controlling the response of mammals and Drosophila to fungi and gram-positive bacteria (Kim and Kim, 2005). Nine genes encode for Toll-related receptors, denoted Toll-1 to Toll-9. Toll-5 specifically interacts with Toll and Pelle, a negative regulator (Valanne et al., 2010). Peptidoglycan recognition protein (PGRP) –SA, PGRP-SD, and GNBP-1 are receptor complexes upstream of the Toll pathway and multimerize after binding Lys-type peptidoglycan on gram-positive bacteria.
to form the Toll receptor complex. This differs from most Toll family members which are normally activated by microbial motifs (Charroux and Royet, 2010).

Activation of the Toll pathway starts by initiation of extracellular recognition factors that begin protease cascades. These cascades lead to the Toll receptor ligand Spatzle, which undergoes proteolysis causing a conformational change. The change allows for Toll receptor binding (Valanne et al., 2010). Downstream components of the Toll signaling pathway includes dMyD88, a homolog to the human MyD88, a kinase Pelle, and an adaptor molecule named Tube (Horng and Medzhitov, 2001). The cascade results in degradation of Cactus, an IκB-like protein, and nuclear translocation of NF-κB-like factors Dorsal, Dif, and Relish (Kim and Kim, 2005).

MyD88, Tube, and Pelle form a heterotrimeric complex as a result of Spatzle binding Toll. Death domains in Tube attach to Pelle and MyD88 independently to form the complex, forwarding the signal to phosphorylate, and then degrade, Cactus in two N-terminal motifs. When the Toll pathway is not activated, Cactus is bound by Dorsal and Dif which serves to inhibit their activity and ability to pass through the nuclear membrane. After phosphorylation, the nuclear translocation of Dorsal and Dif activate transcription of AMP genes by binding κB-related sequences. Dorsal is expressed in the fat body of Drosophila, increasing in expression during infection. Dif is a dorsal-related immune responsive gene that mediates induction of antifungal peptides for the Toll-pathway (Valanne et al, 2010).
**The IMD Pathway**

Contrary to gram-positive bacteria or fungal cell wall chitin, gram-negative bacteria do not activate the Toll pathway but instead stimulate the immune deficient pathway known as IMD. This pathway is similar to one producing the mammalian tumor necrosis-alpha (TNF-α), an inflammatory cytokine responsible for several innate immune phenotypes (Condorelli et al., 2002). The result is that Attacin, Cecropin, and Diptericin are accumulated to bestow substantial immunity against gram-negative bacteria.

The mechanism of the IMD pathway is not completely known, but recent research shows many of the molecular machines responsible for its activation (Figure-1), and shares a great deal of homology with the mammalian TLR pathway (Kim and Kim, 2005; Valanne et al., 2010). The IMD pathway is initiated by peptidoglycan recognition protein (PGRP) activation (Kim and Kim, 2005). DAP-type peptidoglycan (Top of Figure-1), found in the cell walls of Gram-negative and some Gram-positive bacteria, triggers the IMD pathway (Silverman, 2011). Peptidoglycan (PGN) is an important backbone in bacterial cell walls, formed of alternating N-acetylmuramic acid and N-acetylglucosamine, with additional stability from cross-linking amino acid chains of 4-5 residue lengths. The third residue in the amino acid chain determines the type of PGN, and when it is meso-diaminopimelic acid it is referred to as DAP-type PGN (Kleino, 2010).
Figure-1: Diagram of the Mammalian and Drosophila Immune Deficient Pathway (IMD). This pathway responds to DAP-type peptidoglycans present in the cell walls of gram-negative bacteria to induce signal transduction and eventually transcription of antimicrobial peptide genes of the innate immune system. (Silverman, 2011)

The DAP-type PGN is recognized by the PGRP family, specifically PGRP-LC. Three variants of PGRP-LC exist, denoted a, x, and y. The function of PGRP-LCy is unknown, but PGRP-LCx and PGRP-LCa are known to have identical forms, except for
the PGRP domain. PGRP-LCx binds polymeric PGN, whereas PGRP-LCy recognizes monomeric PGN known as tracheal cytotoxin (TCT) (Kleino, 2010). These PGRP-LC molecules recognize microbial patterns using their PGRP family domain to initiate a signal using intracellular tails. PGRP-LE, another IMD pathway receptor, synergizes with PGRP-LC to form the Gram-negative receptor complex which recognizes DAP-type peptidoglycan, specifically the monomeric TCT. PGRP-LE dimerizes when binding TCT, starting a signal that results in IMD pathway activation, but is still mechanistically unknown (Kleino, 2010).

The signal leaving PGRP-LC arrives at IMD (dark green in the diagram), a protein containing a death domain, and which is homologous to mammalian RIP1, a receptor interacting protein (Kleino, 2010; Meylan et al., 2004). Other death domain containing proteins are recruited by IMD, including the Fas-associated death domain (FADD) (green-purple in the diagram) which recruits a caspase named Death-related ced-3/Nedd2-like protein (Dredd) (purple). Dredd has two functions: the first is cleavage of IMD to cause activation, and the second is to cleave phosphorylated transcription factor Relish (green).

Relish is activated in two ways, through cleavage by Dredd and phosphorylation by the IKK complex (Kleino, 2010). The cleaved and phosphorylated N-terminal end of Relish then enters the nucleus and binds target antimicrobial peptide genes to upregulate transcription for the infection (Silverman, 2011).
PGRP-LC and PGRP-LE Function in the IMD Pathway

In *Drosophila*, 13 PGRP family members have been identified, and 4 have been identified in humans. PGRPs are characterized by their polypeptide length. Long PGRP members contain extra domains in addition to their PGRP domain, and often have a transmembrane region. PGRP-SA and PGRP-SD variants play roles in the Toll pathway, while PGRP-LC and PGRP-LE are the main focus in the IMD pathway.

The main receptor of the IMD pathway is PGRP-LC (Figure-2) (blue), made of a cytoplasmic N-terminal region, a transmembrane region, and extracellular PGRP domains. The 3 variants of PGRP-LC all contain identical cytoplasmic domains that interact with the death domain of IMD, but their ectodomains share less than 40% of their sequence. Based on the model of TCT-binding to the docking groove of PGRP-LE it is suggested that PGRP-LC recognize both the polymeric and monomeric forms of DAP-type PGN in gram negative bacteria. The coating of TCT molecules on Gram-negative bacteria could be bound by PGRP-LCx, creating a clustering of cytoplasmic domains and inducing a signal. It is suggested that PGRP-LCa is recruited to bind the ligand and enhance the binding affinity of the heterodimer.
Figure 2: Diagram of the Function of PGRP-LC and LE in the IMD Pathway. This pathway responds to DAP-type peptidoglycans present in the cell walls of gram-negative bacteria to induce signal transduction and eventually transcription of antimicrobial peptide genes of the innate immune system. (Kurata, 2009)

PGRP-LE (light blue, right side of Figure-2) is made of two domains, one N-terminal acidic domain that lacks a known function and the C-terminal PGRP domain that preferentially binds gram-negative DAP-type PGN. PGRP-LE does not have a transmembrane domain, but aids in recognition of PGN and most likely as an intracellular receptor for TCT. The prophenoloxidase cascade, the first response to infection, is also activated by PGRP-LE.

As the major chemical difference between types of PGN, the third residue of the peptide stem also plays an important part in structural binding. An arginine residue was found to be conserved among all PGN-interacting PGRP members. It provides the guanidine group needed to balance the charge of the carboxylate group found in the
bottom PGN-binding groove and mutation of this arginine residue leads to severely reduced protein-ligand binding (Lim et al., 2006).

**Transcription of Antimicrobial Peptides by Relish Activation**

Transcription of anti-microbial peptides (AMPs) for innate defense relies on NF-κB-like transcription factor Relish. The two parts of Relish includes an N-terminal Rel homology domain and a C-terminal IκB-like domain that resembles mammalian p100 and p105. Upon infection, the IMD pathway cascades lead to Relish being endoproteolytically cleaved. The N-terminal NF-κB-like fragment then translocates through the nuclear membrane to begin transcription.

The two branches of IMD that lead to Relish activation are the cleaving and phosphorylating segments. In the phosphorylating end, TAK1 and the IKK complex kinases are activated. Tak1 functions downstream of IMD, and activates the IKK complex, composed of a kinase called Immune response deficient-5 (Ird5) and the regulatory subunit Kenny. Two serine residues on Relish denoted 528 & 529 were identified as IKK-mediated sites for phosphorylation, and are required for strong activation, but not for cleavage. The cleaving arm of the IMD pathway utilizes IMD, FADD, DREDD, and the IKK Complex. The IKK complex is suspected to serve as a structural aid in DREDD mediated cleavage. Both branches are required for full expression of the AMPs activated by Relish (Erturk, 2009; Silverman, 2011).
Drosophila’s Yin Gene

The Yin transporter was identified in recent research as muramyl dipeptide (MDP) transporter in *Drosophila* (Charriere et al., 2010). Nucleotide-binding oligomerization domain containing 2 (NOD2) is a cytosolic pattern recognition receptor responsible for NF-κB activation. It is poorly understood how MDP molecules enter and exit the cytosol to activate NOD2.

Activation of NOD2 requires bacterial ligands like MDP to be transported into the cytosol from the phagosome, which requires a transporter. Yin was identified as a relative of SLC15A transporters and a strong candidate for MDP transporters, as a similar family member was proven in MDP transport. TCT has been shown to be structurally related to MDP, and is potentially able to be transported by Yin (Charriere et al., 2010).
PROJECT PURPOSE

The complete mechanism for the innate immune deficient (IMD) pathway is still unknown, which has relevance to virtually all animals. Studying the mechanism for IMD signal transduction in *Drosophila* will hopefully reveal greater detail about our innate immune system responses. Cloning and mutagenizing newly characterized members of this pathway, such as PGRP-LC, PGRP-LE, TCT, and others, will allow required functional domains to be identified. Greater knowledge of the innate IMD pathway will potentially allow its control during overreactions from innate immune system responses, which significantly contribute to mortality. The first step in this process is to clone and mutagenize the genes encoding various members of the IMD pathway.
METHODS

Site Directed Mutagenesis

Site Directed Mutagenesis was conducted using a modified QuikChange II Site-Directed Mutagenesis Kit protocol (Stratagene). The control reaction was prepared by mixing 5 μl of 10x reaction buffer New England Buffer #3, 2 ul of pWhitescript control plasmid (5 ng/μl concentration), 1.25 μl of oligonucleotide control primer #1 (100 ng/μl concentration), 1.25 μl of oligonucleotide control primer #2 (100 ng/μl concentration), 1 μl of dNTP mix, and 38.5 μl of ddH2O to bring the final reaction volume to 50 μl. 1 μl of PfuUltraHF DNA polymerase was then added at a concentration of (2.5 U/μl).

Sample reactions were prepared by mixing 5 μl of 10x New England Buffer #3, 0.5 μl of dsDNA template, 1.25 μl of oligonucleotide primer #1, 1.25 μl of oligonucleotide primer #2, 1 μl of dNTP mix, and 46 μl ddH2O to bring the reaction volume to 50 μl. 1 μl of PfuUltra HF DNA polymerase was then added to the reaction. In this prior step, the primers used were DP247, DP248, DP251, and DP252, depending upon the mutant being created. Template DNA was pBS-Bgl2_WTΔ generated previously in the lab. Each sample was then added to the thermal cycler and run for one cycle of 30 seconds at 95°C, followed by 18 cycles of 30 seconds at 95°C, 1 minute at 55°C, and 1 minute per kb of plasmid length at 68°C.
**Bacterial Transformation**

PCR amplification products were digested by Dpn I restriction enzyme as 1 μl was added to each reaction. After mixing each reaction and centrifuging for 1 minute, all tubes were incubated at 37°C for 1 hour. XL1-Blue supercompetent cells were thawed on ice, aliquoting 100 μl of cells to a 14 ml tube. 10 μl of Dpn I-treated DNA was added from each control and sample to separate aliquots of XL1-Blue cells. An optional control was conducted, adding 1 μl of pUC18 control plasmid (at 0.1 ng/μl concentration) to a 50 μl aliquot of the cells.

All reactions were incubated on ice for 30 minutes, and then heat pulsed for 45 seconds at 42°C before being placed back on ice for 2 minutes. 0.5 ml of LB broth was heated to 42°C and added to the transformation reactions, before incubating at 37°C for 1 hour on a shake rack.

The entire pWhitescript mutagenesis control and 5 μl cells in 100 μl LB of pUC18 transformation control were plated onto LB agar plates containing 100 μl of 10 mM IPTG and 100 μl of 2% X-gal 30 minutes prior to transformation plating. All of the cells from the mutagenesis reactions were spread. All plates were incubated at 37°C for ~17 hours.

**Plasmid Minipreps**

The QIAprep Protocol: Plasmid DNA Purification Using the QIAprep 8 Miniprep Kit (Qiagen) was used with some modifications. 1.5 ml of inoculation from transformation were used for every sample and centrifuged for 2 minutes at 9,000 rpm. After decanting the solution, the pelleted cells from the transformation reactions were resuspended in 250 μl of P1 buffer for neutralization. Next, 250 μl P2 buffer was added
to each sample and mixed through inverting before adding 350 μl N3 buffer and inverting again several times. After waiting one minute, samples were centrifuged for 9 minutes at 12,000 rpm.

Supernatants from the reaction were moved to spin columns, and centrifuged for one minute at 12,000 rpm to remove excess flow. 0.75 ml of wash PE buffer was added to each sample and centrifuged for one minute. After discarding the flow through, columns were exchanged into clean tubes and given 50 μl of EB buffer for elution of DNA. After letting stand for one minute, samples were centrifuged again for one minute at 12,000 rpm, and the run-through containing DNA was collected. Plasmid sequencing was handled by a different section of the lab, and results were returned to us.

Plasmid Maxipreps

The Maxiprep procedure was conducted according to the QIAprep Spin Midi/Maxiprep Kit Protocol (Qiagen) with minor changes. Colonies from each plate were taken and inoculated in 5 ml LB medium. The cultures were diluted by inoculating 200 μl of culture into 150 ml of LB medium, and left to grow at 37°C for 12-16 hours on a shake rack. The cells were centrifuged at 3500 rpm for 10 minutes at 4°C and resuspended in 10 ml of P1 Buffer. 10 ml of P2 buffer was added one minute later and mixed thoroughly and left to incubate at room temperature for 5 minutes. 10 ml of chilled P3 buffer was then added and mixed thoroughly before incubating on ice for 20 minutes.

After a round of centrifuging at 3,500 rpm for 30 minutes at 4°C, the supernatant was filtered by a compress and recovered in a separate tube. The column was given 10 ml of QBT buffer and allowed to empty by gravity flow before adding the recovered filtrate
by gravity. QC buffer was added twice in 30 ml increments by gravity to wash the QIAGEN tip before adding 15 ml QF buffer to elute. DNA was precipitated using 10.5 ml room temperature isopropanol and centrifugation at 15,000 x g for 30 minutes at 4°C.

After carefully decanting the supernatant, the DNA pellet was washed with 5 ml of 70% ethanol at room temperature, and centrifuged again for 10 minutes at 15,000 x g. The pellet was then allowed to air dry for 10 minutes before being redissolved in 200 μl of buffer for transport.

**Restriction Digestions**

The various PGRP-LE mutant plasmids were restriction digested to isolate the bands for gel extraction, purification, and isolation for ligation. Each sample was given 2.0 μl of 10x BSA, 2.0 μl of 10x New England Buffer #3, 5.0 μl of appropriate PGRP-LE mutation DNA (at 100 ng/μl concentration), 0.4 μl of two enzymes (NcoI, Acc65I, or NotI), and 10.2 μl of dH2O to bring the final volume to 20.0 μl. Enzymes were added last and kept in cold storage until use for maximum efficiency. Gently tapping and centrifugation were used to mix the sample prior to incubation at 37°C for 2 hours.

**Electrophoresis**

All agarose gels were made as 0.8% by adding 0.8 grams of agarose into 100 ml of dH2O and mixing during heating. After cooling all gels were given 8 μl of ethidium bromide. 6 μl of a 1000 bp marker was loaded for comparison, and samples were mixed with 4 μl of 6x orange loading dye. Electrophoresis was used for isolating fragments of
restriction digestions and in checking DNA fragment sizes of successful ligations. Gels were run at 130 volts for 40 minutes.

**DNA Band Extraction and Purification**

DNA fragments were recovered from agarose gels using a QIAGEN apparatus and placed into clean tubes. Each sample was given 3 volumes of QG buffer to 1 volume of gel (estimated as 100 μl of gel) and incubated at 50°C for 10 minutes while vortexing occasionally. 100 μl of isopropanol was added to the mix. Spin columns were placed in 2 ml collection tubes and allowed to pass through slowly. 0.75 wash PE buffer was added and centrifuged for one minute to remove excess. The columns were then transferred to clean 1.5 ml microcentrifuge tubes and given 50 μl EB buffer for elution. After letting stand for two minutes, all tubes were centrifuged for one minute and supernatants collected from the tubes after removing spin column.

**DNA Ligations**

Ligation reaction tubes contained 2.0 μl of 10x T4 DNA ligase buffer, 4.0 μl of the desired vector (pattB), 5.0 μl of the desired insert (PGRP-LE mutants), 0.5 μl of T4 DNA ligase, and 8.5 dH2O to bring each reaction to a total volume of 20 μl. When conducting the double mutant, 5.0 μl of the second insert was added and only 3.5 μl of dH2O to maintain final reaction volume.
RNA Isolation and qRT-PCR

RNA isolation followed the protocol from TRIzol Reagent Isolation Protocol (Life Technologies). Fly tissue samples were from 6 female and 6 male flies. The tissue was added to 600 μl of TRIzol reagent and homogenized. The homogenized sample was then left to incubate at room temperature for 5 minutes to allow for dissociation. 0.12 ml of chloroform was added to the homogenization step and shaken vigorously before left to incubate for 2 minutes at room temperature. The samples were then centrifuged at 12,000 x g for 5 minutes at 4°C. The aqueous phase was removed through pipetting and placed into a new tube.

In RNA precipitation, 10 ug of RNase-free glycogen was used before adding 0.12 ml of 100% isopropanol and incubating for 10 minutes at room temperature. Centrifugation at 12,000 x g for 10 minutes at 4°C then formed a RNA pellet to proceed to RNA washing. The RNA pellet was washed with 0.6 mL of 75% ethanol, vortex, and centrifuged at 7,500 x g for 5 minutes at 4°C. After discarding the wash, the RNA pellet was air dried for 1 minute. Last, the RNA pellet was resuspended in 40 μl of 0.5% SDS solution and incubated in a water bath set to 55°C for 10 minutes. The prepared RNA was then stored at -80°C.

The stored RNA samples were used in real time RT-PCR (qRT-PCR) for generating cDNA through reverse transcription and amplification through PCR. All samples loaded into the qRT-PCR machine were done in triplicate alongside standards of SYBR green ranging from $10^0$ to $10^{-3}$ in concentration. The qRT-PCR ran 35 cycles of 95°C for 3 minutes, 95°C for 10 seconds, and 55°C for 30 seconds. Samples were measured in real-time RT-PCR against the SYBR green fluorescent standard.
RESULTS

Although innate immunity plays an important role in protecting against infection, its overreaction also has a high rate of mortality. Understanding the mechanisms of how the innate immune system accomplishes its tasks will be the start to new procedures that increase or decrease the innate immune response as needed in controlling illnesses. *Drosophila* has a comparable innate immune system to humans, composed of similar pathways, and utilizes molecules of the same family, a relatively simple and well known genome, and short generation periods making it the perfect candidate for genetic research.

Creating Yin “Null” Drosophila

To help elucidate different pieces of the IMD pathway, we began by investigating the target transporter Yin. In order to determine if Yin is a required transporter, it was necessary to create a strain of *Drosophila* that was a null mutant for the Yin gene. Creating this strain then allowed testing of activity of antimicrobial transcription upon challenge with the TCT molecule. To cause expression within every cell of *Drosophila* as opposed to just in the fat body, the daughterless-Gal4 system was incorporated into the strain. This allowed for a more reliable reading of transcription levels due to the lack of complicating factors when testing solely the fat body. Simultaneously, a PGRP-LC mutant was to be introduced into the mix to remove interaction with TCT and compare results between the mutants. Both PGRP-LC and Yin gene mutations were a form of deletion using FLP-FRT recombination, silencing their gene expression.
The wild type strain of lab *Drosophila* used was w118. Two controls were generated for UAS-only and Gal4-only. **Figure-3** shows the scheme used to generate the Gal4 mutant. The second generation crosses are shown on the right side. The correct strains were then separated into samples of 10, made of 5 males and 5 females, with all samples conducted in triplicate.

**Fly crosses shown as Female x Male**

- w118 x da-Gal4 -> da-Gal4/+ [Positive Control]
- w118 x yin RNAi -> yin RNAi/+ [Positive Control]
- yin RNAi x da-Gal4 -> da-Gal4+yin RNAi
- PGRP-LC RNAi x w118 -> PGRP-LC RNAi/+ 
- PGRP-LC RNAi x da-Gal4 -> da-Gal4>PGRP-LC RNAi

**Figure-3. Scheme for Drosophila Genetic Crosses to Generate the da-Gal4 Null Mutant.** The second generation crosses are shown on the right side.

After performing the crosses, the offspring were challenged with TCT by injection. RNA was isolated from the fly samples, and qRT-PCR was performed on the da-Gal4 control, PGRP-LC and Yin mutants, and PGRP-LC and Yin mutants driven with ubiquitous daughterless-Gal4. **Figure-4** shows the knock-down efficiency of Diptericin in these strains and the transcript levels of Yin and PGRP-LC separately. It has been shown that PGRP-LE is an intracellular sensor for TCT (Royet and Dziarski, 2007), and Yin is being tested as a potential transporter candidate. If Yin was the intracellular transporter, deletion of PGRP-LC and Yin would result in lowered expression. The qRT-PCR results (Figure-4) show that Yin is likely not the transporter responsible for TCT induction because levels of transcription were within normal ranges, so TCT is most likely being activated through an unknown transporter.
Beyond this point, the null mutation *Drosophila* line was no longer necessary for the experiments, but was a missing factor in the Silverman Lab. Since performing this experiment, the fly lines are being incorporated into other research projects conducted by different teams on other potential transporters.

**Figure-4. qRT-PCR Experiments.** These experiments were performed to verify the knockdown efficiency of Dipterulin induction in RNAi *Drosophila* lines with Daughterless-Gal4.

**Site-Directed Mutagenesis of PGRP-LE**

After the Yin experiment, the focus in the project shifted to the theory that the DAP-type peptidoglycan binding receptors caused oligomerization to initialize the IMD pathway. To test this hypothesis, and to prevent the oligomerization of the receptors, mutations were induced in the PGRP-LE gene. Four mutations were created using site-directed mutagenesis: S2322E, E252S, R254T, and one double mutant S2322E/R254T.
The wild type PGRP-LE gene was provided, and it was used in site-directed mutagenesis with prepared primers designed to target key base pairs essential for oligomerization between PGRP-LC and PGRP-LE. The resulting four mutants were denoted S2322E, E252S, R254T, and S2322E/R254T. After verifying by sequence analysis that the mutant plasmids were successfully made, the mutant plasmids were transformed into competent E. coli to isolate colonies containing them. Colonies selected from the transformants were used in miniprep procedures to create a culture for sequencing. Correctly identified mutants were then stored for further analysis.

For use in transgenesis of Drosophila, final plasmid constructs were created that incorporated the four PGRP-LE mutants into a plasmid capable of creating Drosophila transgenics. The vector pattB-ird5 was recombined with the inserts of pBS-PGRP-LE mutants through restriction digestion by using NotI, Acc65I, and NcoI. Two restriction enzymes were used in each reaction, and the specific enzymes were chosen depending upon the vector and insert. All enzymes were single cutters, yielding only two fragments from each reaction. After restriction digestion, the correct fragments were excised from gel and ligated to create the final constructs. The final constructs were then transformed into E. coli and grown on agar plates. Four colonies of each construct were mini-prepped and then digested using the same enzymes to confirm correct recombinants. Samples with the best results were used in maxiprep procedure to obtain large quantities of purified plasmid DNA, and aliquots of the DNA were sent off-site to be used to create transgenic Drosophila.

To prepare for the arrival of the transgenic flies, a qRT-PCR assay was created by using wild-type flies and measuring gene activity through Diptericin transcription levels.
Samples (in triplicate) of the WT w118 fly line were challenged with *E. coli*, Schneider’s medium, or not challenged at all, prior to homogenization and RNA isolation and cDNA synthesis. Real-time qRT-PCR was conducted using all samples and SYBR green standards (Figure-5). The standard curve was generated using the negative and positive controls, including the low levels of activity generated by challenge with Schneider’s medium. The generated curve fit the data strongly with a linear correlation of 0.999 (R2 value).

![Figure-5: Standard Curve of Diptericin Levels in WT w118 Drosophila Challenged with E. coli. The curve had a R2 value of 0.999, showing strong correlation to the standard.](image)
DISCUSSION

The results from the Yin deletion mutant suggest that Yin is not likely the transporter responsible for TCT induction. With PGRP-LC and Yin successfully deleted, our detection of normal activity provides evidence that TCT is still being transported into the cell by another molecule to cause transcription of the antimicrobial products. The expression of AMPS during qRT-PCR was within a normal level so Yin is unlikely to be the transporter. Likely the lowered expression in daughterless-Gal4 PGRP-LC transcription reflects only the change from the deletion of the main receptor (PGRP-LC) and not a reduction in TCT transporter into the cell. Though a good candidate transporter, Yin is not responsible for TCT passage and other transporters need to be tested. The results from the Charriere lab on identification of Yin were well done, but the data contradicts the possibility of Yin’s role with tracheal cytotoxin. Other SLC15A family members involved in MDP transport are also potential transporters worth investigating (Charriere et al., 2010).

The final mutagenized PGRP plasmid constructs were used off-site to create transgenic *Drosophila*, and these lines are now ready for qRT-PCR analysis for Diptericin mRNA to determine whether the mutants show altered activation of the IMD pathway and oligomerization in DAP binding, or will have no changed expression and show the pathway is activating normally without interaction between the PGRP receptors. This data will further the research conducted in the Silverman Lab on PGRP-LC and PGRP-LE interaction regarding the IMD pathway (Silverman, 2011).

Conducting research in the Silverman lab had a learning phase, involving acclimatization to the techniques, location, and flow of the lab. After the initial weeks
and start of project work, handling and identification of *Drosophila* began. Fly gender identification and common laboratory phenotypes were learned, and played a constant role during crossing, requiring equal samples of male and female of appropriate wild type characteristics. Crosses were always created using virgin females, and no contamination or abnormal offspring were recovered.

During the time spent working on plasmid constructs, many errors occurred and had to be resolved. Originally many SDM results were flawed due to improper fragment sizes being recovered. It was found through trial and error that the template strand used had been contaminated and a clean sample was prepared to fix the problem.

Errors continued with SDM when attempting to create correct transformants. Often each mutation had little to no true transformants growing on the selection media. Later a typo was discovered in the SDM protocol that was limiting incubation time of the DNA and cell mix, incubated at only 3 minutes instead of 30 for the slowly occurring process. After this point, SDM handled correctly and yielded the correct transformants.

At the beginning of plasmid construct creation, several fragments were recovered consistently that were not in line with expected fragment numbers and fragment sizes. After more research it was found that the initial restriction enzymes being used weren’t single cutters due to a secondary site found within the pattB vector. The restriction digestion was then redesigned to use Acc65I, NocI, and NotI, all of which are compatible single cutters and yield the correct fragments. Further in the experiment it was also found that the restriction sites were interpreted wrong after one reaction; the use of Acc65I and NotI had been switched and was adjusted to return the correct results.
With respect to the transgenic fly lines created, their use will allow continued research into the mechanisms that operate the IMD pathway in the Silverman lab. Continued research will explore the mechanism of induction of the IMD pathway and potential transporters for TCT molecules that upregulate transcription of anti-microbial peptides. The experience in the lab will be indispensable in future work in combination with the lessons learned about time management, problem solving, and refreshed technique with both new and old protocols.
BIBLIOGRAPHY


