Modulation of innate immunity by the cGMP signalling pathway in the *Drosophila* Malpighian tubule

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By

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The research reported within this thesis is my own work except where otherwise stated, and has not been submitted for any other degree

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Abstract

The *Drosophila* innate immune system is one of the most widely characterised of all metozoan defense systems, and shares many similar characteristics to the innate immune systems of higher organisms. As such, *Drosophila* has become the model organism of choice for many researchers with regards to the study of the general mechanisms and regulatory elements of innate immunity. There are a number of mechanisms that *Drosophila* employ in order to combat infection, and these include both humoral and cellular responses. However, perhaps the most widely characterised of these mechanisms is the systemic production of anti-micorobial peptides (AMPs) via the activation of two specific immune signalling pathways – Toll and Imd (Lemaitre et al. 1995a; Belvin and Anderson 1996).

In *Drosophila*, a number of recent studies have identified a role for the diffusible second messenger nitric oxide (NO) in the positive regulation of the Imd pathway, a pathway that is fundamental to host defence against Gram-negative bacteria (Lemaitre et al. 1995a; Nappi et al. 2000; Foley and O'Farrell 2003; McGettigan et al. 2005). To date, the exact mechanism by which NO is mediating its effects on the Imd pathway has not yet been determined. However, it can be suggested that this effect is mediated through activation of the cGMP signalling pathway, via interaction with one of its upstream components, soluble guanylate cyclase (sGC), the main intracellular target for NO (Marletta and Spiering 2003).

Therefore, the aim of this study was to determine the potential role of the cGMP signalling pathway on regulation of the *Drosophila* Imd immune pathway. To do this, the *Drosophila* Malpighian (renal) tubule was used as a model system. The Malpighian tubule is a very well characterised, extensively studied epithelial tissue and for a number of years has comprised the model system of choice with regards to the study of the epithelial roles of signalling and transport genes (Dow and Davies 2001). The suitability of this tissue as a model system for this study is two-fold: Firstly, for many years, the NO/cGMP signalling pathway has been deemed as critical to tubule function (Dow et al. 1994a). Secondly, a recent study has identified the tubule as an important autonomous immune-sensing tissue where, upon immune challenge with Gram-negative bacteria, Imd pathway-associated AMPs are systemically produced in the tubule principle cells. Importantly, it has been demonstrated that activation of the Imd pathway

in the principle cells is regulated via the autocrine production of NO (McGettigan et al. 2005).

Data obtained from this study has demonstrated a completely novel role for cGMP signalling in the tubule. Expression analysis has revealed that cGMP acts to modulate the expression of Imd pathway-associated AMPs in a dose-dependent manner; whereby low nanomolar concentrations are shown to stimulate diptericin expression and higher micromolar concentrations of cGMP are shown to inhibit it. This effect does not appear to extend to the fat body, the canonical tissue involved in the systemic induction of AMPs, thus suggesting a completely tissue-specific mechanism.

Importantly, it is shown here that the cognate cGMP-dependent protein kinases (cGKs), DG1 and DG2 (MacPherson et al. 2004a; 2004b), mediate differential effects on AMP production in the tubule. Targeted modulation of the expression of these kinases to the principle cells of the tubule using the GAL4/UAS system demonstrates that activation of DG1 mediates positive modulation of diptericin expression in the tubule. By contrast, negative modulation of diptericin expression is shown to occur following the activation of the two main isoforms of DG2, DG2P1 and DG2P2. These data therefore describe a completely novel role for each of these kinases. Significantly, the effects of these kinases on diptericin expression in the tubule are sufficient to impact on survival of the whole fly in response to septic infection with Gram-negative bacteria, as well as contribute significantly to bacterial clearance in the gut following natural infection with *E.coli.* This study has therefore revealed a critical novel role for both the tubule and cGKs in the regulation of defence mechanisms in response to both septic and natural infection in the adult fly.

Interestingly, Q-PCR has revealed that DG1 mediates its effects downstream of Imd. Additionally, studies have revealed that both DG1 and DG2 act to regulate the Imd pathway via modulation of Relish activation, the NFκB transcription factor responsible for the induction of AMPs following activation of the Imd pathway (Hedengren et al. 1999). Translocation assays have demonstrated that targeted over-expression of *dg1* to the principal cells of the tubule results in enhanced translocation of activated Relish into the nucleus, whereas targeted knock-down of this kinase by RNAi results inhibition of Relish activation. In contrast to DG1, overexpression of either *dg2P1* or *dg2P2* to the principal cells of the tubule results in inhibition of Relish activation, even in the

presence of immune challenge. However, this study has not revealed the exact mechanism by which these kinases mediate their effects on Relish activation, and therefore it is not clear whether DG1 and/or DG2 are acting directly on Relish, or indirectly via phosphorylation of an, as of yet, unidentified substrate(s). Despite this, a completely novel function for each of these kinases is described here for the first time.

Importantly, data described in this study also identifies that, with regards to Imd pathway regulation, DG1 and DG2 may be activated via different sources of cGMP within the cell. Data shows that stimulation of the Imd pathway in the tubule is facilitated by the activation of sGC via interaction with NO. Alternatively, inhibition of the Imd pathway in the tubule is shown to be facilitated by the activation of a receptor guanylate cyclase (rGC). Additionally, it is demonstrated by this study that cGMP-mediated inhibition of the Imd pathway in the tubule is regulated by the dual-specificity, tubule-enriched phosphodiesterase (PDE), PDE11 (Day et al. 2005), thus describing a functional role for this regulatory enzyme for the first time in *Drosophila*.

In conclusion, this study further validates the role of the tubule as a critical immune-sensing tissue in *Drosophila melanogaster*. In addition, a completely novel role for the cGMP signalling pathway, as a differential regulator of Imd pathway activation in the tubule, is described here for the first time. In particular, an important novel functional role for each of the *Drosophila* cGKs, DG1 and DG2, is revealed. The data shown in this study therefore contributes to fuller understanding of not only Imd pathway regulation in *Drosophila*, but also provides a significant advance in the understanding of the complexities of cGMP signalling and its regulation of tubule function.

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Abbreviations

β-Gal	β-galactosidase
μM	micromolar
·OH	hydroxyl
AMP	anti-microbial peptide
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BSA	bovine serum albumin
Bsk	Basket
cAMP	adenosine 3',5' cyclic monophosphate
cDNA	complementary DNA
CFU	colony forming unit
cGK	cGMP-dependent protein kinase
cGMP	guanosine 3',5' cyclic monophosphate
CNG	cyclic nucleotide-gated ion channel
CREB	cAMP response element binding
CSαβ	cysteine-stabilised α-helix/β-sheet
CSM	complete Schneider's medium
$CuSO_4$	copper sulphate
CyO	curly
DAP	diaminopimelic acid
DAPI	4, 6-diamidino-2-phenylindole
DD	death domain
dDuox	*Drosophila* dual oxidase enzyme
DED	death effector domain
dFADD	*Drosophila* Fas-associated death domain protein
DIAP2	*Drosophila* inhibitor of apoptosis 2
di-butyryl cGMP	dibutyrylguanosine 3', 5'-cyclic monophosphate
Dif	Dorsal-like immune factor
DmIKK	*Drosophila melanogaster* inhibitor of κB kinase
DmPDE	*Drosophila melanogaster* phosphodiesterase
DNA	deoxyribonucleic acid

dNOS	*Drosophila* nitric oxide synthase
dNTP	deoxyribonucloetide triphosphate
Dome	Domeless
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
eNOS	endothelial nitric oxide synthase
FADD	Fas-associated death domain protein
FITC	Fluorescein isothiocyanate
for	*foraging*
for^R	*foraging – rover*
for^S	*foraging – sitter*
FRET	fluorescence resonance energy transfer
GC	guanylate cyclase
GFP	Green Fluorescent Protein
H_2O_2	hydrogen peroxide
HBS	Hepes buffered saline
HCl	hydrogen chloride
Hep	Hemipterous
HOCl	hypochlorous acid
Hop	Hopscotch
IκB	Inhibitor of κB
ICC	immunocytochemistry
IKK	Inhibitor of κB kinase
IL-1R	Interleukin-1 receptor
Imd	immune deficiency
iNOS	inducible nitric oxide synthase
IP	immunoprecipitation
IRC	immune-regulated catalase
IP_3	inositoltriphosphate
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	c-Jun N-terminal kinase
JNKK	JNK kinase

JNKKK	JNKK kinase
KAc	potassium acetate
KCl	potassium chloride
Key	Kenny
KH_2PO_4	di-pottasium hydrogen orthophosphate
LiCl	lithium chloride
luc	luciferase
M	molar
MAPK	mitogen activated protein kinase
$MgCl_2$	magnesium chloride
mM	millimolar
Na_2HPO_4	di-sodium hydrogen orthophosphate
NaCl	sodium chloride
NADPH	nicotinamide adenine dinudeotide phosphate
NEMO	NFκB essential modulator
NFκB	nuclear factor-κB
nM	nanomolar
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
O_2	oxygen
$O_2^{-\cdot}$	superoxide
ODQ	1H-(1,2,4) oxadiazolo-(4,3-a) quinaxalin-1-one
Omps	outer membrane proteins
ONPG	ortho-nitrophenyl-β-D-galactopyranoside
OrR	Oregon R
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterases
PGN	peptidoglycan
PKG1	cGMP-dependent protein kinase 1
ProPO	prophenoloxidase

PRR	pattern recognition receptor
Q-PCR	quantitative polymerase chain reaction
rGC	receptor guanylate cyclase
RHD	Rel-homology domain
RING	really interesting new gene
RIP	receptor interacting protein
RLB	reporter lysis buffer
RNA	ribonucleic acid
RNS	reactive nitrogen species
$RO^{\cdot}$	alkoxyl
$RO_2^{\cdot}$	peroxyl
ROS	reactive oxygen species
rp49	ribosomal protein 49
RT-PCR	reverse transcriptase polymerase chain reaction
S2	Schneider line 2
sb	stubble
SDS	sodium dodecyl sulfate
SEM	standard error mean
sGC	soluble guanylate cyclase
SNAP	S-Nitroso-N-acetylpenicillamine
SP	serine protease
TAB2	TAK1-binding protein 2
tb	tubby
Th2	T-helper cell 2
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-R	tumour necrosis factor receptor
TNFα	tumour necrosis factor α
tot	turandot
TRAF6	tumour necrosis factor receptor-associated factor
UAS	upstream activating sequence
Ub	ubiquitin
Upd	Unpaired
VASP	Vasodilator-Stimulated Phosphoprotein

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Chapter 1

Introduction

1.1 Innate immune response

1.1.1 Introduction

There are two systems that eukaryotes utilise to combat microbial invasion – the acquired immune system and the innate immune system. The acquired immune system operates by producing receptors through somatic gene rearrangement that recognise specific antigens, therefore allowing organisms to develop immunological memory. Alternatively, the innate immune system relies on germline-encoded receptors for detection of microbes (Janeway 1989). Vertebrates are capable of activating both of these systems, where the innate immune system acts as the first line of defence against microbial attack. However in invertebrates, innate immunity is the sole mechanism employed to combat infection (Brennan and Anderson 2004). Understanding the mechanisms which regulate innate immune responses is therefore of major importance.

1.1.2 *Drosophila melanogaster* as a model organism for innate immunity

Over the years, insects have emerged as ideal model organisms for the study of innate immune function. Many important discoveries were made initially in Lepidopteron insects such as the silkworm *Bombyx mori* and later in moths such as *Hyalophora cecropia, Trichoplusia ni* and *Manduca Sexta,* where their large size made them suitable for biochemical work (Steiner et al. 1981; Yoshida et al. 1996). However, in recent years, studies using the fruit fly *Drosophila melanogaster* have emerged as fundamental to the contribution of knowledge within the field. This was first illustrated by Lemaitre et al (1995b) who identified a key regulatory signalling pathway involved in innate immune response in *Drosophila.* These findings subsequently led to the discovery that components of this pathway are highly conserved amongst all metazoans, thus establishing insects as the ideal models to study general innate immune mechanisms in higher animals.

Since this discovery, *Drosophila* has by far become the preferred and most potent model organism for studies into innate immunity and with good reason. Firstly, *Drosophila* has been studied as a model organism for over 100 years and as a result more is known about the genetics of this insect than any other multi-cellular animal. The wealth of information that has been gathered about *Drosophila* over the past century has subsequently led to the

development of an array of both molecular and genetic techniques that make *Drosophila* the most easily manipulated of any model organism. Additionally, the completion of the *Drosophila* genome project has only further enhanced the benefits of using *Drosophila* as the model of choice. *Drosophila* has therefore proved invaluable in the last 10 years with regards to studies in innate immunity and has been instrumental in the rapid progress of the field.

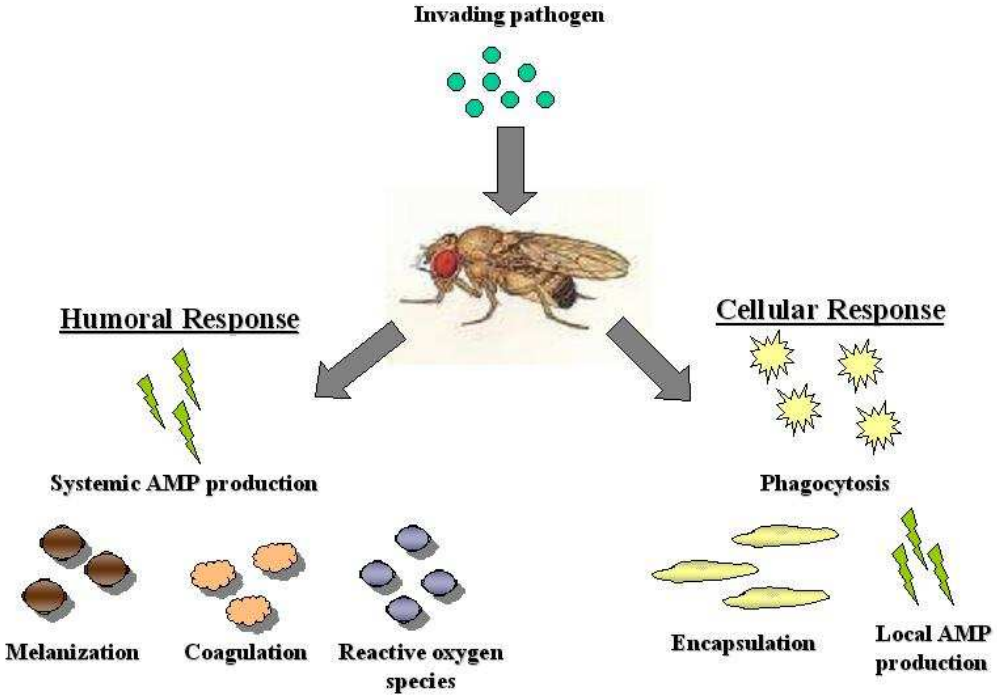


Figure 1.1 - Immune response mechanisms of *Drosophila melanogaster.* To combat infection, *Drosophila* employs a plethora of defence mechanisms that can be divided into both humoral and cellular responses. Humoral responses include the systemic production of anti-microbial peptides, which are secreted into the hemolymph to directly kill invading pathogens; melanization and coagulation around the wound site via specific signalling cascades; and production of reactive oxygen species in response to natural infection. Cellular responses mainly involve the haemocytes, which play a part in both encapsulation and phagocytosis of invading pathogens. Anti-microbial peptides are also produced locally in epithelial tissues as a result of natural infection

1.1.3 Innate Immune Response in *Drosophila melanogaster*

The innate immune response in *Drosophila* is known to manifest itself in a number of ways, many of which are shared with higher organisms, and can be divided into both humoral and cellular responses (Figure 1.1). Humoral responses include the systemic production of anti-microbial peptides (AMPs) via specific signalling pathways (Lemaitre

et al. 1995a), melanization and coagulation at the site of injury (Nappi and Vass 1993; Muta and Iwanaga 1996) and production of reactive intermediates of oxygen or nitrogen in response to natural infection (Nappi et al. 2000; Ha et al. 2005b). Cellular responses in *Drosophila* are mainly mediated by blood cells where invading microbes are encapsulated and destroyed by specific haemocytes (Meister 2004). In addition, barrier epithelia such as the gut and trachea, which are in constant contact with large numbers of microorganisms through natural infection, are known to produce AMPs locally (Ferrandon et al. 1998; Tzou et al. 2000). Together, the above responses encompass a sophisticated defence mechanism to combat infection. The immune response mechanisms that form the focus of this thesis, namely the systemic immune response, the mechanisms involved in natural infection and the involvement of nitric oxide in immunity, are described in detail below.

1.1.3.1 Systemic Immune Response

1.1.3.1.1 Overview

The systemic immune response is by far the most extensively characterised of innate immune mechanisms in *Drosophila* and consists of three distinct steps: detection of pathogen, activation of the appropriate NFκB signalling pathway (known as the Toll and Imd pathways) and production of anti- microbial peptides (Silverman and Maniatis 2001). Over the years, considerable progress has been made in identifying and characterising the various components of the signalling cascades involved in the systemic immune response, resulting in an enhanced understanding of both its activation and regulation.

1.1.3.1.2 Detection of invading pathogens

Drosophila detects the presence of invading microorganisms through molecules known as pathogen-associated molecular patterns (PAMPs), which are absent on host cells and therefore serve as discriminators between self and non-self (Janeway 1989). Examples of PAMPs include such molecules as β-1,3-glucan of fungi, phosphoglycan of parasites and peptidoglycan (PGN) and lipopolysaccharide (LPS) of bacteria, although the effect of LPS has been questioned (Leulier et al. 2003). Each of these molecules contains repetitive patterns in their structure, e.g. alternating chains of *N*-acetylmuramic acid and

N-acetylglucosamine residues in PGN, that are recognised by specific host pattern recognition receptors (PRRs) (Medzhitov and Janeway 1997).

To date, most pathogen recognition studies have focused on PGN recognition in bacteria as opposed to the recognition of other PAMPs. PGN is an essential glucopeptidic polymer consisting of long glycan chains, cross-linked to each other by short peptide bridges. There are marked differences in the PGN between different bacteria, i.e. gram-positive and gram-negative bacteria, and it is these differences that ultimately determine how the bacteria are recognised (Mengin-Lecreulx and Lemaitre 2005). In recent years a significant breakthrough has been achieved with the identification of PGN recognition proteins or PGRPs (Steiner 2004). PGRPs were first discovered in 1996 by Yoshida et al where a 19kDa protein was purified from the hemolymph of *Bombyx mori* and found to bind strongly to PGN, as well as play a role in activation of the prophenoloxidase (ProPO) cascade involved in melanisation (Ashida 1990; Yoshida et al. 1996). Since this initial discovery a whole family of PGRPs has emerged and have found to be highly conserved from insects to mammals.

In *Drosophila,* there are 13 PGRP genes that are spliced into 19 different transcripts. These transcripts are spilt into two different classes of either short (S) or long (L) transcripts and can exhibit either enzymatic or recognition properties (Werner et al. 2000). Those that exhibit, or are predicted to exhibit, enzymatic properties (PGRP-SC1, LB, SB1, SC2, SC2) are referred to as catalytic PGRPs. These PGRPs are known to demonstrate zinc-dependant amidase activity that reduces the biological activity of PGN by removing peptides from the glycan chains, thus converting the PGN into non-immunostimulatory fragments (Mellroth et al. 2003; Zaidman-Remy et al. 2006). Studies have indicated that catalytic PGRPs act as scavengers to control levels of PGN (Mellroth et al. 2003). The result of this is a negative feedback loop that controls Imd and Toll pathway activity levels and ensures the appropriate degree of immune activation.

Alternatively, other PGRPs (PGRP-SA, SD, LA, LC, LE, LF) lack amidase activity but still bind strongly to PGN and act solely as recognition proteins (Werner et al. 2000). Of these PGRPs, epistatic and phenotypic analysis has indicated membrane-bound PGRP-LC as the major recognition protein for the activation of the Imd pathway, through the recognition of DAP-type PGN from Gram-negative bacteria (Gottar et al. 2002). Three

different splice variants of PGRP-LC exist (LCa, LCx and LCy) where each share the same intracellular signalling domain but have different extracellular sensing domains (Kaneko et al. 2004). Additionally, another PGN recognition protein, PGRP-LE, is thought to be involved in activation of the Imd pathway. PGRP-LE also has an affinity to DAP-type PGN and is expressed both extracellularly, where it enhances PGRP-LC PGN recognition, and intracellularly, where it interacts with any monomeric PGN in the cytoplasm that has passed through the cell membrane due to its small size (Takehana et al. 2002; 2004).

Alternatively, activation of the Toll pathway in response to bacteria is thought to be mediated by PGRP-SA. PGRP-SA is a secreted PGRP that is present in the hemolymph and recognises the Lys-type PGN of Gram-positive bacteria (Michel et al. 2001). Recent studies have indicated that PGRP-SA may form a complex with another type of pattern recognition receptor known as GNBP1 (Gram-negative binding protein), which was originally thought to bind to the LPS of Gram-negative bacteria (Gobert et al. 2003). GNBP1 shares sequence homology with bacterial β-glucanases and current hypothesis suggests that it plays a role in degrading Gram-positive PGN. The degraded PGN is then thought to be recognised by PGRP-SA (Wang et al. 2006). Additionally, another secreted PGRP, PGRP-SD, is thought to play a roll in Toll pathway activation where it is thought to cooperate with PGRP-SA and GNBP1 to allow the detection of some Gram-positive bacteria such as *Staphylococcus aureus* (Bischoff et al. 2004).

In addition to recognition of bacteria, specific PRRs are utilised to recognise other forms of potential pathogen such as fungi. In *Drosophila,* some types of fungi are recognised by the presence of β-1,3-glucan and recent studies have suggested that the GNBP family are involved in this recognition (Bangham et al. 2006). As mentioned earlier, the GNBP family share a high sequence homology to bacterial glucanases and in *Drosophila* there are 3 members of this family (Kim et al. 2006). Of these, GNBP3 has been shown to contain a highly similar sequence to that of the Lepidopteran β-glucan recognition proteins, which are known to bind to fungal β-1,3-glucans (Ochiai and Ashida 1988). It is therefore suggested that GNBP3 acts as a fungal PRR, further supported by the reported sensitivity of *GNBP3* mutants to fungal infection. These mutants are also unable to

activate the Toll pathway, known to be the signalling pathway activated as a response to fungal infection (Gottar et al. 2006).

1.1.3.1.3 NFκB immune signalling: The Toll pathway

Throughout the 1980's and into the early 1990's, significant progress was made in the insect immunity field, with the discovery of a battery of anti-microbial peptides and the genes that encode them (Steiner et al. 1981; Hultmark et al. 1983; Wicker et al. 1990). The mechanisms that regulate the production of these peptides remained elusive, however subsequent sequencing of these genes resulted in the discovery of sequence motifs similar to those recognised by the mammalian nuclear factor-κB (NFκB)/REL family of transcription factors (Lemaitre 2004). Since that time, NFκB transcription factors and the signalling pathways that control them have been established as fundamental to the regulation of *Drosophila* systemic immune response, and represent the crucial link between microbial recognition and the anti-microbial response that follows.

The first of these pathways to be identified in *Drosophila* is known as the Toll pathway, so called after one of its main components – the Toll receptor (Lemaitre et al. 1996). The gene encoding Toll was discovered in the early 1980's, however it was originally classified as an essential component in the establishment of the dorso-ventral axis of the *Drosophila* embryo, as well as several other developmental processes (Belvin and Anderson 1996). The connection between the Toll pathway and immune response was not made until the mid-1990s when several research groups identified a number of similarities between the *Drosophila* Toll pathway and the interleukin-1 receptor (IL-1R) signalling cascade in mammals (Lemaitre et al. 1996). The Toll and IL-1R receptors are highly similar and share an intra-cytoplasmic homology domain, known as a Toll/IL receptor (TIR) domain. Activation of these receptors results in a signalling cascade leading to the eventual nuclear translocation of NFκB transcription factors (Gay and Keith 1991). The IL-1R pathway was already known to induce the expression of several immune effector genes in mammals, suggesting that the Toll pathway might play a role in immune response in *Drosophila* (Gay and Keith 1991). This hypothesis was supported by the fact that NFκB transcription sites had already been identified in *Drosophila* AMP gene sequences (Engstrom et al. 1993).

Since that time, the Toll pathway has emerged as the key pathway involved in both anti-fungal and, to a degree, anti-bacterial (Gram-positive only) responses (Rutschmann et al. 2000b; 2002). Over the years, most of the main components of the pathway have been identified and its activation and regulation has been widely characterised (Figure 1.2) (Lemaitre 2004). The pathway is dependent on an extracellular cysteine-knot polypeptide dimer known as Spaetzle, which is activated after cleavage via specific serine protease (SP) cascades (Weber et al. 2003; Hu et al. 2004). With regards to immunity, the serine proteases involved in the cleavage of Spaetzle differ depending on the nature of the invading microbe, and are initiated by recognition molecules such as PGRP-SA/GNBP-1, PGRP-SD (Gram-positive bacteria) and GNBP-3 (fungi) (Michel et al. 2001; Gobert et al. 2003; Gottar et al. 2006; Wang et al. 2006). In addition, certain types of entomopathogenic fungi are thought to be able to activate an SP cascade directly, via the serine protease Persephone (Ligoxygakis et al. 2002). Once activated, Spaetzle binds to the Toll receptor and induces dimer formation (Weber et al. 2003). This leads to a cytoplasmic cascade that begins with recruitment of three Death-domain containing proteins - the adaptor proteins MyD88 and Tube, and the kinase Pelle (Lemaitre et al. 1996; Tauszig-Delamasure et al. 2002). Cactus, an NFκB- inhibitor protein recognised by its ankyrin repeats and similar to mammalian IκB, is then phosphorylated and subsequently targeted by the proteasome for degradation (Nicolas et al. 1998). It should be noted that the mechanism of this step is not well characterised, however it is known that Cactus is not directly phosphorylated by Pelle, but is thought to be phosphorylated by an as of yet unidentified kinase (Nicolas et al. 1998). Removal of Cactus results in subsequent activation of the NFκB/Rel transcription factors Dorsal and/or Dorsal-like immune factor (Dif), which are analogous to the Rel family of proteins in mammals and contain an N-terminal Rel-homology domain (RHD (Lemaitre et al. 1995b; Manfruelli et al. 1999). Activation of these transcription factors results in their translocation into the nucleus as dimers, where the production of specific AMPs such as drosomycin, metchnikowin (fungi) and defensin (Gram-positive bacteria) is induced (Ip et al. 1993; Reichhart et al. 1993; Rutschmann et al. 2000a; 2002).

Since the initial discovery that the Toll pathway was involved in the systemic immune response, eight other Toll receptors have been identified in *Drosophila*, although none of

these have yet been clearly implicated as playing a role in immunity (Tauszig et al. 2000). In fact, most of these receptors are expressed strongly during normal embryonic and pupal development, and are therefore presumed to have important developmental functions. Additionally, the discovery of Toll as an immune receptor in *Drosophila* has led to a breakthrough in mammalian immunity studies, with the identification of a whole family of Toll-like receptors (TLRs) and their ligands. Remarkably, it has been demonstrated that mammalian TLRs are important in the control of both innate and adaptive immune responses. In addition, unlike the *Drosophila* Toll receptor, TLRs have been identified to function as recognition receptors in their own right, and interact directly with invading microbes (Hoebe et al. 2006).

1.1.3.1.4 NFκB immune signalling: The Imd pathway

The Imd pathway was initially defined via the serendipitous discovery of a mutation, subsequently named *immune deficiency (imd)*, which impaired the expression of several characterised AMP genes (Lemaitre et al. 1995a; Georgel et al. 2001). Since its identification, Imd has been characterised as a 30kDa adaptor protein, containing a C-terminal death domain and shown to share similarities to a mammalian protein, Receptor Interacting Protein (RIP). RIP is involved in the tumour necrosis factor receptor (TNF-R) signalling pathway and is known to be essential for both NFκB and mitogen activated protein kinase (MAPK) activation (Georgel et al. 2001). Therefore, rather unsurprisingly, Imd has since been recognized as a key component of an NFκB signalling cascade, now known as the Imd pathway. In recent years, the Imd pathway has emerged as fundamental to the production of AMPs in response to Gram-negative bacteria, therefore providing a complimentary role to that of the Toll pathway. So far, due to a combination of both genetic screens and reverse genetic approaches, eight additional canonical components of the Imd pathway have been identified (Figure 1.2) (Lemaitre et al. 1995a; Dushay et al. 1996; Lu et al. 2001; Leulier et al. 2002; Silverman et al. 2003; Kleino et al. 2005).

Unlike Toll, Imd is an intracellular protein and is therefore not dependent on an extracellular ligand for its activation. Instead, Imd interacts directly through both its N-terminal and C-terminal domains with the cytoplasmic domain of the membrane-bound PGRP-LC, which, as mentioned previously, acts as a recognition receptor for DAP-type

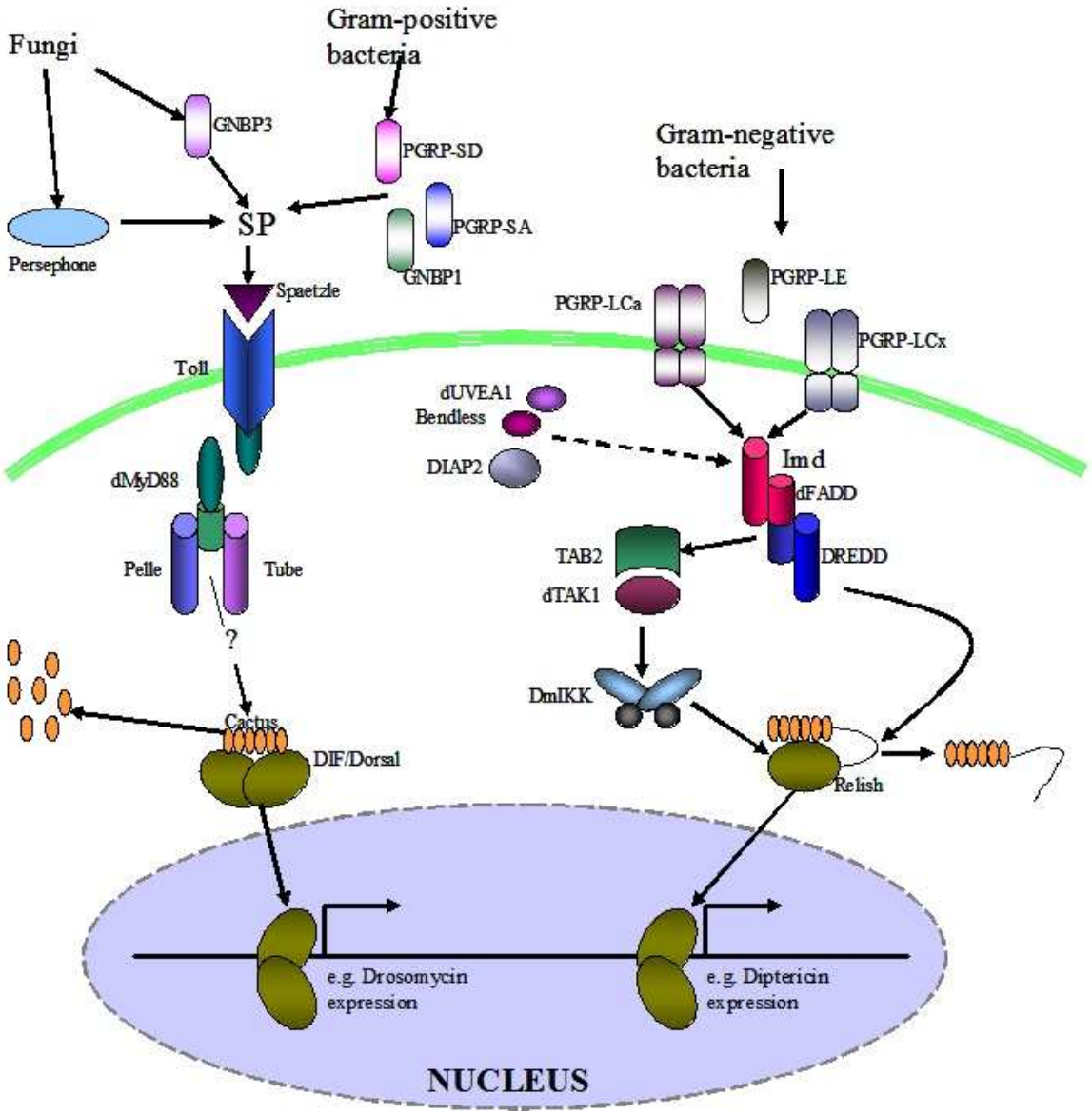


Figure 1.2 – The *Drosophila* Toll/Imd Immune Pathways. The Toll pathway (left) is activated in response to gram-positive bacteria and fungi and the Imd pathway (right) is activated by gram-negative bacteria. Activation of these pathways leads to signalling cascades that results in the activation of NFκB/REL transcription factors known as Dif, Dorsal (Toll pathway) and Relish (Imd pathway). These transcription factors are known to translocate into the nucleus as dimers where they initiate the expression of anti-microbial transcription factors. SP = serine protease; ⇢ = mechanism not confirmed.

PGN (Gottar et al. 2002). Once activated, Imd interacts with another death-domain adaptor protein known as *Drosophila* Fas-associated death domain protein (dFADD) (Naitza et al. 2002). dFADD is the *Drosophila* homologue of the mammalian protein FADD, which is known to play a critical role in apoptosis and has two conserved domains that can act in homotypic protein-protein interactions. Of these domains, the C-terminal death-domain interacts with homologous domains in other death proteins, whereas the N-terminal death effector domain (DED) is needed to recruit apical caspases, such as caspase-8, to receptor adaptor complexes (Hu and Yang 2000). Once activated by Imd, dFADD therefore initiates caspase interaction and recruits the *Drosophila* caspase-8 homologue DREDD to the complex (Hu and Yang 2000; Leulier et al. 2000). Formation of this complex is then thought to lead to the activation of *Drosophila* transforming growth factor-activated kinase 1 (dTAK1) via its adaptor protein TAK1-binding protein 2 (TAB2), the apoptosis inhibitor protein DIAP2 (*Drosophila* inhibitor of apoptosis 2) and the E2 proteins Bendless and dUEVA1 (Vidal et al. 2001; Silverman et al. 2003; Kleino et al. 2005; Zhuang et al. 2006). The mechanism by which this occurs has not yet been characterised, however it is hypothesised that dTAK1 is activated via an ubiquitination step (Zhou et al. 2005). In mammals, homologues of Bendless and dUEVA1, Ubc13 and UEVA1 respectively, are known to interact to form an E2-ubiquitin (Ub) conjugating enzyme. These enzymes are responsible for the ubiquitination of substrate proteins and are known to carry out this process via interaction with the RING (really interesting new gene)-finger domain of E3 Ub ligases (Zhou et al. 2005). DIAP2, which has been identified as an essential component of the Imd pathway, is known to contain a RING-finger domain and is therefore a likely candidate as an E3 ligase (Leulier et al. 2006). The substrate for this ubiquitination step has not yet been identified, however it is likely to be a component of the Imd/dFADD/DREDD complex as the mammalian homologues of DIAP2, c-IAP1 and c-IAP2, are known to promote ubiquitination of RIP, the mammalian homologue of Imd, and are also known to interact with caspases such as DREDD (Leulier et al. 2006). Following this ubiquitination, it's hypothesised that TAB2, the dTAK1 adaptor protein, is recruited to the complex via an N-terminal CUE domain that is able to bind specifically to K63-polyUb chains. Consequently, dTAK1 is recruited to the complex where it is activated, although this step has yet to be confirmed (Kleino et al. 2005).

In mammals, the dTAK1 homologue, TAK1, is implicated in a number of signalling processes, including the activation of the mammalian IKK signalling complex (Wang et al. 2001). The mammalian IKK complex is made up of a number of IκB kinases (IKKs), which together are capable of phosphorylating and de-activating IκBs, thus leading to the activation of NFκB transcription factors (Mercurio et al. 1997). A *Drosophila* homologue of the mammalian IKK complex, the *Drosophila melanogaster* IKK (DmIKK) complex, has been identified in the Imd pathway and is made up of four subunits containing dimers of two identified IKKs, Kenny (Key) and ird5 (Rutschmann et al. 2000b; Lu et al. 2001). Kenny, a homologue of human IKKγ, is thought to provide a purely structural role in the complex, whereas ird5, homologous to human IKKβ, is known to contain the catalytic component (Silverman et al. 2000). Epistatic analysis has identified that the DmIKK complex is located downstream of dTAK1 and therefore suggests that dTAK1 mediates its activation, although direct interaction of the two has yet to be demonstrated (Silverman et al. 2003). Once activated, the DmIKK complex is known to interact directly with the NFκB/Rel transcription factor Relish (Silverman et al, 2000).

Relish, a 110kDa protein similar to mammalian p105 and p100, was first demonstrated to play a role in immunity by Dushay et al (1996) and since then has been identified as the downstream NFκB component of the Imd pathway (Dushay et al. 1996; Hedengren et al. 1999). Relish is a compound protein consisting not only of an N-terminal Rel-homology domain (RHD) but also a C-terminal IκB inhibitory domain. Consequently, Relish is not regulated by interaction with an IκB protein, such as Cactus in the Toll pathway, but instead is regulated by the presence of its own inhibitory domain (Dushay et al. 1996; Cornwell and Kirkpatrick 2001). Activation of Relish is initiated by the phosphorylation of the IκB domain via the ird5/Kenny IKK complex, which subsequently leads to its endoproteolytic cleavage from the Rel-homology domain (Silverman et al. 2000). Rather surprisingly, studies have shown that this cleavage step is carried out by the caspase-8 homologue DREDD, already implicated further up the pathway (Stoven et al. 2000; Stoven et al. 2003). It is suggested that DREDD therefore plays two roles in the Imd pathway, one downstream in cleaving Relish and the other upstream in the activation of dTAK1. Once cleaved, activated Relish translocates into the nucleus in dimeric form, where it initiates the production of AMPs such as diptericin, cecropin, attacin and drosocin (Cornwell and Kirkpatrick 2001).

1.1.3.1.5 Interaction between the Toll and Imd pathways

To date, it has generally been accepted that the Toll and Imd pathways serve independent functions and therefore mediate the specificity of *Drosophila* immune responses towards different microorganisms. The components of each of the pathways are clearly distinct and it has been demonstrated that some AMPs, such as defensin in the Toll pathway and diptericin in the Imd pathway, exclusively respond to only one of the two pathways (Dimarcq et al. 1994; Hedengren et al. 2000). However, some AMP genes have been shown, to different extents, to be regulated by both pathways. It has also been demonstrated that knocking out both pathways can often have a greater phenotypic effect than knocking out Toll or Imd alone (De Gregorio et al. 2002; Hedengren-Olcott et al. 2004).

As mentioned earlier, AMP expression is induced by dimeric NFκB transcription factors, where Relish is induced by the Imd pathway and Dif or dorsal are induced by the Toll pathway. It was originally accepted that each of these transcription factors remained distinct from one another and operated as homodimers (Dushay et al. 1996; Meng et al. 1999). However, it has been demonstrated that these transcription factors are able to form both homodimers and heterodimers, and that the production of many AMPs can be induced by various dimer combinations (Han and Ip 1999). The reason for this appears to be the presence of a combination of different κB binding sites within the promoters of the AMPs. AMP promoters are known to contain clusters of κB binding sites rather than one distinct binding site and, in a recent bioinformatic study, κB binding sites were identified that were specific for certain dimer combinations (Senger et al. 2004; Tanji et al. 2007). For example, studies in *Drosophila* S2 cells, using deletion mutants of the promoter region of the AMP drosomycin, have shown that there are three κB binding sites in the drosomycin promoter, and that each of these sites respond differently to immune stimulation. Results show that site 1 responds to Toll pathway stimulation and can be bound by Dif or Dorsal homodimers. Alternatively, site 2 responds mainly to Imd pathway stimulation, with a very low partial response to Toll pathway induction. Results demonstrated that, at this site, relish was able to bind in both homodimeric and heterodimeric (DIF/Relish, Dorsal/Relish) forms. Finally, site 3 appeared to play an

auxiliary role by demonstrating a very minor response to both pathways. Additionally, expression of drosomycin could be induced by sole activation of either site 1 or site 2 (although to a much lesser extent by site 2 alone), however the highest levels of drosomycin activity was seen when both sites were activated together. Therefore, it appears that although the Toll pathway primarily induces drosomycin expression, simultaneous activation of the Imd pathway acts to enhance expression levels. These results therefore suggest a synergistic effect of the two pathways (Tanji et al. 2007).

Although the subject of Toll and Imd pathway synergy is still under investigation, and a synergistic effect has not been demonstrated for all *Drosophila* AMPs, it appears likely that the NF-κB transcription factors of the two pathways may cooperate to enhance the production of some immune-response genes. The level at which each AMP is induced by either pathway is likely to be determined by the ratio of different κB binding sites within each of their promoter regions.

1.1.3.1.6 Anti-microbial peptides

Anti-microbial peptides were first discovered by Hultmark et al (1980) using dormant pupae of the moth *Hyalophora cecropia*. Since this discovery, it has been found that AMPs exist as immune effectors across all organisms and, to date, approximately 800 AMPs have been identified in a diverse range of organisms such as bacteria, plants, insects and higher animals, including mammals (Reddy et al. 2004).

Currently, at least 20 AMPs have been characterised in *Drosophila* (Table 1.1). These AMPs can be arranged into seven different groups known as cecropin, diptericin, attacin, drosocin, defensin, drosomycin and metchnikowin (Imler and Bulet 2005). Following infection, they are rapidly produced (within 0.5-1 hr) by immune tissues such as the fat body, some blood cells and the Malpighian tubules, and are secreted into the hemolymph where they can reach concentrations between 1 and 100 μM (Uttenweiler-Joseph et al. 1998). In general, all of these AMPs can be described as small (<10 kDa), with exception of the 22 kDa attacins, and cationic (Imler and Bulet 2005). Additionally, as the expression of each of these peptides is induced via NF-κB transcription factors, they can also be characterised by the existence of NF-κB regulatory domains within their promoter

regions, although the number of these domains differs depending on the peptide (Engstrom et al. 1993). Despite sharing many common characteristics, these peptides differ in both their mode of action and their activity against different types of invaders such as gram-positive and gram-negative bacteria and fungi. For example, it's considered that AMPs, due to their highly basic nature, recognise invading microbes via the anionic phospholipids on microbial outer surfaces. It's thought that variations in the net-positive charge of AMPs may, to a degree, account for their specificity towards different types of microbes (Reddy et al. 2004). Additionally, some other biophysical properties of the peptides, such as structural arrangement and hydrophobicity, are known to influence the specificity and mechanism of each AMP and they are grouped accordingly (Meister et al. 1997).

Table 1.1 – Summary of *Drosophila* anti-microbial peptides. Presented in the table is the name, number of genes, estimated concentration in the hemolymph after infection and common structural motifs (nd – not determined) (adapted from Lemaitre and Hoffmann 2007)

AMP	Number of genes	Main activity	Concentration	Structural Motif
Cecropin	4	Gram-negative bacteria	20μM	α-helices
Diptericin	2	Gram-negative bacteria	0.5μM	Glycine-rich
Attacin	4	Gram-negative bacteria	nd	Glycine-rich
Drosocin	1	Gram-negative bacteria	40μM	Proline-rich
Defensin	1	Gram-positive bacteria	1μM	CSαβ
Drosomycin	7	Fungi	100μM	CSαβ
Metchnikowin	1	Gram-positive bacteria, fungi	10μM	Proline-rich

The most studied *Drosophila* AMPs are the cecropins and in *Drosophila* there are four known transcripts (CecA1, A2, B and C). Cecropins are 31-39 residue peptides that consist of an amphipathic N-terminal helix and a hydrophobic C-terminal helix, separated by a short flexible hinge (Kylsten et al. 1990; Quesada et al. 2005). They are predominately induced after activation of the Imd pathway and are mainly active against gram-negative bacteria, however some studies have shown that cecropins are also active

against some types of fungi and can be induced, to a degree, by the Toll pathway (Ekengren and Hultmark 1999). The exact mechanism by which these AMPs function remains somewhat elusive, however its considered that these types of AMPs operate by destroying the membrane integrity of potential pathogens. This is thought to occur via the α-helix structures of cecropin, which are capable of interacting with membrane lipopolysaccharides, thus embedding within the membranes to create aqueous pores (Christensen et al. 1988).

Diptericin is also induced by the Imd pathway and is active against gram-negative bacteria (Lemaitre et al. 1995a). It does not share the same structural motifs as cecropin but instead is identified for its higher than average proportion of glycine and proline residues. There are two isoforms of diptericin in *Drosophila* (DiptA and B), each approximately 9kDa in size, consisting of a long C-terminal glycine–rich G domain and a short N-terminal proline-rich domain (Wicker et al. 1990). In addition, *Drosophila* diptericin contains an O-glycosylation site within its proline domain, resulting in the presence of a disaccharide side-chain. This side-chain does not appear to contribute to the antibacterial activity of diptericin (Cudic et al. 1999; Winans et al. 1999). The exact mechanism of diptericin is not clear, however experiments on *E.coli,* using a synthetically generated peptide, have shown that diptericin may function by disrupting both the inner and outer membrane of the bacteria. Since diptericin lacks the required secondary structure to create pores in the cell membrane, it's hypothesised that it may instead interact with a target protein to interrupt protein synthesis (Winans et al. 1999).

The attacins are the largest of the AMPs, approximately 19-22kDa, with four known isoforms (AttA-D) in *Drosophila*. As with diptericin, they can be recognised for their high proportion of glycine residues and consist of two long C-terminal glycine-rich G domains (G1 and G2) (Dushay et al. 2000; Hedengren et al. 2000). Attacins are induced predominately by the Imd pathway and are active against gram-negative bacteria. The large size of this peptide renders activity studies difficult, however work carried out on *H.cecropia*, the moth where attacin was originally isolated (Hultmark et al. 1983), has shown that attacin inhibits the growth of gram-negative bacteria and increases the permeability of the outer membrane (Engstrom et al. 1984). This appears to occur via the indirect inhibition by attacin of several outer membrane proteins (Omps) (Carlsson et al.

1991). Amazingly, it does not appear to be a requirement that attacin actually enters the bacterial cells to carry out this mechanism. Instead, attacin is thought to partially embed into the outer membrane where it's thought to interact with LPS receptor sites. This interaction then somehow initiates a signal cascade within the cell that results in the shutdown of Omp synthesis (Carlsson et al. 1991; 1998).

Drosocin is a short-chain proline-rich peptide (approx. 3kDa) and can be characterised by repeated Pro-Arg-Pro tri-peptide fragments that are symmetrically distributed along its length (Bulet et al. 1993). Additionally, due to an O-glycosylation site on Thre-11, a disaccharide side-chain is present in the middle of the peptide. Unlike diptericin, it appears that this side-chain is necessary for anti-bacterial activity, as studies have shown that drosocin activity is significantly decreased in the absence of the disaccharide (Bulet et al. 1993). The exact mechanism of drosocin remains elusive however it has been found that all D-isoforms of this peptide are inactive, thus leading to suggestions that native drosocin is bactericidal through a mechanism that involves stereoselective elements (Bulet et al. 1996). As with the AMPs described above, drosocin is induced by the Imd pathway and is primarily active against gram-negative bacteria (Bulet et al. 1993). However, unlike the other acute-phase AMPs induced by the Imd pathway, which are active within around 1hr of infection and can kill invading bacteria very rapidly, drosocin does not exhibit AMP activity until between 6 and 12hr after infection and can take up to 24hr to kill bacteria (Bulet et al. 1996). On the other hand, drosocin can be detected in the hemolymph up to two weeks after infection (Uttenweiler-Joseph et al. 1998). This suggests that drosocin is responsible for ensuring a sustained resistance to gram-negative bacteria as opposed to being involved in the acute response of the other Imd pathway-induced AMPs.

Defensin was first reported from cell cultures of the flesh fly *Sarcophaga peregrina* and has since been found in every insect species investigated to date (Ando et al. 1987). In *Drosophila*, there is one defensin gene encoding a 40-residue (4kDa) peptide. This peptide can be characterised by the presence of an α-helical domain linked to anti-parallel β-strands by two disulphide bridges, known as a cysteine-stabilised α-helix/β-sheet motif (CSαβ) (Dimarcq et al. 1994). As with cecropin, the mechanism of defensin is thought to involve a direct interaction and disruption of the bacterial cell membrane

through the creation of aqueous pores. Studies carried out using another Dipteran fly species, *Phormia teranovae,* have shown that this disruption results in a loss of cytoplasmic potassium, a partial depolarisation of the inner membrane, a decrease in cytoplasmic ATP and an inhibition of respiration (Cociancich et al. 1993). Defensin is induced by the Toll pathway and is a highly potent anti-bacterial peptide. It is active against a broad spectrum of gram-positive strains of bacteria and has been shown to have the ability to kill bacteria within less than a minute, even at very low (0.1 - 1μM) concentrations (Hoffmann and Hetru 1992).

Drosomycin was first discovered in *Drosophila melanogaster* by Fehlbaum et al (1994) and, since its discovery, six other *drosomycin*-like genes have been identified in *Drosophila* alone (Jiggins and Kim 2005). It is expressed as a 44-residue (4kDa) peptide and shares a high sequence homology with the antifungal plant defensins first isolated from seeds of Brassicaceae (Terras et al. 1992). As with insect defensin, drosomycin demonstrates a CSαβ structural motif consisting of a central α-helix linked to an anti-parallel β-sheet by two disulphide bridges. However, it also has an additional short β-strand at its N-terminal and contains four disulphide bridges in total, similar to plant defensins (Landon et al. 1997). Drosomycin expression is induced mainly by the Toll pathway and, even at high concentrations, possesses no antibacterial activity whatsoever. Instead, this peptide is active against a broad spectrum of filamentous fungi (Fehlbaum et al. 1994). Studies have shown that it inhibits spore germination at high concentrations and can delay the growth of hyphae at lower concentrations, resulting in abnormal morphology (Fehlbaum et al. 1994). The exact mechanism of drosomycin is unknown, however, studies using plant defensins Rs-AFP2 and Dm-AMP1 from the seeds of *Raphanus sativus* and *Dahlia merckil* respectively, have shown that anti-fungal defensins appear to induce rapid ion fluxes and membrane potential changes in invading fungi (Thevissen et al. 1996). However, despite the CSαβ structure of these defensins, it doesn't appear that they operate by creating aqueous pores in fungal membranes, but rather may work via interaction with specific membrane binding sites (Thevissen et al. 1999). Drosomycin is induced rapidly following infection (1hr) and can be potent towards fungi at concentrations as low as 0.6μM, despite the fact that it can be detected in the hemolymph at concentrations up to 100μM (Fehlbaum et al. 1994). As with drosocin, drosomycin can also be detected in the hemolymph for 2-3 weeks after

infection suggesting a role in a sustained anti-fungal response (Uttenweiler-Joseph et al. 1998).

The last of the characterised *Drosophila* AMPs, metchnikowin, was first isolated in *Drosophila melanogaster* by Levashina et al (1995). Metchnikowin is expressed as a 26-residue proline-rich (>25%) peptide and shares high sequence homology to abaecin, a long-chain proline-rich AMP isolated from the honey-bee (Casteels et al. 1990; Levashina et al. 1995). Unlike short-chain proline-rich AMPs, which exhibit potent activity against gram-negative bacteria, metchnikowin can be characterised by its antimicrobial activity against both gram-positive bacteria and fungi (Lemaitre et al. 1997). The exact mechanism of this peptide is unknown, however studies on lebocin, a long proline-rich peptide isolated from *B.mori* and also homologous to abaecin, have shown that these peptides may function by increasing the permeability of the microbial plasma membrane (Hara and Yamakawa 1995). As with many other *Drosophila* AMPs, metchnikowin is expressed within 1-2hrs of infection and is bactericidal and fungicidal at concentrations as low as 0.5μM (Levashina et al. 1995). However, as with drosocin and drosomycin, metchnikowin can be detected in the hemolymph for 2-3 weeks after infection, suggesting a role in maintaining a sustained resistance to infection (Uttenweiler-Joseph. 1998). Studies have demonstrated that metchnikowin expression can be induced by either the Toll or the Imd pathway (Levashina et al. 1998).

Although each of the AMPs described above are highly potent against certain types of microorganism, only defensin and drosomycin are able to combat infection when expressed alone *in vivo* (Tzou et al. 2002b). Studies using *imd;spaetzle* flies, which are deficient for both the Toll and the Imd pathways but are able to constitutively express individual AMPs under the control of a non-inducible promoter, have shown that defensin and drosomycin are able to confer wild-type resistance to gram-positive bacteria and fungi respectively when expressed individually (Tzou et al. 2002). However, there aren't any *Drosophila* AMPs that are able to confer wild-type resistance in response to infection with gram-negative bacteria when expressed alone. This suggests that gram-negative bacteria may require the differential activity of multiple AMPs for elimination (Tzou et al. 2002). Rather unsurprisingly therefore, *Drosophila* AMPs are expressed as a battery of peptides in response to infection and are present in different combinations

according to the identity of the invading microorganism. This response ensures the efficient elimination of invading pathogens and comprises a sophisticated mechanism of deterring the development of pathogen resistance to attack.

1.1.3.1.7 Other pathways involved in systemic immune response

There are two other important pathways that have been associated with *Drosophila* immune response – the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, and the c-Jun N-terminal kinase (JNK) pathway.

The first of these, the JAK/STAT pathway, was first identified in mammals and shown to transduce a variety of cytokines and growth factor signals (Darnell 1997). In insects, the JAK/STAT pathway was originally identified through its role in embryonic segmentation and consists of four main components - the ligand Unpaired (Upd), the receptor Domeless (Dome), the JAK Hopscotch (Hop), and the transcription factor STAT92E/Marelle (Agaisse and Perrimon 2004). The first evidence for an involvement of this pathway in insect immunity was obtained from studies using the mosquito *Anopheles gambiae*, where it was demonstrated that the STAT transcription factor accumulates in the nucleus following infection (Barillas-Mury et al. 1999). Since that time, gene expression profiles have identified the JAK/STAT pathway as the pathway responsible for the regulation of a number of immune-responsive genes, including those encoding the complement-like protein Tep2, which is strongly activated in the fat body upon immune challenge (Lagueux et al. 2000), and the *turandot* (*tot*) stress genes, which accumulate in the hemolymph in response to various stress conditions, including septic injury (Ekengren and Hultmark 2001). The precise role of this pathway remains to be established however it has been proposed that the JAK/STAT pathway could respond to tissue damage encountered during infection (Agaisse and Perrimon 2004).

The JNK pathway in *Drosophila* is a highly conserved MAPK signal transduction module and is known to play a role in a variety of different processes such as proliferation, differentiation, morphogenesis, apoptosis and immune response (Sluss et al. 1996; Leppa and Bohmann 1999; Stronach and Perrimon 1999; Boutros et al. 2002; Dong et al. 2002). In this pathway, the main components are the Jun N-terminal kinase kinase (JNKK) Hemipterous (Hep), the JNK Basket (Bsk) and the transcription factor Jun (Glise

et al. 1995; Holland et al. 1997). Once activated, Hep phosphorylates and activates the downstream substrate, Bsk. Bsk then goes on to stimulate the activity of Jun, which, together with a separate protein Fos, constitutes the transcription factor AP-1 (Kockel et al. 2001). Activation of the pathway upstream of Hep is thought to involve a number of different JNKK kinases (JNKKKs) and in the *Drosophila* genome there are six known genes encoding for putative JNKKKs (Stronach 2005). It's hypothesised that the variety of kinases acting at this level may account for the vast array of different processes regulated by the JNK pathway by linking specific upstream signals with the downstream signalling module (Stronach 2005). The involvement of the JNK pathway in immune function was first demonstrated by its induction in response to LPS stimulation (Sluss et al. 1996). Since that time, using a combination of gene expression studies and epistatic analysis, studies have demonstrated that the JNK pathway (i.e. Hep) is actually activated in response to infection via the Imd pathway kinase dTAK1 (Boutros et al. 2002; Silverman et al. 2003). Despite sharing a common component, studies have shown that the JNK pathway and the Imd pathway remain distinct from one another downstream of dTAK1 and regulate the transcription of separate sets of genes. However, in a study by Park et al (2004) it was demonstrated that JNK signalling exhibited a sustained activation in response to infection when the Imd pathway was blocked downstream of dTAK1. This data suggests that the JNK pathway may be negatively regulated by the Relish branch of the Imd pathway (Park et al. 2004). The exact role of the JNK pathway in immune response hasn't yet been confirmed however it is suggested that the JNK pathway may play and important role in stress response and wound repair following infection (Silverman et al. 2003).

1.1.3.2 *Drosophila* immunity in response to natural infection

The systemic immune response has been proven critical to host survival. However, most of the studies carried out regarding this response have been carried out using septic injury as a means of infection, i.e. direct injection of microbes into the hemocoel (Silverman and Maniatis 2001; Boutros et al. 2002; Tzou et al. 2002a; Hoffmann 2003; Hultmark 2003; Brennan and Anderson 2004). In nature, it is far more common for an insect to become infected via the ingestion of microbe-contaminated food, known as natural infection. During this type of infection, the barrier epithelia, and not the fat body, are believed to be the first line of defence and are critical to host survival (Ferrandon et al.

1998; Tzou et al. 2000). There are two main defence mechanisms that the epithelial tissues are known to employ in response to natural infection - firstly, the production of reactive oxygen species (ROS) and, secondly, local production of AMPs.

Reactive oxygen species can be defined as oxygen-derived small molecules. These include oxygen radicals such as superoxide ($O_2^{-\cdot}$), hydroxyl ($^{\cdot}OH$), peroxyl ($RO_2^{\cdot}$) and alkoxyl ($RO^{\cdot}$), as well as non-radicals that can easily be converted such as hypochlorous acid (HOCl), ozone (O_3) and hydrogen peroxide (H_2O_2) (Brown and Borutaite 2006). Once created, ROS can interact with a number of different molecules, such as proteins, lipids, carbohydrates and nucleic acids, and act to irreversibly destroy or alter the function of these molecules (Bedard and Krause 2007). In *Drosophila*, ROS synthesis is induced rapidly in the gut following natural infection and acts to eliminate invading pathogens by causing oxidative damage in the form of both lipid peroxidation and protein carbonylation (Ha et al. 2005a; 2005b). The generation and elimination of ROS is a finely regulated process, allowing for enough ROS production to combat the pathogen whilst at the same time eliminating any residual ROS in order to protect the host (Geiszt et al. 2003). This fine redox balance is mediated by *Drosophila* dual oxidase enzyme (dDuox) and immune-regulated catalase (IRC) (Figure 1.3). Studies have shown that flies deficient in dDuox are unable to generate ROS and succumb rapidly to oral infection by the gram-negative bacteria *Erwinia carotovora* (Ha et al. 2005a). Conversely, flies deficient in IRC produce higher levels of ROS, resulting in fly death due to irreversible oxidative damage (Ha et al. 2005b). Studies have shown that ROS-dependent immunity is critical to host survival and is the primary mechanism by which *Drosophila* combats natural infection.

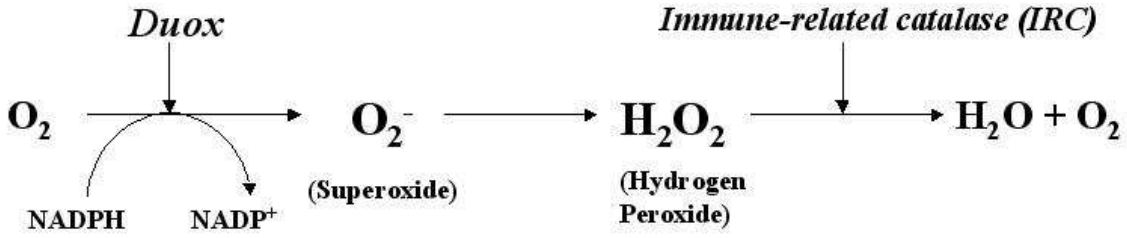


Figure 1.3 – Production and elimination of reactive oxygen species. The production of superoxide from O_2 is catalysed by dual oxidase in the presence of NADPH. Superoxide is then able to spontaneously form hydrogen peroxide. Hydrogen peroxide is then broken down into oxygen and water by immune-related catalase (Ha et al. 2005a; 2005b).

In addition to ROS-dependent immunity, several barrier epithelia, including the epidermis, digestive tract, Malpighian tubules, trachea and reproductive tissues, are known to produce AMPs locally in response to natural infection (Ferrandon et al. 1998; Tzou et al. 2000). Studies using β-galactosidase (β-Gal) and Green Fluorescent Protein (GFP) reporter transgenes have shown that at least two AMPs with complementary activity spectra are expressed in each of these tissues (Basset et al. 2000; Tzou et al. 2000). For example, in the respiratory tract, antibacterial drosocin is expressed alongside the antifungal drosomycin. Similarly, in the Malpighian tubules, antibacterial AMPs diptericin and cecropin are expressed alongside antifungal metchnikowin (Tzou et al. 2000). To date, a response to natural infection in *Drosophila* has only been demonstrated using gram-negative bacteria such as *E.carotovora* and the entomopathogenic *Pseudomonas emtomophila* (Basset et al. 2000; Vodovar et al. 2005; Liehl et al. 2006). There is no evidence at present that suggests AMPs are induced in epithelia in response to gram-positive bacteria or fungi, despite the fact that antifungal AMPs are expressed in epithelial tissues (Ferrandon et al. 1998; Tzou et al. 2000). Interestingly, it appears that the Imd pathway acts as the sole mediator of AMP regulation in epithelia, with no evidence to date to suggest that the Toll pathway has any involvement in local AMP production (Ferrandon et al. 1998; Tzou et al. 2000; Onfelt Tingvall et al. 2001; Ryu et al. 2006). This is particularly interesting in the case of the antifungal peptides drosomycin and metchnikowin, which, in *imd* mutants, are not upregulated in the epithelial tissues following natural infection but remain fully inducible by the Toll pathway in the fat body following systemic infection (Tzou et al. 2000). Finally, studies have shown that although the systemic immune response can be activated after natural infection, via an as of yet unknown signalling mechanism between the gut and the systemic immune tissues, it does not appear to contribute whatsoever to host survival (Liehl et al. 2006; Zaidman-Remy et al. 2006). Instead, bacterial clearance after natural infection is achieved solely through local AMP expression and ROS production (Liehl et al. 2006; Ryu et al. 2006). Together, both ROS-dependent immunity and local AMP production encompass two complimentary inducible defence mechanisms that are critical to host survival and act as the first line of defence following natural infection.

1.1.3.3 Nitric oxide and immunity

Nitric oxide (NO) is a soluble gas that has been demonstrated to play role in a variety of biological processes in both vertebrates and invertebrates. These include vascular smooth muscle relaxation, neurotransmission, apoptosis, cell motility and immunity (Davies 2000; Bogdan 2001b). NO is produced by a group of enzymes known as nitric oxide synthases (NOS), which act to convert $_{L}$-arginine into citrulline (in the presence of NADPH and O_2), producing NO in the process (Figure 1.4). In vertebrates, there are three NOS enzymes, neuronal NOS (nNOS/NOS1), endothelial NOS (eNOS/NOS2) and inducible NOS (iNOS/NOS3) where NOS1 and NOS2 are constitutively expressed, calcium-dependent enzymes and NOS3 is an inducible enzyme with activity independent of cellular calcium levels (Stuehr 1999). Over the years, several NOS homologues have been identified in insects such as *A.stephensi*, *A.gambiae*, *M.sexta* and *B.mori* (Luckhart et al. 1998; Nighorn et al. 1998; Imamura et al. 2002; Dimopoulos 2003). In *Drosophila* there is one gene encoding NOS (*dNOS*), which is most closely related to vertebrate NOS1 (Regulski and Tully 1995).

Figure 1.4 – Generation of NO by the NOS enzyme. NOS-catalysed generation of NO occurs using the substrates $_{L}$-arginine, NADPH and molecular oxygen. NO is produced alongside citrulline and $NADP^+$.

Once generated, NO is able to diffuse rapidly across cell membranes and can interact as a signalling molecule with a number of biological targets such as heme groups, cysteine residues and iron and zinc clusters (Bogdan 2001b). Many of these targets are regulatory molecules, such as transcription factors and components of various signalling cascades, and therefore NO is able to exert heterogeneous and diverse phenotypic effects (Bogdan 2001b). Additionally, NO is able to act as a reactive nitrogen species (RNS) and is able to

form a number of RNS derivatives including peroxynitrate ($ONOO^-$), nitrogen dioxide (NO_2), S-nitrosothiols and dinitrogen trioxide (N_2O_3) (Brown and Borutaite 2006).

In mammals, NO has been implicated in a number of immune response processes and is considered a critical component of mammalian immunity. These responses include antimicrobial activity, pathogen tissue damage, anti-inflammatory activity, T helper cell deviation and anti-tumour activity (Bogdan 2001a). Similarly, in recent years a role for NO in *Drosophila* immunity has emerged (Nappi et al. 2000; Foley and O'Farrell 2003; Silverman 2003; McGettigan et al. 2005). Studies have shown that NO contributes to *Drosophila* immunity in two ways. Firstly, NO (and its derivatives) are known to act as RNS at the site of infection and are able to directly destroy invading microbes via a combination of nitrosylation, nitration and oxidation of essential microbial components (Nappi et al. 2000). Secondly, various studies have shown that NO acts as an important signalling molecule in response to infection. This effect was first indicated by Nappi et al (2000), where it was demonstrated that there was a significant increase in diptericin expression in NO-treated larvae. Similarly, NO levels in infected larvae were significantly higher than controls. Since that time, studies have shown that NO is critical to survival in response to both septic and natural infection with the gram-negative bacteria *E.caratovora caratovora* and is a crucial component in upstream activation of the Imd pathway (Foley and O'Farrell 2003).

The mechanism of action of NO as a signalling molecule in immunity is still not clear, however it has been suggested that NO mediates signalling between immune tissues upon infection. In this model it is proposed that NOS is up-regulated in any particular immune tissue that has come into contact with an invading pathogen. The resulting NO produced is then thought to mediate a signal, possibly via hemocytes, to other immune tissues in order to alert them to activate their own defence systems (Basset et al. 2000; Foley and O'Farrell 2003; Silverman 2003). This model is supported in a recent study by McGettigan et al (2005), which has shown that dNOS activity is significantly increased in the Malpighian tubules upon immune challenge. Similarly, targeted over-expression of *dNOS* to the principal cells of the Malpighian tubules was seen to confer increased survival to adult flies upon infection, suggesting an enhancement of AMP expression within the fly (McGettigan et al. 2005). To date, the identity of the downstream effector of NO with regards to immune function has not yet been confirmed. However, it is

possible that this effect is mediated via the cGMP pathway (Figure 1.5). The main intracellular receptor for NO is the heme moiety of soluble guanylate cyclase, one of the enzymes responsible for the generation of cGMP and subsequent activation of cGMP-dependent protein kinases (cGKs), ion channels and cGMP-dependent phosphodiesterases (PDEs) (Schulz et al. 1989; Davies 2000). It is clear, therefore, that the role of cGMP signalling in *Drosophila* immunity requires further investigation.

1.2 cGMP signalling

1.2.1 Introduction

The intracellular second messenger guanosine 3'5' cyclic monophosphate (cGMP) was first discovered by in rat urine in 1963 (Ashman et al.). In the years since this discovery cGMP has been recognised as a key signalling molecule responsible for mediating a wide variety of physiological responses in both vertebrates and invertebrates. These responses include the regulation of smooth muscle relaxation, phototransduction, renal function, neuronal plasticity and development of the nervous system (Beavo and Brunton 2002; Pilz and Casteel 2003).

cGMP is produced as part of a specific signalling pathway (Figure 1.5), where intracellular levels of cGMP are elevated after the activation of a family of enzymes known as guanylate cyclases (GCs). These enzymes exist as either soluble or membrane bound proteins that are stimulated by specific ligands (Drewett and Garbers 1994; Lucas et al. 2000). Once activated, guanylate cyclases work by catalysing the conversion of GTP into cGMP. The cGMP produced goes on to interact with intracellular receptor proteins such as cGMP-dependent protein kinases (cGK), cGMP-regulated ion channels or cGMP-regulated phosphodiesterases (PDE) - with PDE acting to regulate cGMP levels by catalysing the conversion of cGMP to 5'GMP (Lincoln and Cornwell 1993; Vaandrager and de Jonge 1996; Omori and Kotera 2007). Interaction of cGMP with these effectors ultimately determines the physiological response of the cell.

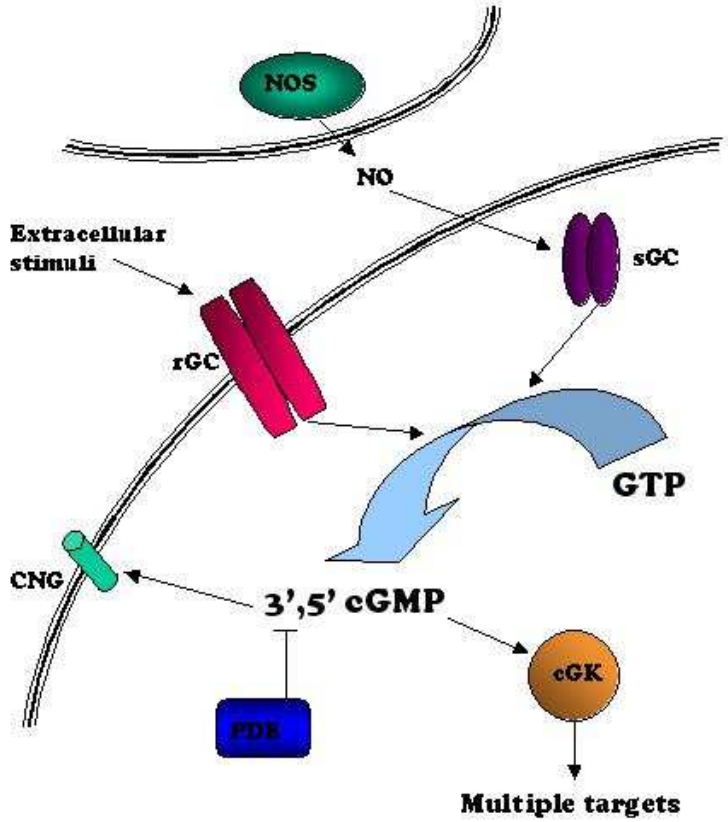


Figure 1.5 – The cyclic GMP signalling pathway. cyclic GMP is generated through the activation of guanylate cyclases. Raised levels of cGMP have then been shown to interact with either ion channels, or cGMP-dependent kinases (cGK) to mediate physiological responses. cGMP production is regulated by c-GMP-dependant phosphodiesterases which convert cGMP into 5' GMP.

1.2.2 Guanylate cyclases

The guanylate cyclase family of enzymes was discovered in 1969 and were found to exist in both soluble and particulate fractions, suggesting the occurrence of two different forms of the protein (Hardman and Sutherland 1969). Since this discovery, two classes of GC have emerged.

The first of these, soluble guanylate cyclase (sGC), exists as a heterodimer made up of both alpha and beta subunits (Kamisaki et al. 1986). Although very similar, studies have shown that co-expression of both subunits is required to produce an enzymatically active protein (Stone and Marletta 1994). Soluble guanylate cyclases are typically activated by nitric oxide, which binds to a heme moiety within the N-terminal regulatory domain of the enzyme. Upon NO binding, a ferrous-nitrosyl-heme complex is formed, leading to a conformational change within the catalytic domain of the sGC (Craven et al. 1979).

Studies have shown that activation of sGC results in an increase in guanylate cyclase activity of up to 200-fold (Friebe and Koesling 2003). In *Drosophila,* there are two sGC genes, Gycα99B and Gycβ100B, encoding for the alpha subunit and the beta subunit respectively. So far, a number of different transcripts of these genes have been identified (Yoshikawa et al. 1993; Liu et al. 1995; Shah and Hyde 1995). Interestingly, a variety of atypical soluble guanylate cyclases have also been identified in recent years. The first of these, discovered by Nighorn et al (1999) in *Manduca sexta* (MsGC-β3) is reported to act as a homodimer that can function in the absence of nitric oxide (Morton and Anderson 2003). Since this discovery, three genes encoding atypical NO-insensitive sGCs have been identified in *Drosophila,* and it has been demonstrated that they play an oxygen-sensing role (Morton 2004; Vermehren et al. 2006).

The second class of guanylate cyclases, known as receptor guanylate cyclases (rGC), are made up of single transmembrane proteins acting as receptors for a variety of ligands (Lucas et al. 2000). Receptor guanylate cyclases act primarily as homodimers, exhibiting highly conserved domain structures. The C-terminal catalytic domain of rGCs is highly conserved, however rGC isoforms are found to differ remarkably in their extracellular binding domains depending upon the ligand to which they bind. Unfortunately, the extracellular ligands of many rGCs still remain unknown, thus making it difficult to define specific function. Receptor GCs were first discovered in the sea urchin *Arbacia punctulata* in 1981 (Suzuki et al.), and since that time a number of rGCs have been identified in both vertebrates and invertebrates. In mammals, seven receptor guanylate cyclases have been identified (GC-A – GC-G), three of which are well characterised and possess identified ligands (Chang et al. 1989; Chinkers et al. 1989; Lowe et al. 1989; Schulz et al. 1989; Yuen et al. 1990). For example, GC-A is known to be activated by the extracellular ligands atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) and has been implicated in such processes as natriuresis, diuresis and hypertension (Kishimoto et al. 1996; 2001). Alternatively, GC-B is activated by C-type natriuretic peptide (CNP), and is thought to play a role in bone development and the inhibition of smooth muscle and fibroblast proliferation (Garbers et al. 2006). Lastly, GC-C has a number of identified ligands such as heat-stable enterotoxin (STa), guanylin, uroguanylin and lymphoguanylin (Schulz et al. 1990; Hamra et al. 1993; Forte et al. 1999), and has been implicated to play a role in intestinal function (Steinbrecher et al. 2001).

Unfortunately, GC-D – GC-G remain orphan receptors with no identified ligands and therefore unknown function (Schulz et al. 1998). In *Drosophila,* there are at least nine genes encoding receptor guanylate cyclases (Flybase – http://flybase.bio.indiana.edu). Three of these genes, *gyc32E, gyc76C* and *gyc89A*, have been cloned in previous studies using rat GC-A cDNA as a probe (Gigliotti et al. 1993; Liu et al. 1995; McNeil et al. 1995). However, as of yet there are no identified ligands of receptor GCs in insects and therefore these genes still remain to be characterised.

1.2.3 Phosphodiesterases

Cyclic nucleotide phosphodiesterases (PDEs) were first discovered by Butcher and Sutherland (1962). They comprise a superfamily of metallophosphohydrolases that act as negative regulators of cyclic nucleotide signalling and are therefore pivotal in maintaining the role of cyclic nucleotides in cellular function (Omori and Kotera 2007). PDE families can be specific to either cGMP or cAMP, or can exhibit a dual specificity for both, and function by cleaving the phosphodiester bonds of either cyclic nucleotide in order to produce a 5'-nucleotide. To date, a number of PDE genes and their numerous splice variants have been identified in both vertebrates and invertebrates, each with unique kinetic and regulatory properties. Over the years, it has become clear that any single cell type can express multiple different PDEs and that the nature and localisation of these PDEs is likely to be a major regulator of local cGMP or cAMP concentrations in each cell (Francis et al. 2001). Cyclic nucleotides are generated in a limited space within the cell, in close proximity to both their activating enzymes and their downstream effectors. It is suggested therefore that the localisation of each PDE acts to control the specific 'pools' of cyclic nucleotides within each cell, i.e. to prevent cyclic nucleotides from spreading to inappropriate areas of the cell, or to regulate the levels of cyclic nucleotides able to activate the downstream effectors in close proximity (Bender and Beavo 2006; Omori and Kotera 2007). PDEs themselves are regulated by diverse biochemical reactions including phosphorylation/dephosphorylation, allosteric binding of cGMP or cAMP, binding of Ca^{2+}/calmoldulin and various protein-protein interactions (Bender and Beavo 2006; Omori and Kotera 2007).

There are six PDEs expressed in *Drosophila*, *Drosophila melanogaster* (Dm) PDE1, DmPDE4 (Dunce), DmPDE6, DmPDE8, DmPDE9 and DmPDE11 (Day et al. 2005;

Davies and Day 2006). Of these, Dunce is the most widely characterised and acts as a cAMP-specific PDE with a role in learning and memory functions (Davis and Dauwalder 1991). Biochemical characterisation of the remaining *Drosophila* PDEs has demonstrated that DmPDE1 and DmPDE11 are dual-specificity PDEs, whereas DmPDE6 is specific to cGMP (Day et al. 2005). Interestingly, despite its dual-specificity, DmPDE11 has been demonstrated to exhibit the highest specificity for cGMP of all other Drosophila PDEs (K_m: 6 $\pm$ 2μM). Unfortunately, due to the lack of appropriate antibodies, DmPDE8 and DmPDE9 have yet to be fully characterised (Day et al. 2005). Expression studies have shown that all DmPDEs are expressed in the adult head and body and, interestingly, DmPDE6, DmPDE8 and DmPDE11 are all significantly enriched in the Malpighian tubules, an important cGMP signalling tissue (Day et al. 2005).

1.2.4 Cyclic nucleotide-gated channels

Cyclic nucleotide gated (CNG) channels were first discovered in the plasma membrane of retinal photoreceptors (Fesenko et al. 1985). CNG channels are activated by cyclic nucleotides that bind to probably four intracellular sites showing differing ligand selectivity towards cyclic AMP and cyclic GMP (Kaupp and Seifert 2002). Activation of CNG channels results in the fluctuation of different ions, such as sodium, potassium and calcium, within the cell. CNG channels are highly sensitive to changes in cytosolic cyclic nucleotide concentration and therefore provide a powerful route by which cyclic nucleotide signalling pathways regulate cellular processes (Zagotta and Siegelbaum 1996).

In vertebrates, the activation of CNG channels has mainly been associated with olfactory and visual processing, where their role has been widely characterised (Yau and Baylor 1989; Zufall et al. 1994). Additionally, CNG channels have been associated with the regulation of synaptic plasticity and neuronal pathfinding, however their role in these processes is not as well understood (Zagotta and Siegelbaum 1996; Kaupp and Seifert 2002). Unfortunately, very little is known about the *in vivo* function of CNG channels in invertebrates, suggesting a wider spectrum of action. In *Drosophila*, there are at least four genes encoding CNG channels, including *cng*, originally shown to be expressed in the eye and the antennae; *cngl*, expressed in the neuronal cells and mushroom bodies in the brain; *CG3536* and *CG17922* (Miyazu et al. 2000).

1.2.5 cGMP-dependent protein kinases

cGMP-dependent kinases (cGKs) belong to a large superfamily of protein kinases. They function by catalysing the transfer of γ-phosphoryl group of ATP to the hydroxyl group of serine, threonine or tyrosine residues of acceptor substrate proteins and, as a result, are able to regulate the activity of numerous proteins (Vaandrager and de Jonge 1996). In general, cGKs can be described as homodimers, with each subunit containing an N-terminal domain that mediates dimerisation and protein-protein interactions; a regulatory domain that contains two identical cGMP binding sites; and a kinase domain responsible for substrate phosphorylation (Takio et al. 1984).

In *Drosophila*, there are two genes encoding confirmed cGKs, *dg1* and *dg2 (foraging, for)* and another putative cGK gene – *CG4389* (Kalderon and Rubin 1989; Davies 2000). At present, there is no functional information available for the *CG4289*-encoded product, however the kinases encoded by *dg1* and *dg2* are quite widely characterised. DG1 and DG2 were first discovered serendipitously as part of a screen to identify cAMP-dependent protein kinases (Kalderton and Rubin 1989). In this study, expression data indicated that DG1 was a head-specific kinase, whereas DG2 was seen in both the head and body of adult flies. Subsequent studies have revealed that *dg1* encodes an 84kDa protein, with *bona fide* cGK activity, which is expressed in the optic lobes and proximal cortex (Foster et al. 1996). It has also been demonstrated in recent years that DG1 is expressed in the Malpighian tubules and appears to be cytosolic in nature, with some association at the basolateral membrane (Dow et al. 1994a; MacPherson et al. 2004b). Interestingly, overexpression studies have implicated a role for DG1 in cGMP-mediated fluid transport (MacPherson et al. 2004b).

DG2 has been widely characterised and has traditionally been implicated as having a role to play in *Drosophila* feeding behaviour (Osborne et al. 1997). *dg2* is a complex gene, comprising several exons encoding a number of major transcripts, which encode proteins of differing sizes (Kalderton and Rubin 1989; http://flybase.bio.indiana.edu/cgi-bin/uniq.html?FBgn0000721%3Efbtr). A role for the *dg2* gene was first assigned after studies into larval food-search behaviour identified it as the source of the naturally occurring rover/sitter *foraging* polymorphism, where rovers (for^R) are seen to be much

more active than sitters (*for*^{S}) when searching for food (de Belle et al. 1989; 1993). Subsequent phosphorylation studies in *for* mutants revealed that a ~10% reduction in cGK activity may account for the *for*^{S} phenotype in larvae (Osborne et al 1997). Interestingly, the *for*^{S} allele has been shown to confer an epithelial phenotype in the Malpighian tubules, where *for*^{S} flies were shown to demonstrate a hypersensitivity to capa-1, a nitridergic neuropeptide known to stimulate the cGMP signalling pathway (MacPherson et al. 2004a). Although all of the studies mentioned above have identified an important role for the *dg2* gene, the proteins encoded by the gene have only recently been investigated. In a recent study, the *in vivo* roles of the transcripts of *dg2* were examined. Results demonstrated that the two major transcripts of *dg2,* DG2P1 and DG2P2, encode *bona fide* cGKs. Additionally, cGK activity in adult flies appears to be enriched in the heads and Malpighian tubules, further suggesting an important renal function for cGKs. Targeted expression of DG2P1 and DG2P2 to the Malpighian tubules showed that DG2P1 appears to localise to the apical membrane of tubule principal cells. Conversely, DG2P2 was shown to be expressed both apically and basolaterally, again only in the tubule principal cells (MacPherson et al. 2004b).

To date, there are no documented phosphorylation targets of cGKs in *Drosophila*, however it appears that both DG1 and DG2 kinases may have important roles to play in the Malpighian tubule. Interestingly, the differential localisation of each cGK or cGK isoform suggests that each has a distinct role from one another, and are probably controlled by different sources of cGMP.

1.3 The *Drosophila* Malpighian tubule

1.3.1 Introduction

Insect Malpighian tubules have been extensively studied in terms of both morphology and function over the years and as a result more is known about this tissue than any other animal epithelia (Dow and Davies 2001). The Malpighian tubule can be defined as a

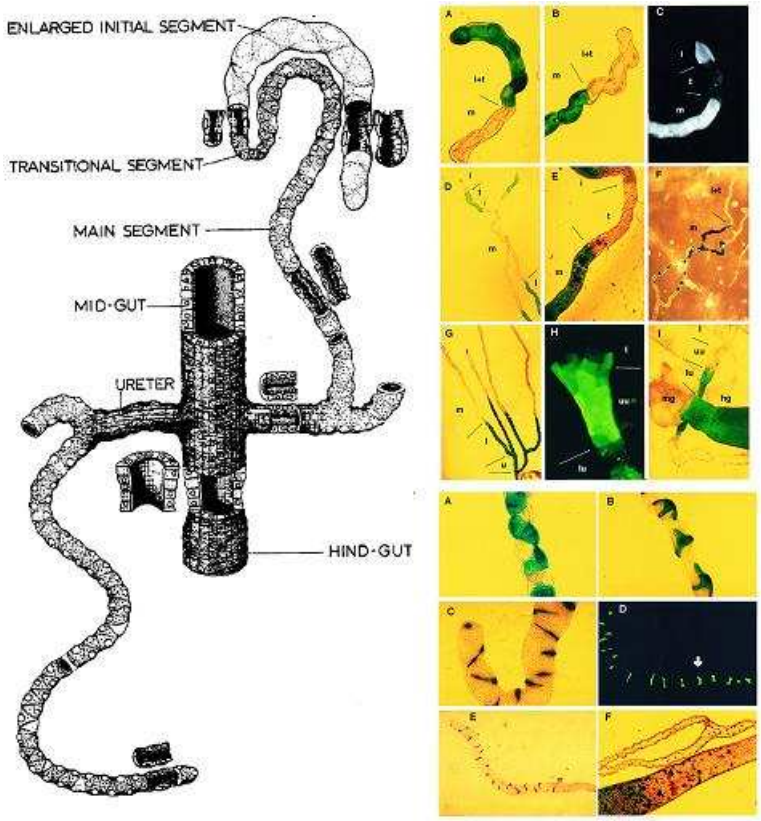


Figure 1.6 – The *Drosophila melanogaster* Malpighian tubule. Left panel: morphology of the Malpighian tubule, showing each of the distinct segments of the tubule as reported by Wessing and Eichelberg (1978). Right panel (top): tubules of enhancer trap fly lines, showing each of the different regions of the tubule. Right panel (bottom): tubules of enhancer trap fly lines, showing various pictures of different cell types within the tubules. Top two pictures show the principal and stellate cell types of the main segment of the tubule (Sozen et al. 1997).

fluid-transporting osmoregulatory epithelium that is critical for detoxification and ion homeostasis in the fly. It has emerged over the years as a potent model system for the study of the signalling and transport genes involved in epithelial fluid transport (Dow et al. 1994b).

1.3.2 Morphology

Insect Malpighian tubules are simple, free-floating, tubular epithelia, joined in pairs through short common ureters to the alimentary canal (Wessing and Eichelberg 1978). *Drosophila* tubules are amongst the smallest ever studied, measuring ~2 mM long by ~35 μM in diameter, and comprising of ~150 cells. Each fly possesses two pairs of tubules, an anterior and a posterior pair, both contributing equally to tubule function. The tubule can be divided into three segments; an enlarged initial segment, a narrower transitional segment and a main segment that leads to the ureter. The main segment is responsible for

fluid secretion and is composed of two cell-types, the columnar epithelial principal cell (type I) and the star-shaped stellate cell (type II). Other cell types, such as bar-shaped cells, are present on the initial and transitional segments (Figure 1.6; Sozen et al. 1997).

1.3.3 The Malpighian tubule and the cGMP signalling pathway

Studies have shown that the Malpighian tubule is responsible for many processes within the fly, including fluid transport, osmoregulation, detoxification and ion homeostasis. All of these processes are known to be regulated by the cGMP signalling pathway in the tubule (Davies 2006). Microarray studies have demonstrated that many genes associated with cGMP signalling are expressed in the tubule and are typically enriched in this tissue compared to the whole fly (Wang et al. 2004). For example, the tubule has been shown to express NOS and sGC, and also shows a high enrichment of the receptor guanylate cyclase Gyc76c. Additionally, three as of yet uncharacterised rGCs, CG4224, CG9873 and CG5719 are expressed in the tubule, and a putative atypical guanylate cyclase CG14885 (Wang et al. 2004). The presence of multiple activators of the cGMP pathway therefore suggests its role in regulation of a variety of physiological processes.

Similarly, as mentioned earlier in the text, the tubule has been demonstrated to express multiple cGMP effector molecules (MacPherson et al. 2004b; Wang et al. 2004; Day et al. 2005). All known *Drosophila* CNG channels, PDEs and cGKs are expressed in the tubule with particular enrichment of DmPDE11 and the cGK DG1 (Wang et al. 2004). Therefore, it would seem that the Malpighian tubule is the ideal tissue to study their effects. Fortunately, following the development of the fluid secretion assay by Dow and colleagues (1994), the tubule has proved to be a robust, quantitative phenotype with regards to the study of not only cGMP signalling, but a whole host of signalling and transport mechanisms (Dow and Davies 2006).

1.3.4 The Malpighian tubule and immunity

One of the most recent developments with regard to tubule function is the identification of the tubule as an important immune sensing tissue. Studies have shown that the tubule constitutes an autonomous immune system that is capable of sensing bacterial challenge and mounting an immune response, entirely independently of the fat body (McGettigan et

al. 2005). Previously, a role for the tubule in local epithelial AMP production has been indicated, where it was shown that diptericin, cecropin and metchnikowin levels are upregulated in the tubule in response to natural infection (Tzou et al. 2000). However, since that time it has been demonstrated the tubule is capable of activating immune mechanisms in response to septic injury, suggesting a role in systemic immunity (McGettigan et al. 2005). In this study, it was demonstrated that tubules, excised and incubated *in vitro* with *E.coli*, show an up-regulation of diptericin expression, confirming that the tubule is capable of sensing and responding to bacterial infection autonomously. Additionally, targeted over-expression of *dNOS* to the tubules (the enzyme responsible for generation of NO, a known activator of immune responses) was seen to confer increased survival to adult flies upon septic infection, thus confirming the involvement of the tubule in systemic immunity and also demonstrating its importance in immune function (McGettigan et al. 2005). In support of these data, microarray studies have also indicated the importance of the tubule in immune response (Chintapalli et al. 2007 - www.flyatlas.org). When comparing expression levels of major components of immune signalling pathways in different tissues of the adult fly, it can be seen that many components of the Imd signalling pathway are highly enriched in the tubule. For example, Relish shows an mRNA signal of 783$\pm$91 in the adult tubule, compared to an mRNA signal of 426$\pm$31 in the adult carcass (consisting of the adult fat body cells, as well as cuticle). Additionally, Relish is also highly enriched in the larval tubule (mRNA signal = 398$\pm$12) although not to the same level as the larval fat body (mRNA signal = 685$\pm$55) (*www.flyatlas.org* - search string Relish). These data therefore suggest that the tubule is very important to Imd pathway activation in the adult fly.

The involvement of the tubule in immune function is not surprising. The morphology of the tubules means that they are spread throughout the body cavity, both anteriorly and posteriorly, which suggests that they are likely to be one of the first tissues exposed to bacteria upon infection. It makes sense therefore, that the tubule may act alongside the fat body in the activation of immune response. Secondly, the tubules open into the gut lumen and are therefore in contact with a large number of microorganisms on a constant basis (Dow and Davies 2006). It is possible therefore that the tubule may act as the signalling tissue between natural infection defence mechanisms and the systemic immune system. To date, investigation into the involvement of the Malpighian tubule in immune function

is still in the early stages, however, from the evidence seen so far it appears that the tubule is a critical immune sensing tissue in *Drosophila*.

1.4 *Drosophila* as a model organism – useful genetic tools

Although traditionally associated with developmental studies, over the years *Drosophila* has become a valuable model organism for studying many physiological processes (Dow and Davies 2003). There are a number of reasons why *Drosophila* is of particular biological significance as a model organism. Firstly, *Drosophila* has a short generation time and is cheap and easy to breed in large numbers, without compromising genetic power. Secondly, *Drosophila* has been studied as a model organism for over 100 years and as a result there is a wealth of genetic markers available. Thirdly, those wishing to use *Drosophila* as their model of choice have access to a sequenced, and very well annotated, genome. Finally, a wealth of available transgenic tools has made *Drosophila* a very powerful model system for integrative organismal studies (Dow and Davies 2003).

An example of this is the adaptation of the *Drosophila* P-element by enhancer trapping, a technique that has allowed rapid identification of genetic domains and genes with expression patterns of interest (Rubin and Spradling 1983; Bellen et al. 1989). P-elements are known to form a classic transposable system whereby transposons are able to 'jump' around the genome, an effect mediated by the transposase enzyme (Robertson et al. 1988). In the enhancer trapping technique, the transposase gene in the P-element is replaced with a reporter gene, consisting of various genetic markers, downstream of a weak promoter. Flies carrying the P-element are then crossed to another fly line carrying Δ2,3 P-element, a defective transposon that can only express transposase in the germ line, and which itself is unable to move. Therefore, as the Δ2, 3 P-element flies provide a source of transposase, the reporter P-element can effectively jump around the genome in the progeny of this cross. Thus, the progeny are allowed to breed and then the subsequent progeny that have lost the Δ2, 3 P-element are selected. These flies are now carrying the reporter gene trapped in a new position (Bellen et al. 1989). This technique is extremely useful as, in a significant fraction of these enhancer trap lines, the reporter P-element may become trapped near a potentially interesting gene and may become activated to produce a similar expression pattern.

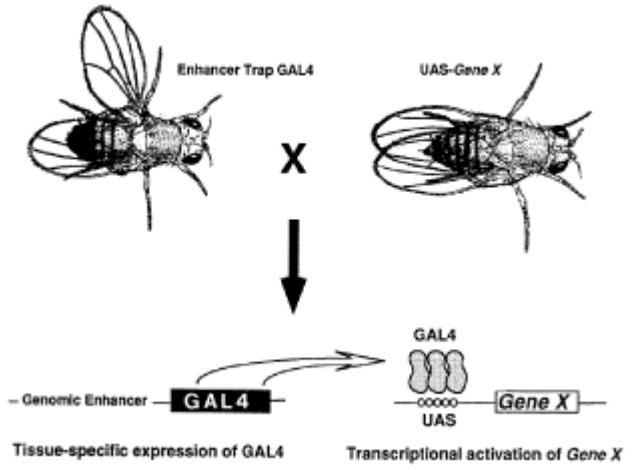


Figure 1.7 – The GAL4/UAS crossing system. In this system an enhancer-trap line expressing GAL4 in a tissue of interest is crossed to flies carrying a UAS-transgene. Expression of the transgene is subsequently driven in the GAL4 tissue in the resulting progeny (Brand and Perrimon 1993)

The genetic toolbox of *Drosophila* has evolved even further with the development of the GAL4/UAS (upstream activating sequence) system (Brand and Perrimon 1993). In this system, the reporter gene is the yeast transcription factor GAL4. This transcription factor is completely inert in the *Drosophila* genome under normal circumstances, however it is capable of driving transgenes under the control of the yeast UAS promoter (Fischer et al. 1988). Therefore, by cloning a gene of interest downstream of the UAS promoter, its expression can be activated in cells where GAL4 is present (Figure 1.7). Targeted expression of genes of interest using this system became possible after the development of an enhancer-trap GAL4 construct (pGAWB) (Brand and Perrimon 1993). Using this construct, it has been possible to develop GAL4 'driver' fly lines for any tissue or cells of interest and, as a result, an astounding array of GAL4 lines are now available for use. The advantages of this system are extensive. For example, once an appropriate GAL4 driver line has been developed, it can be maintained as a parental stock. Targeted expression of any gene can then be achieved in a tissue of interest simply by crossing this GAL4 line to the UAS-transgene fly line of choice, without the need for further enhancer trapping studies. Similarly, a UAS-transgene fly line can be kept as a parental stock without any serious loss of fitness, as there is no source of GAL4 to promote expression. This is particularly useful in fly lines where a gene is silenced using UAS-RNAi, resulting in an easily maintained and viable stock. Overall, the development of the GAL4/UAS system

has been paramount in improving the genetic tractability of *Drosophila* even further, confirming the status of *Drosophila* as a very powerful model organism.

1.5 Project Aims

In recent years, nitric oxide signalling has emerged as a key component of the immune response in *Drosophila,* using both *in vivo* and *in vitro* systems (Weiske and Wiesner 1999; Nappi et al. 2000; Foley and O'Farrell 2003). It has been suggested that NO plays some functional role in activating the Imd pathway upstream of the Imd protein and that NO may act to mediate signalling between immune tissues (Basset et al. 2000; Foley and O'Farrell 2003; Silverman 2003). The mechanism by which this occurs has not been elucidated, however, considering that the main intracellular receptor for NO is soluble guanylate cyclase, it is highly possible that cGMP signalling may play a role.

Recently, the Malpighian tubule has been demonstrated as an important immune sensing tissue (Tzou et al. 2000; McGettigan et al. 2005). Similarly, NO/cGMP signalling has long been reported as critical to normal tubule function (Davies 2006). Consequently, the tubule appears to be the ideal tissue for studying the role of cGMP in immune response. Therefore, using a combination of microarray, Q-PCR and transgenic approaches, the aim of this study was to investigate the role of cGMP signalling in immune response. This was achieved using the *Drosophila* Malpighian tubule as a model system, with particular emphasis being placed on its role in Imd pathway regulation.

Chapter 2

Materials and Methods

2.1 Drosophila melanogaster

2.1.1 *Drosophila* stocks

Table 2.1 – *Drosophila melanogaster* lines used in this study

Strain	Genotype	Description and application	Reference (if applicable)
Oregon R	Wild type	Q-PCR, survival assays.	-
Diptericin-GFP$^{\sigma}$	*w*$^{-}$; +/+; *diptGFP*	Fluorescence studies, natural infection experiments	Tzou et al. 2000
Cecropin-GFP$^{\sigma}$	*w*$^{-}$; +/+; *cecA1GFP*	Flouresence studies	As above
c42	*w*$^{-}$; +/+; c42	Tubule principal cell-specific GAL4 driver	Sozen et al. 1997; McGettigan et al. 2005
c42 (balanced)	*w*$^{-}$;*bl/Cyo*;c42	As above	-
GAL80$^{\delta}$	*w;P{tub-GAL80*^{ts}*}; TM2/TM6b*	GAL4 repressor line, inactivated after heat shock at 30 °C	(Lee and Luo 1999)
GAL80;c42	*w*$^{-}$;*P{tub-GAL80*^{ts}*}*; c42	Inducible tubule principal cell-specific GAL4 driver – used for Q-PCR and survival assays	-
UO	*UO*; +/+; +/+	Tubule principal cell-specific GAL4 driver	Terhzaz et al, in preparation
c564*	*w*$^{-}$; c564; +/+	Fat body specific driver	
UAS-*dg1*	*w*$^{-}$;+/+;UAS-*dg1*	Overexpressor of *dg1*, crossed to either c42 or c564 GAL4 drivers – used for Q-PCR, survival assays and natural infection experiments	MacPherson et al. 2004a ; 2004b
UAS-*dg1*RNAi	*w*$^{-}$; UAS-*dg1*RNAi; +/+	Knocks down *dg1* expression by RNAi – crossed to either c42 or c564 and used as above	-
UAS-*dg2P1*	UAS-*dg2P1*; +/+; +/+	Overexpressor of *dg2P1*, crossed to either c42 or c564 and used as above	MacPherson et al. 2004a; 2004b
UAS-*dg2P2*	*w*$^{-}$; UAS-*dg2P1*; +/+	Overexpressor of *dg2P2*, crossed to either c42 or c564 and used as above	As above
UAS-*dg2*RNAi	*w*$^{-}$; UAS-*dg2*RNAi; +/+	Knocks down *dg2* expression by RNAi - crossed to the GAL80;c42 driver, used as above	-
UAS-*PDE11*RNAi	*w*$^{-}$; +/+; UAS-*PDE11*RNAi	Knocks down *PDE11* expression by RNAi - crossed to the c42 GAL4 driver and used for Q-PCR and survival assays	-
UAS-GC-A	*w*$^{-}$; UAS-*GC-A*; +/+	Overexpressor of the rat ANP receptor – crossed to c42 and used for Q-PCR	(Kerr et al. 2004)
dN1-8	*w*$^{-}$; UAS-*dNOS*; +/+	*dNOS* overexpressor – crossed to UrO and used for Q-PCR	McGettigan et al. 2005
Relish E20 *	*w*$^{-}$; +/+; *relish*^{E20}, *e*$^{-}$	Homozygous Relish null mutant carrying the *ebony* marker – used for Q-PCR	Hedengren et al. 1999
Imd^{1} *	*w*$^{-}$; *pr,imd*^{1}*/CyO*; *TM3sb/GFPser*	Balanced Imd mutant carrying the *purple* marker – used for Q-PCR and to generate lines for epistatic analysis	Lemaitre et al. 1995, Georgel et al. 2001
Key^{1} *	*w*$^{-}$; *cn-bw, Key*^{1}*/ CyO*; *TM2tb/TM6e*	Balanced Kenny mutant carrying the *cinnabar-brown* marker – used to generate lines for epistatic analysis	Rutschmann et al. 2000
UAS-*imd* *	*w*$^{-}$; UAS-*imd/CyO*; *TM3sb/TM6e*	Balanced Imd overexpressor – used to generate lines for epistatic analysis	Georgel et al. 2001
UAS-*relish* §	*w*$^{-}$; UAS-*relish*-HIS/*CyO*; *TM3sb/TM6e*	Balanced Relish overexpressor tagged with 6xHIS – used to generate lines for epistatic analysis	Hedengren et al. 1999; Stoven et al. 2000; 2003
Imd^{1};c42	*w*$^{-}$; *pr,imd*^{1}*/CyO*; c42/*TM3sb*	Tubule principle cell-specific GAL4 driver in an *imd* mutant background – crossed to UAS-*dg1* and used for epistatic analysis (Q-PCR)	-
Key^{1};c42	*w*$^{-}$; *cn-bw, Key*^{1}*/ CyO*; c42/*TM2tb*	Tubule principle cell-specific GAL4 driver in an *Kenny* mutant background	-
UAS-*imd*;c42	*w*$^{-}$; UAS-*imd/CyO*; c42/*TM3sb*	Tubule principle cell-specific GAL4 driver overexpressing Imd	-
UAS-*relish*;c42	*w*$^{-}$; UAS-*relish*-HIS/*CyO*; c42/*TM3sb*	Tubule principle cell-specific GAL4 driver overexpressing Relish – crossed to cGK transgenic lines and used for epistatic analysis (ICC)	-

$^{\sigma}$ – Kind gift from Professor J. L Imler, University of Strasbourg, France; $^{\delta}$ – Bloomington Stock Centre; * - Kind gift from Professor S Kurata, Tohoku University, Sendai, Japan; § - Kind gift from Professor D Hultmark, University of Umea, Sweden.

The various *Drosophila* lines used in this study and their genotypes and application are listed in Table 2.1. Unless otherwise stated, fly lines are lab stocks. Relavent references for these lines are also listed where applicable.

2.1.2 *Drosophila* rearing

Flies were reared in vials on standard *Drosophila* medium (appendix 1) at 22-25°C in a 12 h: 12 h light: dark cycle. If large quantities were required (>100), flies were reared in large bottles on standard medium.

2.1.3 Dissection of *Drosophila* tissues

For dissection of tubule and midgut samples, 7-day old adult flies were anesthetized on ice before acute dissection in sterile Schneider's media (Invitrogen). For dissection of fat body, late third-instar larvae were dissected live in sterile Schneider's medium. For RNA samples, approximately 50 tubule pairs (25 flies) or fat body from approximately 10 larvae were dissected for each sample. Where appropriate, samples were then incubated in 3 ml of sterile Schnieder's medium containing the appropriate concentration of either cGMP, dibutyryl-cGMP, cAMP, SNAP, ODQ (all Sigma), or PGN(-) (Invivogen) as stated throughout the text.

2.2 RNA extraction

RNA extraction was carried out using the QIAGEN® RNeasy® Mini kit and a QIAGEN® RNase-free DNase set as according to the manufacturers' instructions. RNA was typically eluted in 25µl RNase-free water and quantified as described in Section 2.5.

2.3 First strand cDNA synthesis

First strand cDNA synthesis was carried out using Superscript ™ II Reverse Transcriptase (Invitrogen). For each 20 µl reaction, 1 µl of $Oligo(dT)_{12-18}$ (500 µg/ml), 1 – 10 µl total RNA (up to 1 µg) and 1 µl of dNTP mix (final concentration 200 µM each) were added to a nuclease-free PCR tube. Each reaction was then made up to a

volume of 12 µl with RNase-free water and incubated at 65 °C for 5 min followed by a quick chill on ice. The contents of each tube were then collected by brief centrifugation, followed by the addition of 4 µl of 5x First strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 2 µl of 0.1 M DTT and 1 µl of RNaseOUT ™ (40 units/µl). Each reaction was then mixed briefly and incubated at 42 °C for 2 min. 1 µl of Superscript ™ II Reverse Transcriptase was then added to each tube and the contents mixed by gentle pipetting. Each reaction was then incubated at 42 °C for 50 min, followed by heat inactivation of the enzyme at 70 °C for 15 min. cDNA concentration was then quantified as described in Section 2.4. Unless otherwise stated, all components used in this reaction were purchased from Invitrogen.

2.4 Preparation of genomic DNA

For PCR procedures that required moderate amounts of genomic DNA, the Berkeley *Drosophila* Genome Project Quick Fly Genomic DNA prep, by E. Jay Rehm, was used (see http://www.fruifly.org/about/methods/inverse.pcr.html). Briefly, 30 anaesthetised flies were collected in a 1.5 ml eppendorf tube and briefly frozen at -70°C. Flies were then ground in 200 µl of Buffer A (100 mM Tris-HCl [pH 7.5], 100 mM EDTA, 100 mM NaCl, 0.5 % [w/v] SDS) using a disposable tissue grinder (Kontes). An additional 200 µl of Buffer A was then added and grinding continued until only cuticles remained. The suspension was then incubated at 65°C for 30 min. 800 µl of LiCl/KAc solution (1 part 5 M KAc: 2.5 parts 6 M LiCl) was then added and the resulting solution incubated on ice for at least 10 min. Samples were then centrifuged at 13,000 g at for 15 min at room temperature. 1 ml of the supernatant was then transferred into a new tube, avoiding floating material. 600 µl of isopropanol was then added, the solution mixed, and centrifuged at 13,000 g for 15 min at room temperature. The supernatant was then carefully aspirated, and the DNA pellet washed with 70 % ethanol (v/v) in H_2O. The washed pellet was air-dried and then resuspended in 150 µl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Genomic DNA samples were then stored at –20°C.

2.5 Quantification of nucleic acids

Nucleic acid concentrations were measured at 260 nm (A_{260}) using a NanoDrop 1000™ (Thermo Scientific) according to manufacturers' instructions. Readings were

zeroed with the solution in which the samples had been diluted and nucleic acid concentrations were displayed as ng/µl. A ratio of A_{260}/A_{280} provided an estimate of nucleic acid purity. Values of 1.8 for DNA and 2.0 for RNA indicated pure preparations.

2.6 Oligonucleotide synthesis

Oligonucleotides were synthesised by the MWG Biotech custom primer service on a 0.01 µmol scale, purified by High Purity Salt Free (HPSF®) technology, and their quality assessed by Matrix Assisted Laser Desorption Ionisation - Time of Flight (MALDI-TOF) analysis. Oligonucleotides were received as a lyophilised pellet, resuspended in H_2O to a stock concentration of 100 µM, and further diluted with H_2O to a working concentration of 6.6 µM. All primers were stored at -20°C. A list of all the primers used in this study is provided in appendix 2.

2.7 Polymerase chain reaction (PCR)

2.7.1 Standard PCR using *Taq* DNA polymerase

Standard PCR protocols were used in the everyday amplification of DNAs. For a small number of PCRs, reactions were set up according to manufacturers' instructions using a pre-aliquoted PCR master mix containing Thermoprime Plus DNA polymerase and 2.5 mM $MgCl_2$ (Abgene). For each reaction, 2 µl each of forward and reverse primers (final concentration 260 nM each) and 1 µl of template DNA (up to 1 ng of plasmid DNA, 100 ng of genomic DNA or 500 ng of cDNA) or dH_2O (no template control) were added to the master mix to make up a final volume of 50 µl.

For large numbers of PCRs, reactions were set up in 0.2 ml thin walled PCR tubes to contain 5 µl of 10x PCR reaction buffer containing 2.5 mM $MgCl_2$, 1 µl of dNTP mix (final concentration of 200 µM each), 2 µl each of forward and reverse primers (final concentration 260 nM), 0.5 µl of *Taq* DNA polymerase (1.25 Units) and 1 µl of template DNA (concentrations as before) or dH_2O (control). Each reaction was then made up to a final volume of 50 µl with dH_2O. All components were purchased from Invitrogen. Cycling was then performed using either a Hybaid OmnE, Hybaid PCR

Sprint or Hybaid PCR Express-Gradient thermocycler. A typical cycling procedure is described in Table 2.2.

Table 2.2 – Typical cycling procedure for DNA amplification using *Taq* DNA polymerase.

Step	Temperature	Time		Comments
Initial Denaturation	94 °C	3 min		To ensure template denaturation
Denaturation	94 °C	30 sec	25 – 30 cycles	-
Annealing	50 – 60 °C	30 sec		Temperature is set depending on the melting temperature of the primers used; typically ~5 °C lower than T_m
Extension	72 °C	30 sec – 5 min		30 sec extension for each 500 bp of DNA to be amplified
Final Extension	72 °C	5 min		-

2.7.2 PCR using *pfu* DNA polymerase

Pfu DNA polymerase (Promega) is a thermostable enzyme from *Pyrococcus furiosus* which catalyses DNA dependent polymerisation of nucleotides into duplex DNA in the 5'→3' direction, exhibits 3'→5' exonuclease (proofreading) activity and is used for PCR reactions requiring high fidelity synthesis such as expression constructs. The reaction mix was set up as follows; 5 µl of 10x *Pfu* DNA polymerase buffer (Promega), 1 µl dNTPs (final concentration 200 µM each - Invitrogen), 2 µl each of forward and reverse primers (final concentration 260 nM each), 1 µl of DNA template (concentrations as before) or dH_2O (control), 0.5 µl *Pfu* DNA polymerase (1.25 Units - Promega), made up to a final volume of 50 µl with dH_2O. Temperature cycling for a typical *pfu* DNA polymerase PCR was carried out as described in Table 2.2, however as *pfu* exhibits a lower extension rate compared to *Taq* DNA polymerase, extension times were increased to 1 min for every 500 bp of DNA to be amplified.

For all PCR protocols, DNAs were separated following amplification by agarose gel electrophoresis (Section 2.7.5).

2.7.3 Reverse-transcription (RT)-PCR

RT-PCR was carried out in two steps in order to facilitate the amplification of a number of different sequences from the same cDNA sample. For this approach, total

RNA was extracted from tissues of interest as described in Section 2.2. cDNA was then synthesised from each sample using Superscript™ II Reverse Transcriptase (Section 2.3). Sequences of interest were then amplified from the resultant cDNA using a standard PCR protocol (Section 2.7.1). Prior to use, the concentration of cDNA was quantified as described in Section 2.5 and equal amounts of cDNA added to each PCR reaction. Additionally, in order to control for possible genomic contamination, primer pairs were designed to span intron/exon boundaries of each gene of interest. Following amplification, DNAs were separated by electrophoresis on a 0.1 % agarose-TBE gel (Section 2.7.5).

2.7.4 Quantatitive (Q)-PCR

To quantify levels of expression of genes of interest, Q-PCR was carried out using the fluorescent double-stranded DNA dye DyNAmo™ SYBR® Green (Finnzymes). As with RT-PCR, total RNA was extracted from tissues of interest as described in Section 2.2. cDNA was then synthesised from each sample using Superscript™ II Reverse Transcriptase (Section 2.3). Prior to setting up each reaction, gene-specific primers were designed to generate an optimal PCR product of <500 bp (Appendix 2). In addition, primers were designed, where possible, to span intron/exon boundaries of each gene of interest in order to control for possible genomic contamination. For each cDNA sample, reactions were set up in triplicate to contain 25 µl 2x SYBR Green Master Mix (*Tbr* DNA polymerase, SYBR Green I, optimised PCR buffer, 5mM $MgCl_2$, dNTP mix), 2 µl each of primers (0.3 µM final concentration) and 1 µl of template cDNA (up to 500 ng), made up to a final volume of 50 µl with dH_2O. Additionally, in order to facilitate quantification of each gene of interest relative to a standard reference gene, reactions were also set up in triplicate for each cDNA sample containing primers specific for the *rp49* gene, known to encode a ribosomal protein of standard expression.

Reactions were set up on ice using optical grade PCR strips (MJ Research) alongside two blanks (1 x SYBR Green Master Mix), primer-only controls (set up in duplicate for each set of primers used) and a range of external standards (in duplicate) for each gene containing 10^{-1} – 10^{-7} ng of template amplicon DNA (obtained from PCR amplification). Cycling was then performed using an Opticon™ 3 thermal cycler according to the protocol described in Table 2.3.

Table 2.3 – Typical cycling conditions for Q-PCR

Step	Temperature	Time		Comments
Initial Denaturation	95 °C	10 min		To ensure template denaturation
Denaturation	95 °C	20 s	35 – 45 cycles	-
Annealing	55 °C	20 s		Temperature is set depending on the melting temperature of the primers used; typically ~5 °C lower than T_m
Extension	72 °C	5 – 20 s		5 s per 100 bp of product
Data Acquisition	-	-		Fluorescence data collection is performed after each cycle
Final Extension	72 °C	5 min		-
Melting Curve	60 – 90 °C	1 s hold per 0.3 °C		Used to check the specificity of the amplified product

Following amplification, each Q-PCR reaction was analysed using Opticon^{TM} 3 software as according to the manufacturers' instructions. Absolute quantification of gene expression was calculated using a standard curve whereby threshold cycle C(t) values of each unknown sample were compared to the C(t) values of gene standards of known DNA concencentrations. Specificity of each amplified product was also analysed using melting curve data.

Following absolute quantification of DNA in each reaction, relative quantification of each sample was determined by calculating a ratio of target gene DNA concentration to *rp49* DNA concentration. Results were then plotted as means $\pm$ SEM (where control = 1) using GraphPad Prism 4.0 software. Statistical significance of data was determined by 2-way ANOVA and/or Student's *t* tests where appropriate.

2.7.5 Agarose gel electrophoresis

DNAs were separated in 1 % agarose in 0.5x TBE [90 mM Tris, 90 mM boric acid (pH 8.3), 2 mM EDTA] containing 0.1 μg/ml EtBr as described in Sambrook and Russell, 2001), using 0.5x TBE as the electrophoresis buffer. Sizes were compared to a 1kb ladder (Invitrogen). Prior to loading, 6x loading dye [0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol, 30 % (v/v) glycerol in water] was added to the samples to a final 1x concentration of loading dye in the sample.

2.7.6 PCR purification

DNA bands were excised from agarose gels using a clean scalpel blade and the DNA extracted using the QIAGEN® QIAquick Gel Extraction Kit according to the manufacturers' instructions. Alternatively, PCR products were purified directly using the QIAGEN® QIAquick PCR purification kit according to instructions. DNA was typically eluted in 20 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) and quantified as described in Section 2.5.

2.8 DNA Cloning

2.8.1 *E.coli* strains and plasmids

Listed below are the *E.coli* strains and plasmids used in this study (Table 2.4).

Table 2.4 – *E.coli* strains and plasmids

E.coli	
Strain	**Genotype**
DH5α™ subcloning efficiency competent cells (Invitrogen)	(F^- ϕ80d*lac*Z ΔM15, Δ(*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *end*A1, *hsd*R17 (r_K-,m^K+), *pho*A, *sup*E44,λ^-, *thi*-1, *gyr*A96, *rel*A1).
Plasmids	
Name	**Purpose**
pGL3-Basic Vector (Promega)	Used to clone AMP promoter sequences of interest upstream of the luc^+ luciferase gene. Also contains the Amp^R gene to confer ampicillin resistance when transformed into *E.coli.*
pAc5.1/V5-His/lacZ (Invitrogen)	Plasmid expressing the *lacZ* β-galactosidase gene. Co-transfected alongside AMP promoter/pGL3 constructs in order to determine transfection efficiency. Also contains the Amp^R gene to confer ampicillin resistance when transformed into *E.coli.*

2.8.2 DNA constructs generated for this study

Table 2.5 lists the DNA constructs generated for this study. Constructs were made according to the protocols listed in Sections 2.8.3 – 2.8.8. DNA inserts were generated by *pfu* DNA polymerase PCR (Section 2.7.2) using forward and reverse primers encoding appropriate restriction sites for ease of cloning. The primer sequences used for this study, including restriction sites, are described in Appendix 2.

Table 2.5 – DNA constructs generated for this study

Construct	Method of cloning	Purpose
Dipt-pGL3	Promoter sequence of diptericin cloned upstream of *luc*$^+$ in the multiple cloning region of pGL3 between the *KpnI* and *HindIII* restriction sites	Measurement of diptericin expression by luminescence in *Drosophila* S2 cells
AttD-pGL3	Promoter sequence of attacin D cloned upstream of *luc*$^+$ in the multiple cloning region of pGL3 between the *KpnI* and *BglII* restriction sites	Measurement of attacin D expression by luminescence in *Drosophila* S2 cells
CecA1-pGL3	Promoter sequence of cecropin A1 cloned upstream of *luc*$^+$ in the multiple cloning region of pGL3 between the *KpnI* and *HindIII* restriction sites	Measurement of cecropin A1 expression by luminescence in *Drosophila* S2 cells

2.8.3 Restriction digests

Restriction digests were set up to contain 10 µl of the appropriate 10 x Buffer for the restriction enzyme(s) used, bovine serum albumin (BSA; final concentration of 100 µg/ml, only used if necessary), up to 1 µg of template DNA and the appropriate restriction enzyme(s) (10 Units/µl) at a ratio of 1 Unit of enzyme/µg of DNA. Each reaction was then made up to 100 µl with dH_2O and incubated for 1-2 h at 37°C. Where a double digest was required but enzymes were not compatible to use in the same buffer, a single digest was first of all performed and the resultant DNA product purified using a QIAGEN® QIAquick PCR Purification kit (Section 2.7.6). Purified DNA was then eluted in an appropriate volume of dH_2O and another digest performed with the second enzyme and appropriate buffer as described above. Following each digest, samples were purified as described above and quantified as described in Section 2.5. For this study, all restriction enzymes and corresponding buffers used were purchased from New England Biolabs.

2.8.4 DNA ligation

For cloning DNA inserts into vectors, the vector and the DNA insert were first digested with the appropriate restriction enzymes and purified as described in section 2.8.3. Ligation reactions were then carried out using the Roche Rapid DNA Ligation Kit according to the manufacturers' instructions. For each reaction, a molecular ratio of 3:1 insert: vector was used (typically 50-100 ng of vector). Ligations were

incubated for 5 - 30 min at room temperature before transformation into competent *E.coli.*

2.8.5 Transformation into *E.coli*

Plasmids were transformed into DH5α^{TM} subcloning efficiency chemically competent cells (Invitrogen) by the addition of 50-100 ng of plasmid to 50 µl of cells on ice. Samples were then incubated on ice for 15 min followed by a heat-shock at 37 °C for 30 s. Following heat-shock, samples were transferred back to ice for a further 2 min, and 950 µl of L-broth added (appendix 1). Each sample was then incubated at 37 °C for 30 min to allow expression of the *amp^R* gene. 100 µl of each transformation was then spread onto L-Agar plates (appendix 1) containing 100 µg/ml ampicillin and incubated overnight at 37 °C.

2.8.6 Identification of positive clones

2.8.6.1 Plasmid selection

Each plasmid used in this study contained the ampicillin resistance gene (*amp^R*) encoding β-lactamase. Therefore, transformed *E.coli* containing the plasmid of interest were selected for by their ability to grow in the presence of 100 µg/ml ampicillin when cultured in L-Broth media or on L-Agar plates. Ampicillin (Sigma) was made as a 100 mg/ml stock solution (w/v) in 50% H_2O, 50% ethanol) and stored at -20°C.

2.8.6.2 Diagnostic PCR

To identify the presence and orientation of a DNA insert in a vector, bacterial colonies could be tested directly using PCR. Reactions were set up according to the protocol described in Section 2.7.1 using one primer that bound to the insert and one primer that bound within the vector (facing into the cloning site). For DNA template, selected colonies were touched with a sterile pipette tip, which was then used to pipette the PCR solution up and down.

2.8.6.3 Diagnostic restriction digest

For diagnostic restriction digests, selected colonies were grown overnight at 37 °C in 5 ml of L-broth supplemented with 100 µg/ml ampicillin. Plasmid DNA was then isolated from each culture as described in Section 2.8.7 and quantified as described in section 2.5. Isolated plasmids were then digested in order to determine the successful insertion and orientation of the DNA insert into the vector. To do this, digestion sites were identified in each construct both within the DNA insert and within the plasmid that, once digested, would result in DNA products of known size. Digestions were carried out as described in Section 2.8.3, however the total volume of each reaction was reduced to 10 µl. The volumes of the components of each digest were also adjusted accordingly. Following digestion, DNA was separated in each sample by agarose gel electrophoresis (Section 2.7.5).

2.8.7 Isolation of plasmid DNA

Small scale plasmid DNA preparation was carried out using the QIAGEN® Qiaprep Spin Miniprep kit. Large scale preparation for germline transformation and cloning was carried out using the QIAGEN® Qiagen Plasmid Maxi, Endofree Maxi or the Hi-Speed™ Plasmid Maxi kit according to the manufacturers' instructions.

2.8.8 Automated DNA sequencing

Automated sequencing was performed at the Glasgow University Molecular Biology Support unit (MBSU). Automated sequencing at the MBSU was performed as a single-stranded reaction with template and primer supplied at 1 µg and 3.2 pmol, respectively, with a PCR mix containing fluorescently labelled dideoxynucleotides. Samples were run on an agarose gel with the nucleotides being detected on an ABI automated DNA sequencer. Analysis was performed using an Applied Biosystems automated sequence analysis programme and the sequences were down-loaded from the server onto Editview (version 1.0, free DNA sequencing software from Perkin Elmer) and further analysed.

2.9 *Drosophila* S2 cell culture

2.9.1 Passaging of S2 cells

Drosophila S2 cells (Invitrogen) were maintained in Complete Schneider's Medium (Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated Foetal Bovine serum) (CSM) at a temperature of 28 ^{o}C. Cells were typically kept in a total volume of 15 ml in 75cm^3 flasks. For general maintenance, cells were passaged at a density of 10^7 cells/ml. To do this, cells were resuspended by gentle pipetting and then diluted 1:2.5 by adding 6 ml of cells into 9 ml of fresh CSM.

2.9.2 Transient transfection of S2 cells

Transient transfection was carried out in tissue culture six-well plates. 24 hours before transfection 6 x 10^6 cells in a volume of 3 ml were seeded into individual wells. For each 600 µl transfection, 19 µg of each plasmid DNA and 36 µl $CaCl_2$ (2 M - Invitrogen) were added to a sterile 1.5 ml eppendorf tube and made up to a total volume of 300 µl with dH_2O. This was mixed well and then added drop-wise over 1-2 min to 300 µl of 2 x Hepes buffered saline (HBS – 50 mM Hepes, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 7.1; Invitrogen) with continuous mixing. Each 600 µl reaction was then left to precipitate for 30 min at room temperature before being added drop-wise to the seeded S2 cells whilst swirling continually to mix. Cells were then incubated for 16 – 24 hr at 28 ^{o}C. Following incubation, cells were resuspended by gentle pipetting and transferred to a 15 ml falcon tube. Each sample was then pelleted by centrifugation at 1500g for 1 min at room temperature and resuspended in 3 ml fresh CSM to wash. This step was repeated twice more before cells were resuspended in 3 ml of CSM and returned to the same six-well plate. If a plasmid encoding a metal inducible promoter was used, protein expression was induced in each 3 ml culture by the addition of 15 µl of 100 mM $CuSO_4$ and expression was allowed to proceed for 40-42 hours. Cells were then incubated with 100 µM di-butyryl-cGMP (Sigma) and/or 10 µg/ml lipopolysaccharide (LPS - Calbiochem) for 3 hr before harvest. Cells were then harvested by centrifugation at 1500g for 1 min, washed once in phosphate-buffered saline (PBS – 135 mM NaCl, 1.3 mM KCl, 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4 [pH 7.4]) and pelleted by centrifugation as before. 100 µl of room temperature Reporter Lysis Buffer (RLB – Promega) was then added to each pellet and cells lysed using a single freeze-thaw cycle. Each cell lysate was then mixed thoroughly and centrifuged

at 13,000g for 5 min at 4 °C. The supernatant of each sample was then transferred to a microcentrifuge tube and mixed thoroughly by pipetting. Cell lysates were then used for further analysis as described in Sections 2.10 and 2.11.

2.10 β-galactosidase assays

In order to account for discrepancy in both cell numbers and transfection efficiency, each cell lysate was assayed for levels of β-galactosidase activity as well as luminescence. For this assay, β-galactosidase activity was measured using an Invitrogen β-gal assay kit as according to manufacturers' instructions. Briefly, 5 µl of cell lysate was added to a sterile microcentrifuge tube and made up to 30 µl using dH_2O. To this, 70 µl of ortho-nitrophenyl-β-D-galactopyranoside (ONPG – stock of 4 mg/ml) and 200 µl of 1x Cleavage buffer containing 200 mM β-mercaptoethanol (Sigma) were added and each sample mixed thoroughly. Samples were then incubated at 37 °C for 30 min. Each reaction was then stopped by the addition of 500 µl of STOP buffer. The absorbance of each sample was read at 420 nm using a standard spectrophotometer (CECIL CE2021 2000 Series) against a blank containing ONPG and 1x Cleavage buffer without cell lysate. β-galactosidase activity was then calculated for each sample using the equation below;

$$\text{nmoles of ONPG hydrolysed} = \frac{(OD_{420})(8x10^5 nanolitres)}{(4500nl/nmoles-cm)(1cm)}$$

where;
4500 = extinction coefficient
1cm = path length
8×10^5 nl = total volume of the reaction

2.11 Luciferase assays

Luciferase assays were carried out using a Promega Luciferase Assay System kit as according to the manufacturers' instructions. For each sample, 20 µl cell lysate was added to 100 µl Luciferase Assay Reagent and transferred to a luminometer tube. Luminescence was then detected in each sample using a standard manual luminometer

programmed to perform a 2 second measurement delay followed by a 10 second measurement read. Luminescence counts were then normalised for each sample by comparison to corresponding β-galactosidase expression levels assayed from same cell lysate (Section 2.10) and expressed as counts/nmole of hydrolysed ONPG.

2.12 cGMP-dependent protein kinase assays

The cyclic GMP-dependent protein kinase activity of Malpighian tubules was ascertained by using direct measurement of radiolabelled phospho-transfer to a short peptide sequence substrate (MacPherson et al. 2004b). Approximately 400 tubules per sample were dissected and homogenised on ice in 20 µl of homogenisation buffer (20 mM Tris [pH 7.5], 250 mM sucrose, 2 mM EDTA, 100 mM NaCl, 50 mM β-mercaptoethanol, 1:100 dilution of protease inhibitor cocktail (Sigma)). Protein concentration of each sample was then determined by Bradford assay (Section 2.13). Two stock solutions of kinase assay buffer were prepared, with and without 1 µM cGMP. This comprised 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM EGTA, 10 mM magnesium acetate, 1 nM PKA inhibitor (TYADFIASGRTGRRNAI-NH_2), 20 µM ATP, 1 mM zaprinast, 1 µM sildenafil, 1 mM DTT, 0.2 µg/ml GLASS-tide (RKRSRAE, a heptapeptide cGK-specific substrate, Calbiochem), 0.5-2 µl of [γ-^{32}P] ATP (370 MBq/ml, to an approximate specific activity of 4000 cpm/pmol ATP).

For each reaction, 40 µl reaction buffer was added to a 5 µl (approximately 30 µg of protein) tubule sample. This was carried out with both cGMP-containing (+cGMP) and cGMP-absent (-cGMP) buffer. Sample blanks were generated using 40 µl reaction buffer and 5 µl of homogenisation buffer. Reactions were incubated for 30 min at 30 °C, after which 35 µl of each sample was spotted onto individual squares of P81 paper (Whatman). These squares of paper are referred to as reaction samples. In order to determine the specific activity of the radiolabelled ATP at the end of the reaction, several reactions were chosen randomly and 5 µl samples (representative of 1/9 of total counts) of each spotted onto individual squares of P81 paper ('total count'), allowed to dry and set aside.

The reaction samples were washed for 3 x 5 min in 75 mM phosphoric acid, then washed once for 15-20 s in ethanol and allowed to dry. All squares of paper, including the total count samples, were then transferred to scintillation vials, with the addition of 3 ml scintillation fluid and counted in a scintillation counter (Beckman) for 60 s. Specific activity of [γ-^{32}P] ATP was calculated (9 x mean c.p.m. of total count squares/[ATP] in reaction) and used to calculate protein kinase activity (pmol ATP min^{-1} μg^{-1} protein).

2.13 Bradford protein assay

The Bradford protein assay was used to estimate the protein concentration of different protein samples. Each assay was carried out on a 96-well plate. Eight standards of 0-5 μg of BSA in water were set up in triplicate in a 50 μl total volume. Between 1 and 3 μl of each protein sample (usually approximately 2 μg of protein) were also set up in triplicate in a final volume of 50 μl. To these were added 200 μl of a 1 in 5 dilution of Bradford reagent concentrate (Biorad). The absorbance at 590 nm of the samples were read on a standard plate reader and standard curve and protein concentrations calculated using Quanta Smart software.

2.14 Survival assays

Cultures of *E. coli*, *B. subtilis* or *P. aeruginosa* (Selectrol freeze-dried pellets, TCS Biosciences) were grown overnight in 5 ml LB-broth to stationary phase at 37 °C. Bacterial challenge was performed by pricking groups of 30 five to seven day old adult flies of the appropriate genotype (as stated throughout the text) with a thin needle (BD Microlance ^{TM} 3, 26 G x $^{5}/_{8}$) dipped in the concentrated bacterial cultures. Control experiments were carried out by mock-stabbing flies with a sterile needle. Following infection, flies were transferred into clean vials and survival monitored over several days. The percentage of survivors was then calculated for each experiment and plotted using a Kaplan-Meier survival curve (GraphPad Prism), as means ± SEM.

2.15 Natural Infection assays

2.15.1 Natural infection of *Drosophila* using *E.coli*

Prior to infection, 7-day old adult flies of appropriate genotype were incubated for 2 hr without food. Flies were then transferred into empty vials containing filter paper hydrated with 5 % sucrose solution contaminated with concentrated ampicillin-resistant *E.coli* (OD_{600} = 1; concentrated to contain ~10^{10} CFU/ml). Control flies were transferred to vials containing filter paper hydrated with 5 % sucrose solution alone. Each vial was then incubated at 25 °C for 24 hr, following which flies were dissected and assessed for either levels of bacterial clearance in the gut (Section 2.15.2) or diptericin expression levels in the tubules, either by Q-PCR (Section 2.7.4) or GFP fluorescence (Section 2.16.1).

2.15.2 Assessment of bacterial clearance

For the assessment of *E.coli* proliferation in the intestine, adult flies of the appropriate genotype were infected as described in Section 2.15.1. Following infection, the midgut of each fly was carefully dissected (10 per sample), rinsed in dH_2O and then dipped in 70 % (v/v) ethanol for surface sterilisation. Midguts from each sample were then transferred into 100 µl of sterilised PBS (pH 7.4) and homogenized using a disposable tissue grinder (Kontes). Each sample was then diluted 1:100 with LB-broth and 150 µl of each mixture spread onto LB-agar plates in the presence of 100 µg/ml ampicillin. Each plate was then incubated overnight at 37 °C and the number of colony forming units (CFUs) monitored. Results were then plotted using GraphPad Prism 4.0 software as means $\pm$ SEM and significance of data determined by Student's *t*-tests.

2.16 Fluorescence Imaging of Tubules

2.16.1 Live imaging of GFP fluorescence

For live imaging of GFP expression in *Drosophila*, tissues were carefully dissected as described in Section 2.1.3 and mounted on pre-treated Poly-L-lysine-coated (100 µl/ml – Sigma) BDH microscope slides in 100 µl PBS for immediate viewing using either the Zeiss Axiocam HRC System or the Zeiss 510 Meta confocal system as stated throughout the text. For samples viewed using the Zeiss Axiocam HRC system, GFP fluorescence was excited using a standard UV source (mercury lamp) and images

recorded at identical exposure conditions using Axiovision 5.0 acquisition software. Confocal analysis of GFP fluorescence is described in Section 2.16.3.

2.16.2 Immunocytochemistry (ICC) of Relish translocation

Malpighian tubules from 7-day old adult flies (either c42;UAS-*relish* or UAS-cGK/c42;UAS-*relish* as appropriate) were dissected and incubated as described in Section 2.1.3 with appropriate concentrations of either cGMP (Sigma) and/or PGN(-) (Invivogen) as stated throughout the text. Tubules were then arranged on pre-treated Poly-L-lysine-coated BDH microscope slides in 100 µl PBS. PBS was then carefully removed and each sample fixed with 4% (w/v) paraformaldehyde in PBS for 15 min. Samples were then washed 5 times in PBS and incubated in 0.2 % (v/v) Triton-X-100 in PBS for 15 min in order to permeabilise the cells. Tubules were then washed in 0.2 % (v/v) Triton-X-100, 0.5 % (w/v) bovine serum albumin in PBS (PAT) for 3 hr and then incubated overnight at 4°C in PAT containing a 1:50 dilution of monoclonal mouse anti-tetra-HIS primary antibody (QIAGEN®). After washing in PAT for 2 hr (changing solution every 30 min) tubules were incubated in PAT containing 2 % (v/v) goat serum for 4 hr and then subsequently incubated overnight at 4°C in PAT containing anti-mouse FITC-labelled secondary antibody (1:500 dilution; Jackson). Tubules were then washed in PAT for 2 hr and incubated in 4, 6-diamidino-2-phenylindole (DAPI) solution (1:5000 dilution of a 5 mg/ml stock - Sigma) for 1-2 min in order to stain the nuclei. After washing three times with PBS for 10 min, tubules were mounted in VectaShield (Vector Labs) using 22 mm square BDH coverslips, and sealed with glycerol/gelatin (Sigma). Samples were then viewed using the Zeiss 510 Meta confocal system.

2.16.3 Confocal microscopy

Samples were imaged using a Zeiss 510 Meta confocal system coupled to a Zeiss microscope. An Argon 488 laser and a 505-530 band pass filter were used for imaging the FITC antibody or GFP-fluorescent proteins. For visualisation of DAPI, a pseudo-DAPI technique was used. The DAPI was excited using the standard UV source (mercury lamp) and the image captured using the confocal photomultipliers. The DAPI image was then merged with the other channels retrospectively, using LSM 510 Meta Browser software. A 63x objective was used in all cases.

Chapter 3

cGMP modulation of Imd pathway-associated antimicrobial peptide expression

3.1 Summary

Recently, NO has been determined as a key regulatory molecule in both mammalian and *Drosophila* immunity (Nappi et al. 2000; Bogdan 2001a; Foley and O'Farrell 2003; McGettigan et al. 2005). In *Drosophila*, studies have identified NO as critical to fly survival in the adult fly in response to infection by gram-negative bacteria (McGettigan et al. 2005), and have implicated NO as playing a crucial role in upstream activation of the Imd pathway (Foley and O'Farrell. 2003). The mechanism by which NO exerts its effects is likely to be mediated by the cGMP signalling pathway via soluble guanylate cyclase, however this has yet to be determined. Therefore, using a combination of microarray, quantitative (Q) PCR and transgenic approaches, the role of cGMP signalling in immune response was investigated. Given the role of the Malpighian tubule as both an immune-sensing tissue and one that utilises NO/cGMP, these experiments were carried out using the adult tubule as the model system. In this chapter, results show that not only does cGMP have a modulatory effect on the expression of Imd pathway AMPs in the tubule, but that this effect is both dose and time-dependent. It is also demonstrated that the effect of cGMP does not appear to occur in all immune-sensing tissues, with no change in Imd pathway AMP expression seen in the larval fat body in response to exogenous cGMP. Additionally, it is indicated that there may be a role for cAMP signalling in *Drosophila* tubule immune response, possibly involving the regulation of an anti-fungal response.

3.2 The Malpighian tubule is a viable model for investigating immune response in *Drosophila*

3.2.1 Introduction

Over the years, NO/cGMP signalling has been established as essential to the regulation of tubule function (Davies 2006). As such, the tubule has emerged as the ideal genetic model for investigation into NO/cGMP signalling *in vivo*. However, the role of the Malpighian tubule as an important immune-sensing tissue has only recently been established (Tzou et al. 2000; McGettigan et al. 2005). In these studies, it is demonstrated that certain AMPs (diptericin, cecropin and metchnikowin) are upregulated in the tubule in response to infection (Tzou et al. 2000) and that all the major immune-associated genes of the Imd pathway are expressed in *Drosophila* tubules (McGettigan et al. 2005). Additionally, experiments using *lacZ* reporter flies have indicated that diptericin

expression is under the control of the Imd pathway in tubules, although this regulation has not yet been demonstrated quantitatively (Tzou et al. 2000). Therefore, using a both a bioinformatic and reverse transcriptase (RT)-PCR approach, further validation of the results of previous studies was carried out, as well as confirmation by Q-PCR that the Imd pathway is responsible for diptericin production in the Malpighian tubule.

3.2.2 Imd pathway-associated genes are strongly expressed in the *Drosophila* Malpighian tubule

The Imd pathway has been established as the main pathway involved in sensing and response to gram-negative bacteria (Georgel et al. 2001). A number of AMPs are also known to have specific activity against gram-negative bacteria and it has been demonstrated that the expression of these AMPs is directly induced through activation of the Imd pathway (Lemaitre et al. 1995a). As mentioned above, in a previous study by McGettigan et al, it was demonstrated that all of the main components of the Imd signalling pathway are expressed in the tubule (Figure 3.1A; taken from McGettigan et al. 2005). However, to date, the only Imd pathway-associated AMPs that have been identified in the tubule are diptericin and cecropin (Tzou et al. 2000; McGettigan et al. 2005). Therefore, in order to further validate that the Imd pathway may be operational in the tubule, the expression of selected Imd pathway-associated AMPs was investigated. This was carried out via an RT-PCR approach whereby gene-specific primers were used to amplify cDNA derived from the tubules of 7-day old Oregon R (OrR, wild type) adult flies. It should be noted that all primers were designed around intron/exon boundaries of the genes so as to control for the possibility of genomic contamination of cDNA preparations. Results show that all of the AMP genes tested are expressed in the tubule, including two cecropin isoforms and two attacin isoforms (Figure 3.1B). Therefore, these data further demonstrate that the tubule has all the relevant components in place to activate a sufficient immune response to gram-negative bacteria via the Imd pathway.

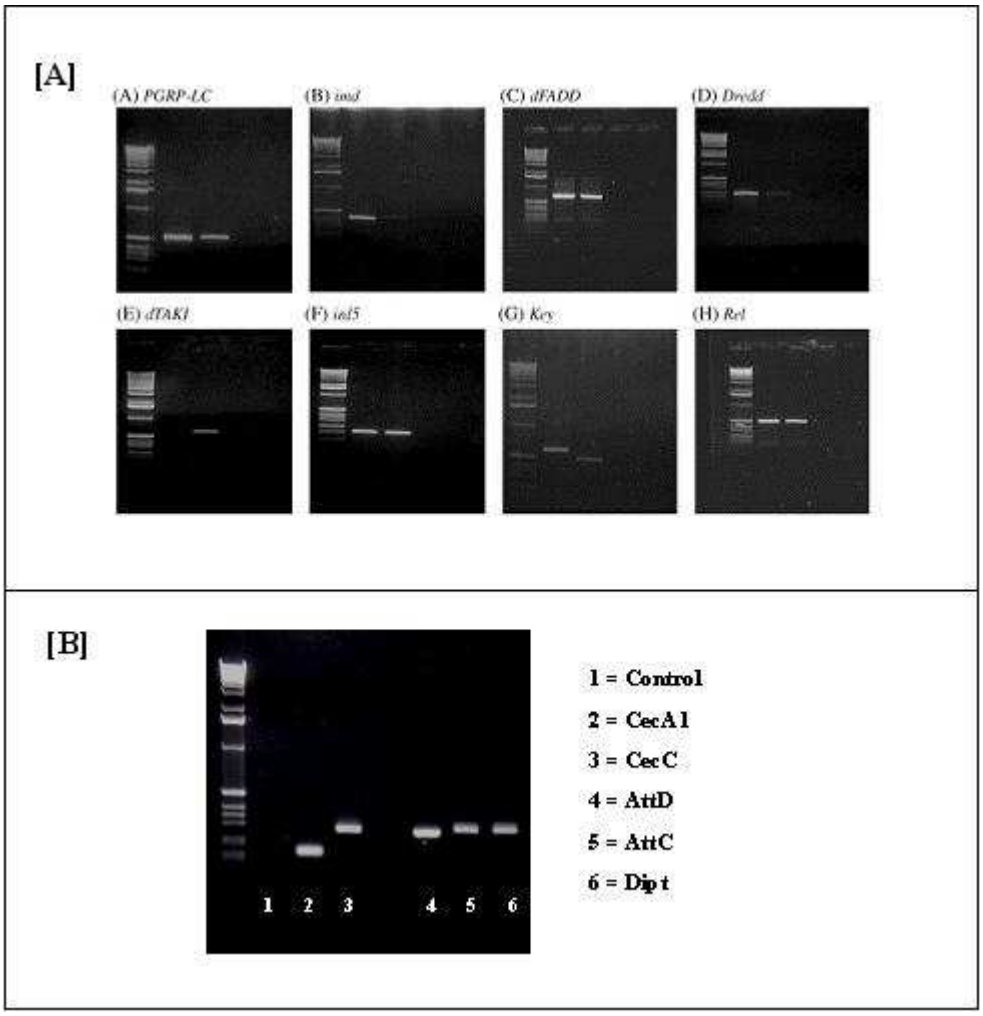


Figure 3.1 - Expression of Imd pathway-associated genes in the Malpighian tubule. [A] Previous studies have demonstrated that all components of the Imd pathway are expressed in the tubule (taken from McGettigan et al. 2005). [B] Imd pathway-associated AMPs are also expressed in the tubule. RT-PCR was performed on tubule cDNA from 7-day old OrR flies using gene-specific primers. Results show that all of the AMPs tested are expressed in the Malpighian tubules. Lane 1 depicts a 'no template' control.

In order to determine the relevance of these data with regards to expression of the Imd pathway in the tubule compared to other tissues, a bioinformatics approach was used. Using the *Drosophila* database *www.flyatlas.org*, a comprehensive view of gene expression in specific tissues can be obtained. Therefore, searches were performed using this database to determine expression levels of Imd pathway components in various tissues of the fly. Interestingly, results show that many components of the Imd pathway are highly enriched in the tubule, especially Dredd and Relish (Table 3.1). In fact, it is demonstrated that Relish, the NFκB/Rel transcription factor involved in AMP induction, is most abundantly expressed in the adult tubule compared to other tissues. Therefore, it

is suggested that not only is the Imd pathway active in the tubule, but that the tubule is a significantly important tissue with regards to Imd pathway activation.

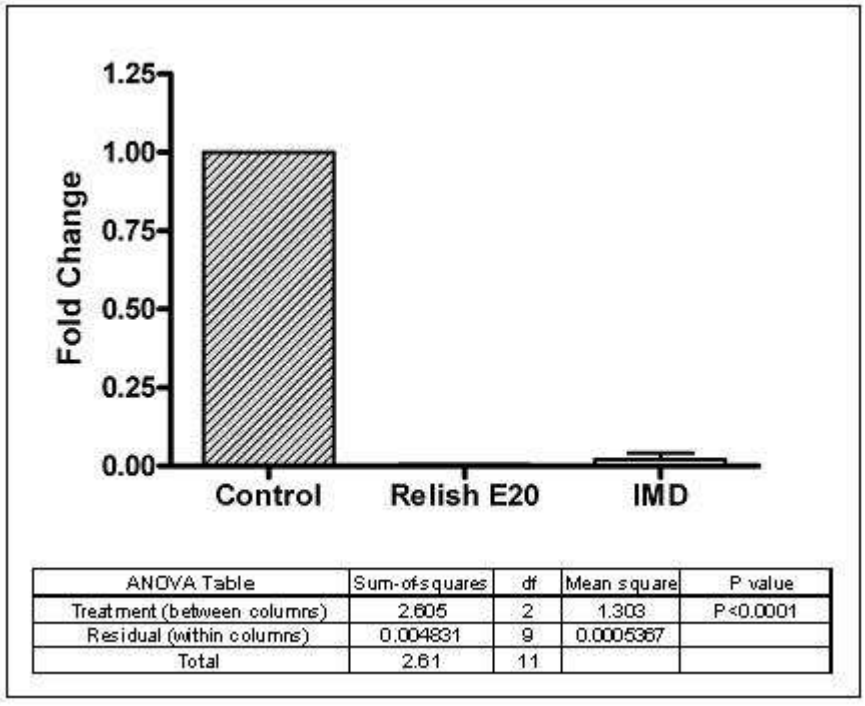
Table 3.1 – Expression of Imd pathway components in *Drosophila* fly tissues. Table shows mRNA abundance of both Dredd and Relish in various fly tissues and indicates their enrichment in comparison to the whole fly signal. Major sites of enrichment are shown in red and tubule data is shown in bold. Dredd and Relish are tabulated here as an example, for expression of other Imd pathway components see www.flyatlas.org

	Dredd			Relish		
Tissue	**mRNA signal**	**Enrichment**	**Affy Call**	**mRNA signal**	**Enrichment**	**Affy Call**
Brain	63 ± 2	0.70	Down	114 ± 6	0.50	Down
Head	92 ± 3	1.00	None	445 ± 35	1.80	UP
Thoracicoabdominal ganglion	82 ± 2	0.90	None	147 ± 5	0.60	Down
Crop	202 ± 5	2.20	UP	724 ± 10	2.90	UP
Midgut	170 ± 7	1.90	UP	517 ± 15	2.10	UP
Tubule	**170 ± 4**	**1.90**	**UP**	**783 ± 91**	**3.10**	**UP**
Hindgut	217 ± 8	2.40	UP	735 ± 33	2.90	UP
Ovary	91 ± 2	1.00	None	211 ± 6	0.80	None
Testis	54 ± 6	0.60	Down	40 ± 3	0.20	Down
Male Accessory Gland	251 ± 7	2.80	UP	380 ± 22	1.12	UP
Adult carcass	140 ± 2	1.50	UP	426 ± 34	1.50	UP
Larval tubule	**189 ± 5**	**2.10**	**UP**	**398 ± 12**	**1.60**	**UP**
Larval fat body	191 ± 9	2.10	UP	685 ± 55	2.70	UP
Whole fly	90 ± 8			250 ± 14		

3.2.3 Diptericin expression in the Malpighian tubule is dependent on the Imd pathway

To date, evidence that the Imd pathway is responsible for the regulation of AMP expression in the tubule has been provided using *diptericin-lacZ* transgenic reporter flies, where it was reported that activation of the diptericin promoter is hindered in *imd* mutant flies (Tzou et al. 2000). To further support this data, experiments were carried out in order to quantify the levels of diptericin expression in the tubule in both wild type and mutant flies. In this experiment, tubules of 7-day old adult flies were excised in Relish E20 and imd^{1} flies, null mutants of the *relish* and *imd* genes respectively (Hedengren et al. 1999; Lemaitre et al. 1995; both kind gifts from Professor S Kurata, University of Tohoku, Japan). Additionally, tubules from wild type Oregon R (OrR) flies were excised to act as controls. Tubule cDNA was then generated for each line and diptericin

expression quantified by Q-PCR using an Opticon 2 thermal cylcer. Results show that diptericin expression is completely abolished in the tubules of *relish* flies and that there is only minimal expression of diptericin in the tubules of *imd* mutant flies (Figure 3.2). These data support the results of previous studies and confirm quantitatively that the Imd pathway is responsible for diptericin expression in the Malpighian tubule.



ANOVA Table	Sum-of-squares	df	Mean square	P value
Treatment (between columns)	2.605	2	1.303	P<0.0001
Residual (within columns)	0.004831	9	0.0005367	
Total	2.61	11		

Figure 3.2– Diptericin expression in tubules is dependent on the Imd pathway. Expression of diptericin was assessed by Q-PCR in OrR tubules (Control) and in tubules from both *relish* and *imd*^{1} mutant flies. Resulting data were normalised against expression of the standard, *rp49*, and expressed as a fold change of diptericin expression where control =1 (N=4, $\pm$ SEM). Significance of data was determined by one-way ANOVA (See table). Data shows that diptericin expression is significantly lower in the tubules of both *relish* and *imd* mutant flies.

3.3 cGMP modulates expression of Imd pathway-associated anti-microbial peptides in the *Drosophila* Malpighian tubule

3.3.1 Introduction

In recent years, NO has been shown to modulate function of the Imd pathway in a number of different *Drosophila* tissues such as the hemocytes, Malpighian tubules and the fat body (Bassett et al. 2000; McGettigan et al. 2005; Foley and O'Farrell. 2003). The main role of NO is to stimulate cGMP production and activate cGMP-dependent signalling processes. As such, it is likely that the mechanism by which NO acts to

regulate immune function occurs via cGMP signalling. To date, a definitive role for cGMP signalling in immunity has not been established in vertebrates or invertebrates, however studies have implicated the involvement of the cGMP pathway in mammalian immune processes. For example, cGMP signalling is suggested to be involved in the proliferation of lymphocytes (Sadighi Akha et al. 1996; Fischer et al. 2001), chemotaxis and adhesion of neutrophils and macrophages (Syrovets et al. 1997; VanUffelen et al. 1998; Lawrence and Pryzwansky 2001), and the gene expression of iNOS and TNFα in macrophages and dendric cells (Harbrecht et al. 1995; Kiemer et al. 2000; Paolucci et al. 2000). Therefore, given the already established role of NO in *Drosophila* immunity and the implication from mammalian studies that cGMP could also be involved in immune regulation, studies were initiated to determine the potential role of cGMP signalling in *Drosophila* innate immunity. This was carried out using the tubule as an *in vivo* model system and achieved using a combination of microarray, Q-PCR and transgenic approaches.

3.3.2 Expression of Imd pathway-associated AMPs is down-regulated in the Malpighian tubules in response to stimulation by cGMP *in vitro*

With the development of microarray analysis it has become possible to generate large amounts of information regarding changes in gene expression in response to specific signals. Therefore, microarray analysis was carried out in *Drosophila* Malpighian tubules in order to ascertain the effect of cGMP on gene expression (Dow, Davies and Day, unpublished). In this study, tubules of 7-day old OrR flies were excised and incubated for 3 hr in either sterile Schneiders medium (control) or sterile Schneiders medium containing 100 μM cGMP. It should be noted that 100 μM cGMP was used in this assay as previous studies have determined this to be the concentration which elicits maximum physiological response from the tissue (Davies et al. 1995). RNA was then extracted from these tubules and applied to Affymetrix *Drosophila* genome array chips according to a standard protocol. Results were then analysed using Affymetrix MAS 5.0 software (Dow, Davies and Day, unpublished).

The effect of cGMP on immune-related genes in the tubule is summarised in Table 3.2. Interestingly, the genes that appear to be modulated by cGMP are those expressed in response to Imd pathway activation. This data further supports the evidence that NO is

involved in Imd pathway regulation and suggests that cGMP is also a key modulator of immune response in *Drosophila*. However, in complete contrast to studies carried out using NO, cGMP appears to modulate AMP expression in a negative manner. Results show that, at this concentration, cGMP is responsible for between approximately a 25-90% downregulation of Imd pathway-associated AMPs.

Table 3.2 – Tubule expression of AMP genes in response to cGMP stimulation. Summary of microarray analysis of gene expression in cGMP-stimulated tubules compared to non-stimulated tubules. Data shows that in the presence of cGMP there is between a ~25-90% downregulation of Imd pathway-associated AMPs compared to controls

Gene	Average mRNA signal (±SEM)		Fold Change
	Control	cGMP-	
Diptericin	734 ± 90	368 ± 65	0.50
Attacin C	216 ± 48	160 ± 49	0.74
Attacin D	9480 ± 615	2839 ± 499	0.30
Cecropin A1	4168 ± 473	789 ± 77	0.19
Cecropin A2	1770 ± 183	363 ± 50	0.21
Cecropin C	2639 ± 221	279 ± 89	0.11

In order to validate this data, both a Q-PCR and transgenic approach was used (Figure 3.3). In these experiments, tubules of 7-day old OrR flies were excised and incubated under exactly the same conditions as the previous microarray experiment. For Q-PCR, cDNA was then derived from RNA extracted from each tubule sample and expression of specific AMP genes quantified as described previously. As can be seen in Figure 3.3A, quantitative analysis of microarray results confirms that Imd pathway-associated AMPs are down-regulated in response to 100 μM cGMP in the tubules. Results show that the tubule expression levels of all the AMPs tested are significantly lower after cGMP stimulation, with the decrease in expression levels compared to controls ranging from approximately 30% (AttC) to 80% (CecA1 and CecC).

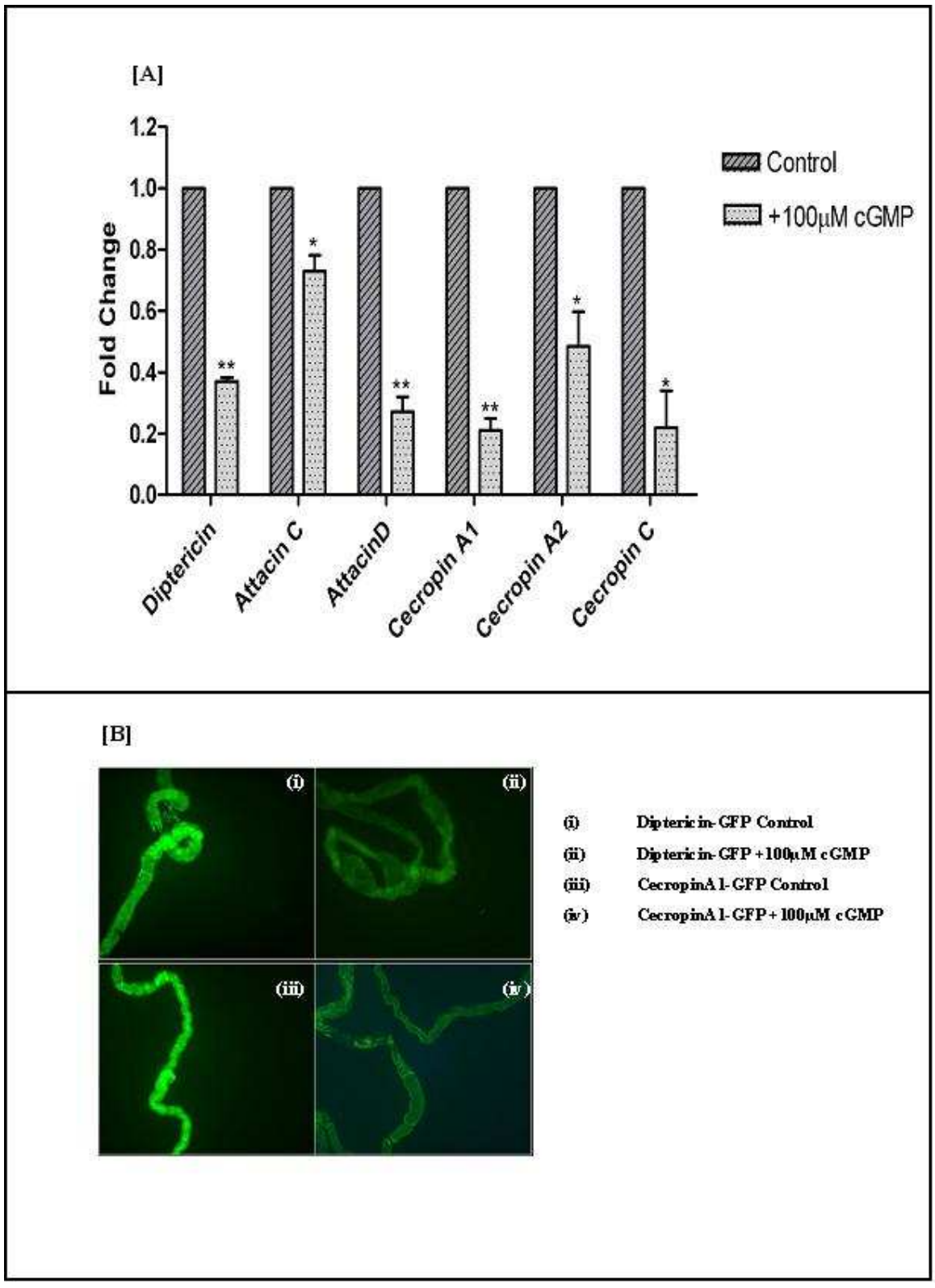


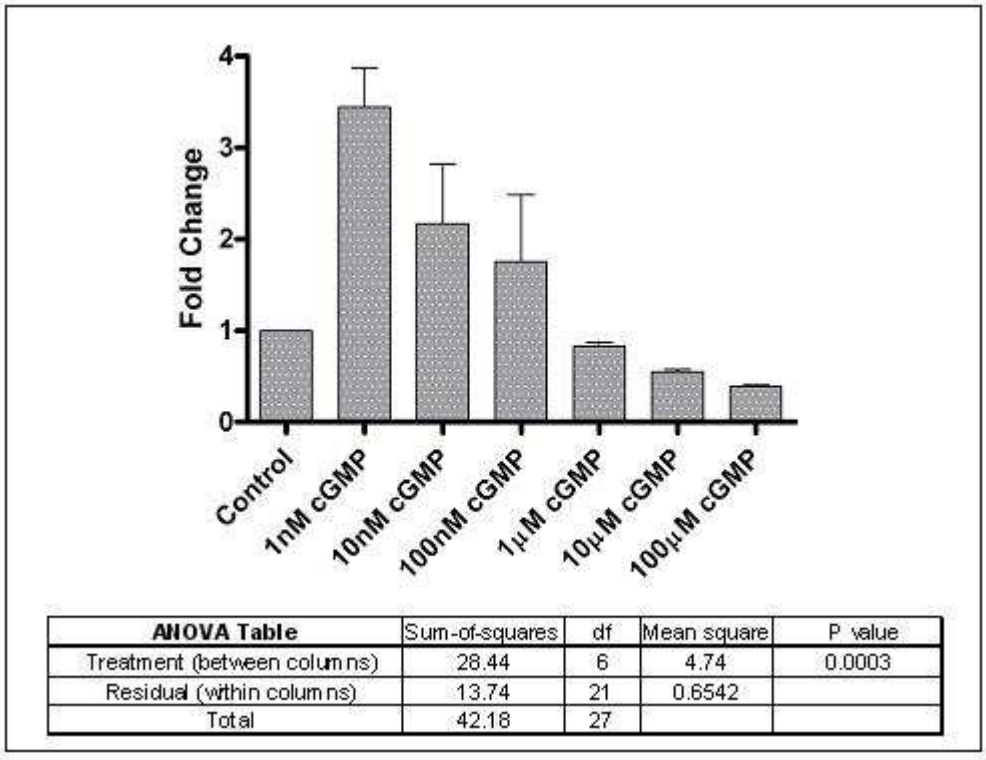
Figure 3.3– Validation of microarray by Q-PCR and transgenic methods. For both approaches, tubules were dissected and incubated under the same condition as microarray. [A] Q-PCR shows approximately between a 30% and 80% down-regulation of Imd pathway-associated AMPs in response to cGMP. Resulting data were normalised against expression of a standard gene, *rp49*, and expressed as a fold change of AMP expression where control =1 (N=4, $\pm$ SEM). Data significant from control are indicated by * ($P<0.05$) or ** ($P<0.01$) (as analysed by Students *t*-test). [B] Fluorescence is reduced in AMP-GFP transgenic flies in response to cGMP, suggesting a down-regulation in expression. All pictures were taken at the same exposure using a Zeiss Axiocam HRC.

To further support this data, AMP expression in response to cGMP was monitored in the tubules of both diptericin-GFP and cecropinA1-GFP reporter flies (Tzou et al. 2000; McGettigan et al. 2005; kind gifts from J.L Imler, University of Strasbourg). In these flies, GFP expression is under the control of the promoter of the corresponding gene. It should be noted that in unchallenged flies, fluorescence levels of both diptericin-GFP and cecropin-GFP are relatively low, however this experiment was carried out merely as further support to both the microarray and Q-PCR data.

As with the Q-PCR, tubules of 7-day old flies were dissected and incubated under the same conditions as those used for the microarray. After incubation, all tubules were mounted in Phosphate Buffered Saline (PBS) for immediate viewing under fluorescence using a Zeiss Axiocam HRC System. All subsequent pictures were taken under exactly the same exposure conditions. As Figure 3.3B shows, expression of both cecropinA1 and diptericin is reduced in the tubules when stimulated with 100μM cGMP. Therefore, these data further support the evidence provided by microarray and Q-PCR that cGMP is a key modulator of Imd pathway activation in the Malpighian tubule.

3.3.3 The effect of cGMP on anti-microbial peptide expression occurs in a dose and time-dependent manner

As mentioned earlier, the original time of incubation and concentration of cGMP used in these assays was determined by results obtained from previous studies (Davies, 1995). However, in an organismal context, due to the presence of a number of different cGMP activators within each cell, as well as a number of downstream effectors, transporters and regulators of cGMP, the basal concentration of cGMP *in vivo* is difficult to determine. In fact, it is known that cGMP is present as specific 'pools' of differing concentrations and frequency within each cell, allowing for its role in a number of distinct physiological processes (Beavo and Brunton 2002; Piggott et al. 2006). Therefore, Q-PCR was carried out in order to determine the effect of different concentrations and incubation times of cGMP on the expression of Imd-pathway induced AMPs. For these experiments, focus was placed on diptericin, an AMP controlled solely by Imd pathway activation (Hedengren et al. 2000).

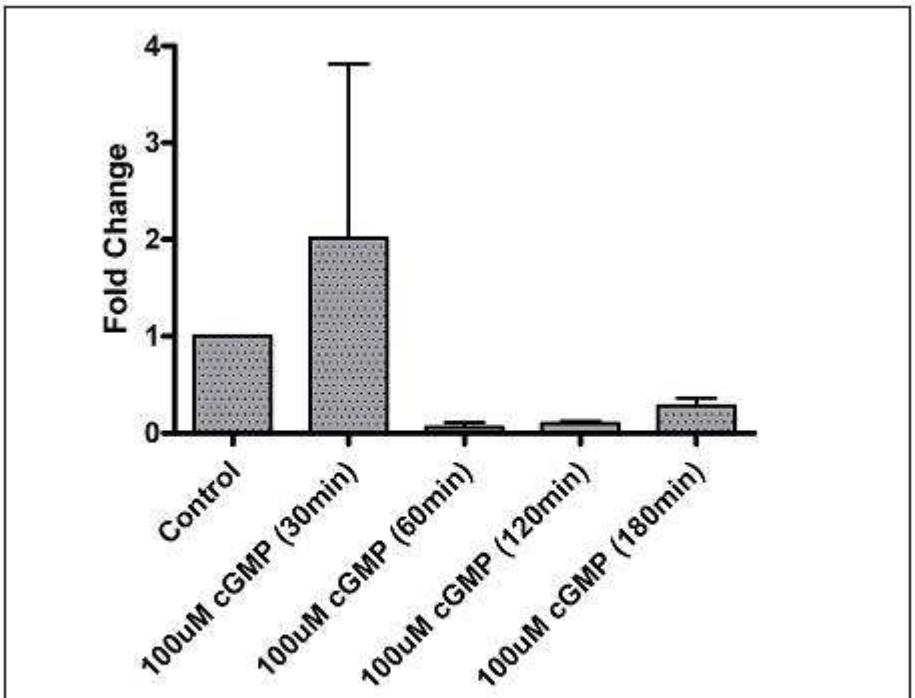


ANOVA Table	Sum-of-squares	df	Mean square	P value
Treatment (between columns)	28.44	6	4.74	0.0003
Residual (within columns)	13.74	21	0.6542	
Total	42.18	27		

Figure 3.4– Dose-dependent effects of cGMP on diptericin expression. Tubules were dissected and incubated under the same conditions as described in the text. Resulting data were then normalised against expression of a standard gene, *rp49*, and expressed as a fold change of AMP expression where control =1 (N=4, ± SEM). Significance of data was determined by One-way ANOVA (See table). Data shows that effect of cGMP on diptericin expression is biphasic depending on concentration used. Analysis by One-way ANOVA shows that this effect is considered statistically significant.

When assaying the effect of different concentrations of cGMP on diptericin expression, tubules were excised under the same conditions as previously described and incubated with an array of physiologically relevant cGMP concentrations ranging from 1 nM – 100 μM. Interestingly, results show that at low concentrations of cGMP (within the nanomolar range), diptericin expression is increased within the tubule, suggesting that cGMP may be involved in activation of the Imd pathway under certain cellular conditions, possibly via NO. In contrast, when tubules are incubated with concentrations of cGMP in the micromolar range, a decrease in diptericin expression is seen relative to increasing cGMP concentration. Therefore, it is implied from these data that cGMP signalling is able to mediate either a stimulatory or an inhibitory effect on Imd pathway signalling and that this effect is dependent on cGMP concentrations within the cell.

When assaying the effect of time on cGMP modulation of diptericin expression, tubules were excised as previously described and incubated in 100 μM cGMP for varying time-points ranging from 0 min-180 min. Additionally, in order to account for any endogenous variation in gene expression over time, control sets of tubules were incubated in the absence of cGMP for each time-point. Q-PCR analysis was then carried out on cDNA derived from each tubule set and resultant data (after initial normalisation to *rp49* expression) was normalised against expression of controls for each time-point (where control equals 1) (Figure 3.5). Results show that, at this concentration, cGMP appears to exhibit a biphasic effect on diptericin expression depending on whether the cGMP signal within the tubule is acute or sustained.



ANOVA Table	Sum-of-squares	df	Mean square	P value
Treatment (between columns)	11.11	4	2.778	P<0.05
Residual (within columns)	38.99	15	2.599	
Total	50.1	19		

Figure 3.5 – Time-dependent effects of cGMP on diptericin expression. Tubules were dissected and incubated under the same conditions as previously described. Resulting data were normalised against expression of a standard gene, *rp49*, and expressed as a fold change of AMP expression where control =1 (N=4, $\pm$ SEM). Separate control data was obtained for each time-point to account for natural changes in gene expression. Significance of data was analysed by one-way ANOVA (See table). Data shows that effect of cGMP on diptericin expression is biphasic depending cGMP incubation time, however statistical analysis does not show that this effect is significant.

To explain further, it appears that when tubules are incubated with 100 μM cGMP there is an initial induction of diptericin expression, followed by a down-regulation of expression between 30 and 60 min. Unfortunately, the initial increase in diptericin expression seen at 30 min was only consistent for the majority of datasets, resulting in a large error. This experiment was therefore repeated multiple times (N = 8), using shorter time-points of 15min, 30 min and 45min, in order to try and pin down the precise time at which the increase in diptericin expression occurs. Unfortunately, although an increase in diptericin expression was seen in every dataset before down-regulation occurred, the exact time of this increase was very transient, occurring at varying times in the first 30 min after incubation (data not shown). Therefore, it can only be indicated by this data that cGMP might exhibit a biphasic effect depending on time, however further work is required to confirm this.

3.4 cGMP modulation of Imd pathway anti-microbial peptide expression in other *Drosophila* tissues.

3.4.1 Introduction

Following validation that cGMP signalling acts to regulate the Imd pathway in the Malpighian tubule, studies were initiated in order to determine the effects of cGMP on AMP production in both the fat body and *Drosophila* Schneider line 2 (S2) cells. The fat body has been described as the canonical immune sensing tissue and therefore the effect of cGMP signalling on the Imd pathway in this tissue is of some interest. Similarly, *Drosophila* S2 cells have emerged as a useful *in vitro* cell-based system for the analysis of the activity of many genes and gene products. Therefore, using a combination of Q-PCR, molecular cloning and reporter assay techniques, the effect of cGMP on Imd pathway activation in fat body and S2 cells were investigated.

3.4.2 cGMP modulation of Imd pathway AMP expression does not occur in the *Drosophila* fat body

To date, most studies of innate immune signalling pathways in *Drosophila* have been carried out in the fat body (Silverman and Maniatis 2001). The fat body originates from the mesoderm during embryogenesis and is critical to a number of processes including

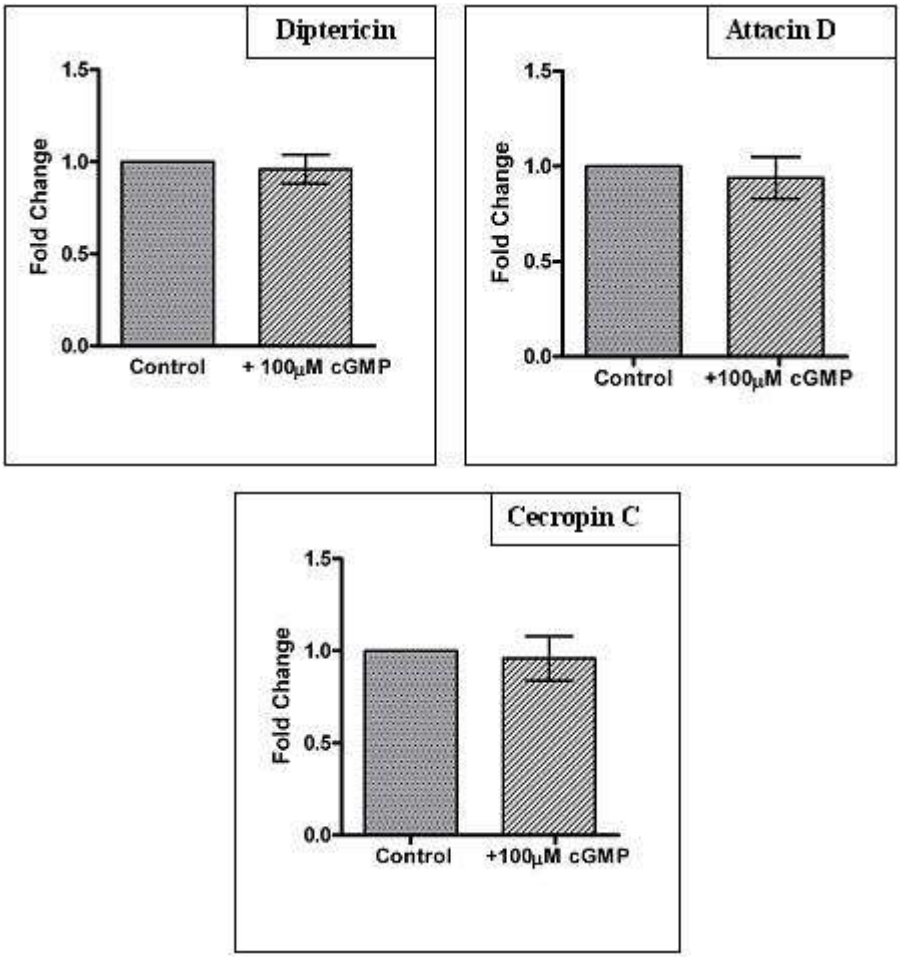


Figure 3.6 - Fat body expression of Imd pathway-associated AMPs in response to cGMP. Fat body was dissected from 3rd instar larvae and incubated with 100 μM dibutyryl-cGMP for 3hr. Resulting data were normalised against expression of a standard gene, *rp49*, and expressed as a fold change of AMP expression where control =1 (N = 4, ± SEM). Data shows no significant change in AMP expression in response to 100 μM cGMP.

nutrient sensing, energy metabolism and 'liver'-related functions (Van Doren 2006). Like the Malpighian tubules, the fat body is present throughout the body cavity of the fly and therefore represents a powerful tissue for sensing and responding to invading pathogens. As such, numerous studies have identified the fat body as an important tissue with regards to the activation of immune signalling pathways and the subsequent production of AMPs. Additionally, in a study by Foley and O'Farrell (2003), NO was demonstrated to play a role in regulation of the Imd pathway in the fat body. This would suggest that cGMP signalling might also play a role in fat body immune responses. Therefore experiments were carried out in order to determine the effect of cGMP on AMP expression in larval fat body. Larval tissue was used for these studies as it is extremely difficult to isolate intact fat body from the adult fly. Experiments were carried out under the same conditions as with tubules however, as there is no known cGMP transporter

present in fat body, a cell-permeable analogue of cGMP was used (dibutyrylguanosine 3', 5'-cyclic monophosphate). As Figure 3.6 shows, there is no significant difference in expression of diptericin, cecropin or attacin in response to 100 μM cGMP. These data therefore suggest that Imd pathway regulation in the fat body may occur by cGMP-independent means, although further investigation is required.

3.4.3 Heterologous expression of Imd pathway AMPs in *Drosophila* S2 cells

Drosophila S2 cells are derived from a primary culture of 20-24 hr old *Drosophila* embryos, and are hemocyte-like in quality (Schneider 1972). They have long been established as an effective cell-line for the *in vitro* analysis of the activity of many genes and their products, and their suitability as a system for the study of *Drosophila* innate immune responses has been demonstrated in a number of previous studies (Foley and O'Farrell 2004; Kallio et al. 2005; Kaneko and Silverman 2005; Thoetkiattikul et al. 2005).

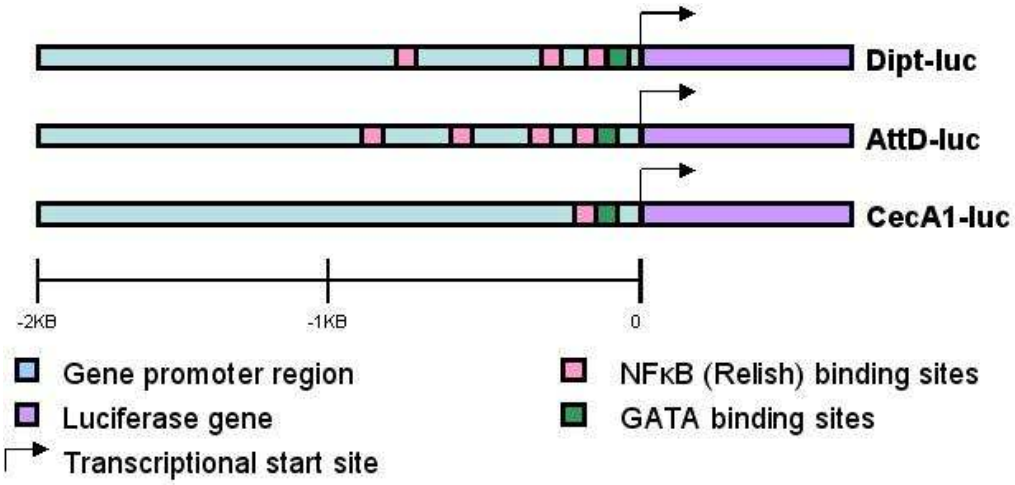


Figure 3.7 – Cartoon map of the promoter regions of diptericin, attacin D and cecropin A1 following downstream cloning of the firefly luciferase gene.

For this study, the promoter regions of *diptericin*, *cecropinA1* and *attacin D* were amplified and cloned into the pGL3-Basic Vector upstream of the firefly luciferase gene, *luc*+ (Figure 3.7). The resultant DNA plasmids were then purified and transfected into S2 cells using a calcium phosphate transfection method. Additionally, in order to control for

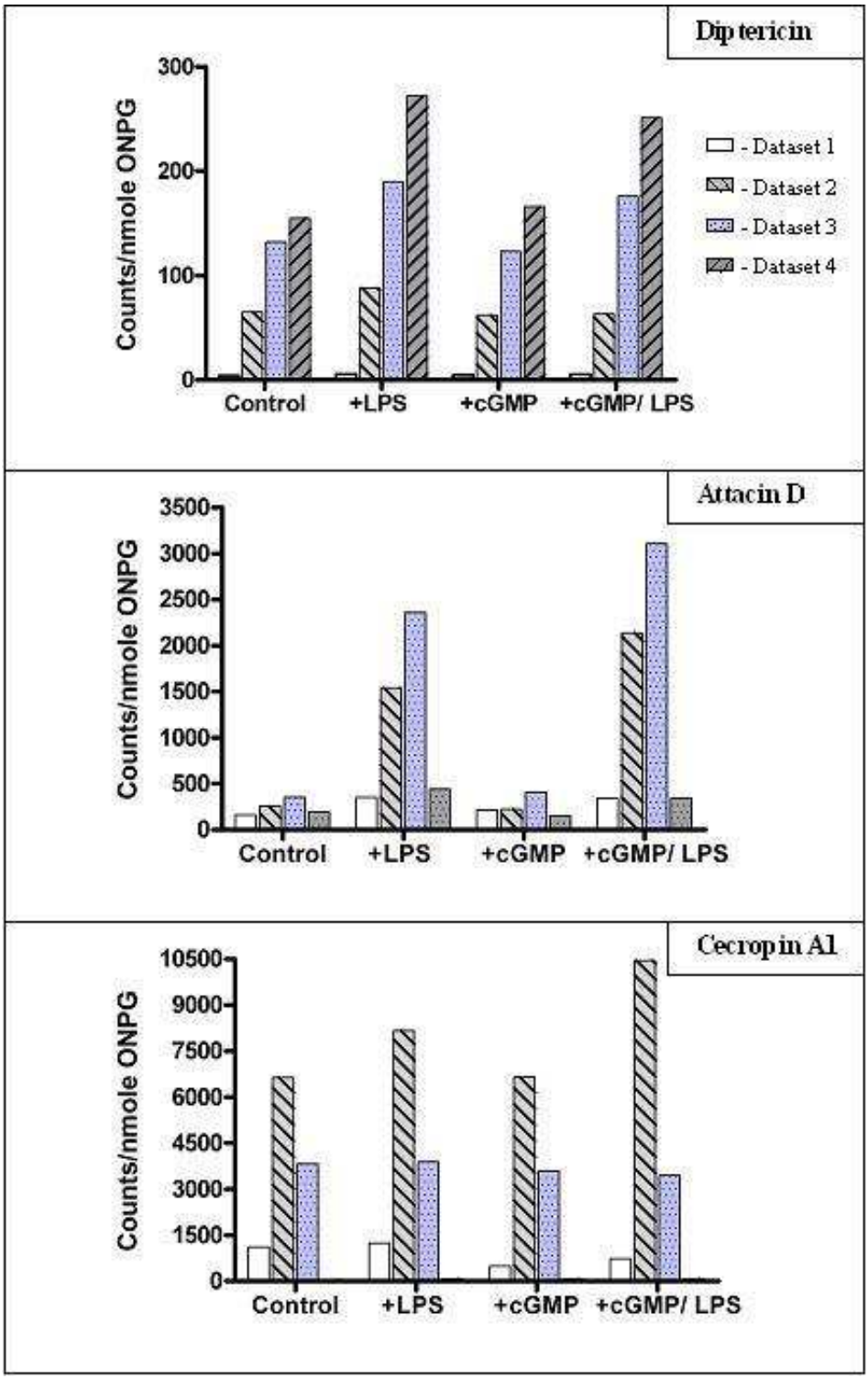


Figure 3.8 – AMP-luciferase gene expression in S2 cells. The effect of cGMP on Imd pathway regulation was monitored in S2 cells after transfection of AMP-luc reporter plasmids. AMP-reporter expression was measured by luminescence and data normalised to corresponding β-gal expression levels. Data is expressed as luminometer counts per nmole of ONPG (see Materials and Methods for assay details). For each reporter gene, 4 datasets of results are depicted above. Results show highly variable expression levels between samples. Key in top panel is representative of all three graphs.

variations in transfection efficiency or cell number, cells were transfected with the β-galactosidase expression vector, pAc5.1/V5-His/lacZ (Invitrogen). Transfected cells were then stimulated with 100 μM cGMP and/or 10 μg/ml LPS for three hours before harvest. It should be noted that again a cell-permeable analog of cGMP, di-butyryl-cGMP, was used in these studies, as there are no known transporters of cGMP expressed endogenously in S2 cells. Once harvested, cells were lysed using the Promega Luciferase Assay system and luminescence detected using a standard luminometer. All data obtained was then normalised to corresponding expression levels of β-galactosidase. Unfortunately, the data obtained from these assays was highly variable and, as a consequence, no significant trends could be demonstrated with regards to role of cGMP in Imd pathway regulation (Figure 3.8). In the case of diptericin-luciferase, it can be seen that expression levels between each individual dataset are extremely variable, with control data alone ranging from 4 – 155 counts/nmole ONPG. This variability is also present for stimulated cells. However, when looking at each dataset individually, a basic trend can be identified whereby diptericin-luc expression is increased in response to LPS, decreased in response to cGMP (though not significantly), and increased after stimulation with both LPS and cGMP, although to a lesser extent than when stimulated with LPS alone. Unfortunately, these data are too variable for this trend to be considered significant. For attacin-luc, both control data and cGMP-stimulated data are fairly comparable between datasets, however data obtained from cells stimulated with LPS or LPS/cGMP is highly variable. When comparing control expression levels to expression levels after cGMP stimulation, it can be seen that there is no significant difference in expression of attacin-luc, suggesting that cGMP does not have an effect on Imd pathway regulation in S2 cells. Finally, expression data obtained from cells transfected with the cecropinA1-luc plasmid shows no significant trends whatsoever and variability between datasets is too vast for interpretation.

Overall, these experiments have proved inconclusive and suggest that S2 cells do not comprise a suitable system for investigation of the role of cGMP in Imd pathway regulation.

3.5 cAMP signalling also plays a role in *Drosophila* immune response in the Malpighian tubule

Another important cyclic nucleotide second messenger is adenosine 3',5'-cyclic monophosphate (cAMP). cAMP was first discovered in the late fifties by two different groups (Cook et al. 1957; Sutherland and Rall 1958) and since that time it has been established as a critical regulator of a number of processes such as glucose and lipid metabolism, cell proliferation, vasodilation, neuronal function and fluid secretion (Sutherland 1972; Beavo and Brunton 2002). The cAMP and cGMP signalling pathways are very closely related, whereby cross talk is known to occur between the pathways via dual-specificity phosphodiesterases. For example, in mammals, cGMP is known to stimulate cAMP hydrolysis by PDE2. Similarly, cGMP is known to act as a competitive inhibitor of cAMP hydrolysis by PDE3 (Omori and Kotera 2007; Zaccolo and Movsesian 2007). Therefore, given the role of cGMP signalling in the regulation of immune response in *Drosophila*, it is suggested that cAMP may also be implicated in this regulation. To date, as with cGMP signalling, a role for cAMP signalling in *Drosophila* immune response has not been investigated. However in mammals, cAMP signalling has been implicated in a number of immune-related processes such as macrophage survival and regulation of T-cell activation (Park et al. 2005; Roach et al. 2005).

In order to investigate a possible role of cAMP in Imd pathway regulation, a Q-PCR approach was used. Tubules from 7-day old OrR flies were dissected as described previously and incubated for 3 hr in sterile Schneiders medium (control) or sterile Schneiders medium containing 100 μM cAMP. Expression levels of selected Imd pathway-associated AMPs were then quantified by Q-PCR using cDNA derived from each tubule sample.

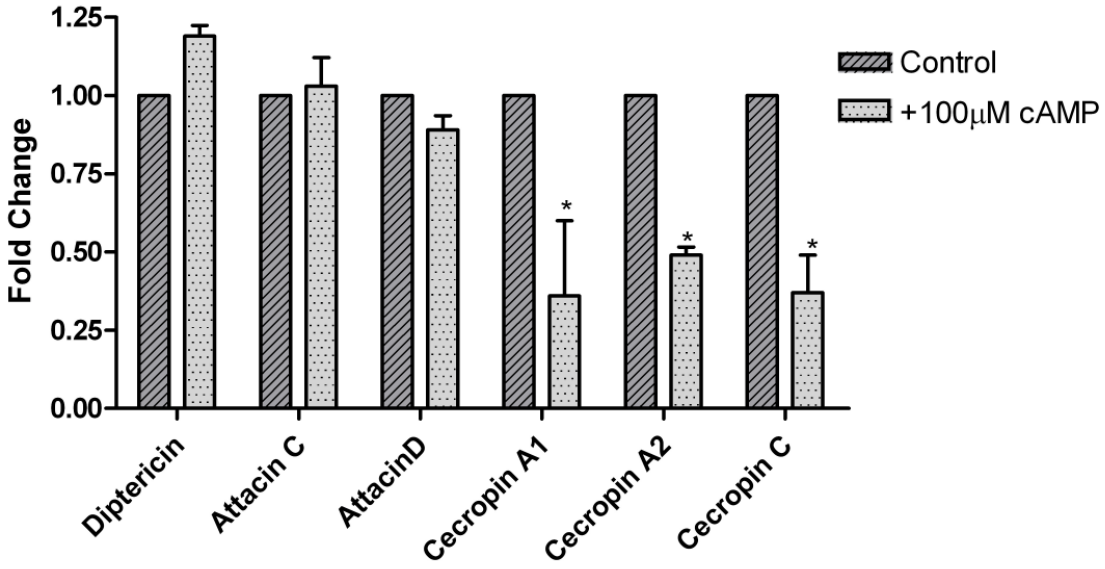


Figure 3.9 - Effects of cAMP on AMP expression. Tubules were dissected and incubated with or without 100μM cAMP for 3hrs. Resulting data were normalised against expression of a standard gene, *rp49*, and expressed as a fold change of AMP expression where control =1 (N=4, ± SEM) Data significant from control are indicated by * ($P<0.05$). Data shows that cAMP does not have an effect on either diptericin or attacin expression but effects all isoforms of cecropin, showing between a 50-60% downregulation of expression for each.

As Figure 3.9 shows, stimulation with cAMP does not significantly effect the expression of diptericin or attacins C and D, however a significant decrease in expression can be seen for all three cecropin peptides. Interestingly, unlike diptericin and attacin, which are solely active against gram-negative bacteria, previous studies have demonstrated that cecropin is active against both gram-negative bacteria and fungi (Ekengren and Hultmark 1999). Data therefore indicates that whilst cGMP regulation may be specific to Imd pathway activation in response to gram-negative bacteria in tubules, cAMP may play a role in regulation of anti-fungal immune response.

3.6 Discussion

In this chapter, a novel role for cGMP signalling has been identified in the *Drosophila* Malpighian tubule. Data shows that cGMP modulates expression of Imd pathway-associated AMPs, and that this effect can be either stimulative or inhibitory depending on concentration levels of cGMP. These data would therefore suggest that Imd pathway regulation by cGMP occurs via two distinct cGMP signals within the cell. As mentioned

earlier, cGMP is known to exist in localised 'pools' of differing concentrations, generated in close proximity to particular upstream activators i.e. soluble or receptor guanylate cyclases. Consequently, the action of each distinct cGMP signal within each cell is determined by its proximity and affinity to particular downstream effectors such as cGKs, PDEs and CNG channels (Beavo and Brunton 2002; Piggott et al. 2006). With regards to *Drosophila* immunity, previous studies have demonstrated a role for NO in the activation of the Imd pathway. Similarly, it is shown here that activation of Imd pathway-associated AMP expression is observed in response to low nanomolar concentrations of cGMP in the Malpighian tubule. Therefore it is suggested that, *in vivo,* activation of the Imd pathway by cGMP may occur via NO-mediated activation of sGC. Alternatively, it can be suggested that the inhibitory effect of cGMP seen at micromolar concentrations is probably mediated via NO-independent means, through the activation of a receptor GC. The differential effects of cGMP on Imd pathway activation in the tubule, as well as the cGMP activator and effector proteins involved in this regulation, form the basis of the remaining chapters presented in this thesis.

When investigating the effect of cGMP signalling on AMP expression in other *Drosophila* tissues, it is demonstrated that cGMP stimulation has no significant effect on AMP expression in the fat body, and, as such, does not appear to play a regulatory role in Imd pathway activation in this tissue. Since the fat body has been determined as a key immune tissue in *Drosophila*, this result would suggest that further investigation is required to determine the relevance of cGMP regulation of the Imd pathway in the tubule with respect to overall immune response in the whole organism. Therefore, this effect is investigated in future chapters, whereby whole animal survival is assayed in response to infection using a transgenic approach.

Unfortunately, when investigating the effect of cGMP on AMP expression in S2 cells, results proved highly variable. The reason for such variability between samples is unknown. Promoter sequences were verified before transfection, and variations in transfection efficiency were controlled for by simultaneous transfection of a *lacZ* expression vector. As mentioned earlier, S2 cells have previously been validated as a suitable model for investigation into immune signalling pathways. However, it is not known whether S2 cells are able to naturally express all of the components involved in cGMP signalling, as previous studies have involved the heterologous expression of

cGMP pathway components (MacPherson et al. 2004b; Day et al. 2005). Therefore, this may result in cells deficient in the proteins needed for cGMP signalling, or alternatively, S2 cells expressing inactive versions of the needed components. Consequently, based on the data obtained, it can be concluded that S2 cells are not a valid system to investigate the effects of cGMP signalling on Imd pathway regulation in *Drosophila*.

Finally, a role for cAMP in regulation of immune response in the *Drosophila* tubule is demonstrated here, where data shows that expression of cecropin (A1, A2 and C) is significantly reduced in response to cAMP. As mentioned earlier, previous studies have implicated a role for cecropin as a potent anti-fungal agent (Ekengren and Hultmark 1999). This would suggest that cAMP might play a role in regulation of anti-fungal response, possibly through interaction with the main anti-fungal signalling pathway in *Drosophila*, the Toll pathway. Further investigation into this effect is required. However, given the similarities between cGMP and cAMP signalling, and the fact that cGMP and cAMP are both regulated by a number of common PDEs, a complementary role for cAMP signalling in immune response to that of cGMP signalling would not be surprising. Therefore, the effect of cAMP signalling on *Drosophila* immune response pathways is a subject for future work.

Chapter 4

Modulation of immune response by cGMP is mediated by the *Drosophila* cGKs DG1 and DG2

4.1 Summary

In the previous chapter, cGMP was shown to modulate expression of Imd pathway-associated AMPs in the Malpighian tubules. In *Drosophila*, cGMP is known to mediate its effects via the cognate cGMP-dependent kinases DG1 and DG2, both of which are expressed in the tubule (Dow et al. 1994a). Therefore, in this chapter, the possible effector role of *Drosophila* cGKs in cGMP-mediated immune regulation was investigated. This was carried out using a transgenic approach, whereby expression of *dg1* and the two main transcripts of *dg2, P1* and *P2*, was modulated in the principal cells of the tubule using the GAL4/UAS binary system (Brand and Perrimon 1993). Transgenic flies were then assessed for changes in diptericin expression in the tubule by Q-PCR. Additionally, in order to determine the impact of cGMP pathway-mediated modulation of AMP expression in the tubules on the whole organism, survival in response to septic infection with both Gram-negative and Gram-positive bacteria was monitored in each transgenic fly line. Furthermore, the potential role of cGKs in the tubule in response to natural infection was investigated by both Q-PCR and bacterial clearance assays. Results demonstrate that targeted over-expression or knock-down via RNAi of cGKs in tubule principal cells of the adult fly results in differential effects of DG1 and DG2 on Imd pathway regulation. It is shown here that diptericin expression is stimulated by DG1 in the tubule of the adult fly. Similarly, the effects of DG1 in the tubule are sufficient to impact positively on whole fly survival in response to septic infection with Gram-negative bacteria, and are also shown to mediate enhanced bacterial clearance in the gut following natural infection with *E.coli*. Alternatively, DG2P1 and DG2P2 are demonstrated to have an inhibitory effect on diptericin expression in the tubule. These effects are shown to have a negative impact on survival of whole flies in response to septic infection with Gram-negative bacteria. Additionally, it is demonstrated that modulation of DG2P1 in the tubule is sufficient to significantly inhibit bacterial clearance in the gut following natural infection with *E.coli*. Interestingly, it is shown that the effects mediated by cGKs on AMP regulation are tissue-specific, as modulation of cGK expression in the fat body does not confer similar survival phenotypes in the adult fly.

4.2 *Drosophila* cGKs, DG1 and DG2, mediate differential effects on diptericin expression in the tubule

4.2.1 Introduction

To date, investigation of functional effects of *Drosophila* cGKs has mainly focussed on neuronal function. For example, expression analysis studies have demonstrated that DG1 and the various isoforms of DG2 are enriched in the head of the adult fly (Kalderon and Rubin 1989; Foster et al. 1996). Additionally, a number of studies involving genetic analysis of the *dg2 (for)* gene have implicated cGKs to play a role in neuronal processes such as feeding behaviour, sensory responsiveness, and learning and memory (Osborne et al. 1997; Scheiner et al. 2004; Mery et al. 2007). However, in recent years, a role for cGKs in tubule function has also emerged (MacPherson et al. 2004a; 2004b). Studies involving the analysis of the gene products of both *dg1* and *dg2* have demonstrated that the tubule contains almost as much endogenous cGK activity as the head (tubules: 10.8 $\pm$ 1.3 pmol of ATP/min/mg; heads: 14.9 $\pm$ 0.9 pmol of ATP/min/mg – data from MacPherson et al. 2004b). Interestingly, a mutation in *dg2* which results in a behavioural phenotype does not result in a dg2-associated phenotype in tubules; suggesting tissue-specific effects of such mutations; and/or effects of other components of the GMP signalling pathway (Osborne et al. 1997; MacPherson et al. 2004a). Additionally, targeted overexpression of DG1, DG2P1 and DG2P2 to the principal cells of the tubule is demonstrated to result in significantly enhanced fluid secretion in the tubule in response to exogenous cGMP (MacPherson et al. 2004b).

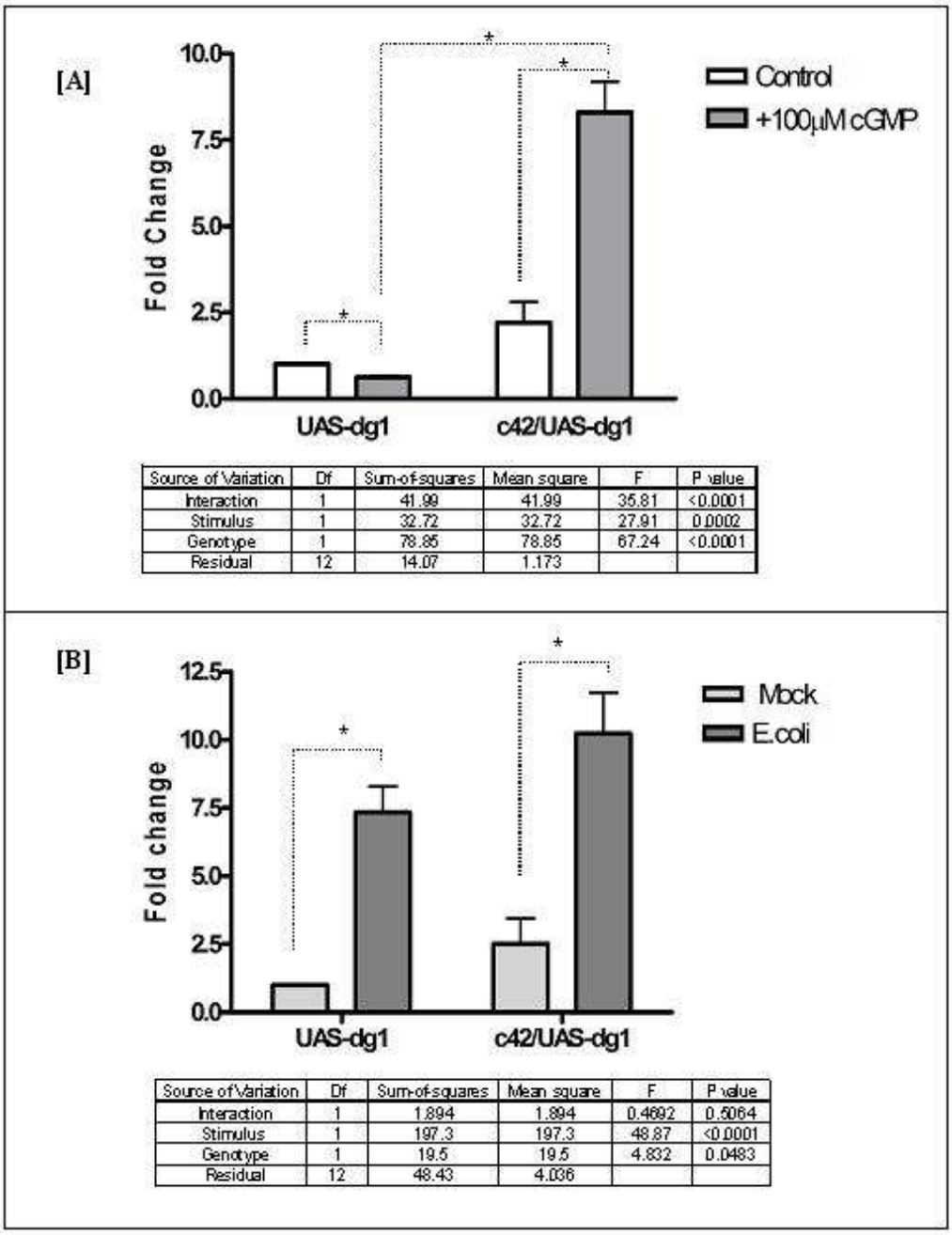
Given the significance of cGK activation in mediating the effects of cGMP in the tubule, it is possible that cGKs mediate the effects of cGMP on the Imd pathway. Interestingly, previous studies have already indicated a role for a mammalian cGK, protein kinase G type 1 (PKG1), as an important immune regulator. For example, studies in mice have shown that PKG1 is highly expressed in a number of murine lymphoid tissues such as the thymus, lymph nodes and the spleen (Kurowska et al. 2002). Additionally, PKG1 has been implicated to play a role in inhibition of T-cell proliferation, and has been identified in a number of studies as an important regulator of neutrophil chemotaxis and granule secretion (Wyatt et al. 1991; Pryzwansky et al. 1995; VanUffelen et al. 1997; Fischer et al. 2001).

In order to determine the involvement of *Drosophila* cGKs in regulation of the Imd pathway, a transgenic approach was used. In this approach, targeted expression or knock-down of cGKs was achieved using transgenic lines for *dg1*, *dg1*RNAi, *dg2P1, dg2P2* and *dg2*RNAi under control of the UAS promoter (flies generated by Dr M. R. MacPherson, University of Glasgow; (MacPherson et al. 2004b)). In order to target overexpression or knockdown of these genes, the UAS-transgene fly lines described above were crossed to the tubule principal cell-specific GAL4 driver line, c42. Specificity of this driver line has been established previously in a number of studies using both UAS-aequorin and UAS-YFP transgenic flies, and counter-staining of cell nuclei (Rosay et al. 1997; Sozen et al. 1997; Broderick et al. 2004; McGettigan et al. 2005). It should also be noted that the UAS-*dg1,* UAS-*dg2P1* and UAS-*dg2P2* transgenic lines used in this study have also been previously validated to show increased expression and cGK activity in the tubule when crossed to c42 driver flies (MacPherson et al. 2004b). Validation of knockdown of cGK activity in the tubules of c42/UAS-*dg1*RNAi and c42/UAS-*dg2*RNAi flies is demonstrated in later in this chapter. Diptericin expression was monitored in acutely-dissected tubules from the resultant progeny of the crosses described above using Q-PCR.

4.2.2 DG1 is a positive regulator of diptericin expression in the principal cells of the tubule

To date, functional roles of *Drosophila* DG1 *in vivo* have not been extensively documented. Previous studies have demonstrated that *dg1* encodes an enzyme with *bona fide* cGK activity that is highly expressed in optic lobes and proximal cortex of the head, suggesting a role in neuronal function (Foster et al. 1996). Additionally, studies have shown that DG1 is highly expressed in the Malpighian tubules, where it has been demonstrated to be important in cGMP-mediated fluid secretion (MacPherson et al. 2004b). Interestingly, microarray studies have demonstrated that DG1 expression is actually approximately 16-fold enriched in the tubule compared to the rest of the fly (http://www.mblab.gla.ac.uk/%7Ejulian/arraysearch.cgi; Wang et al. 2004). Thus, there may be other, yet unexplored, roles of DG1 in tubule function.

In order to determine whether any of the effects of cGMP seen in the previous chapter are mediated by DG1, diptericin expression was monitored in response to various stimuli in



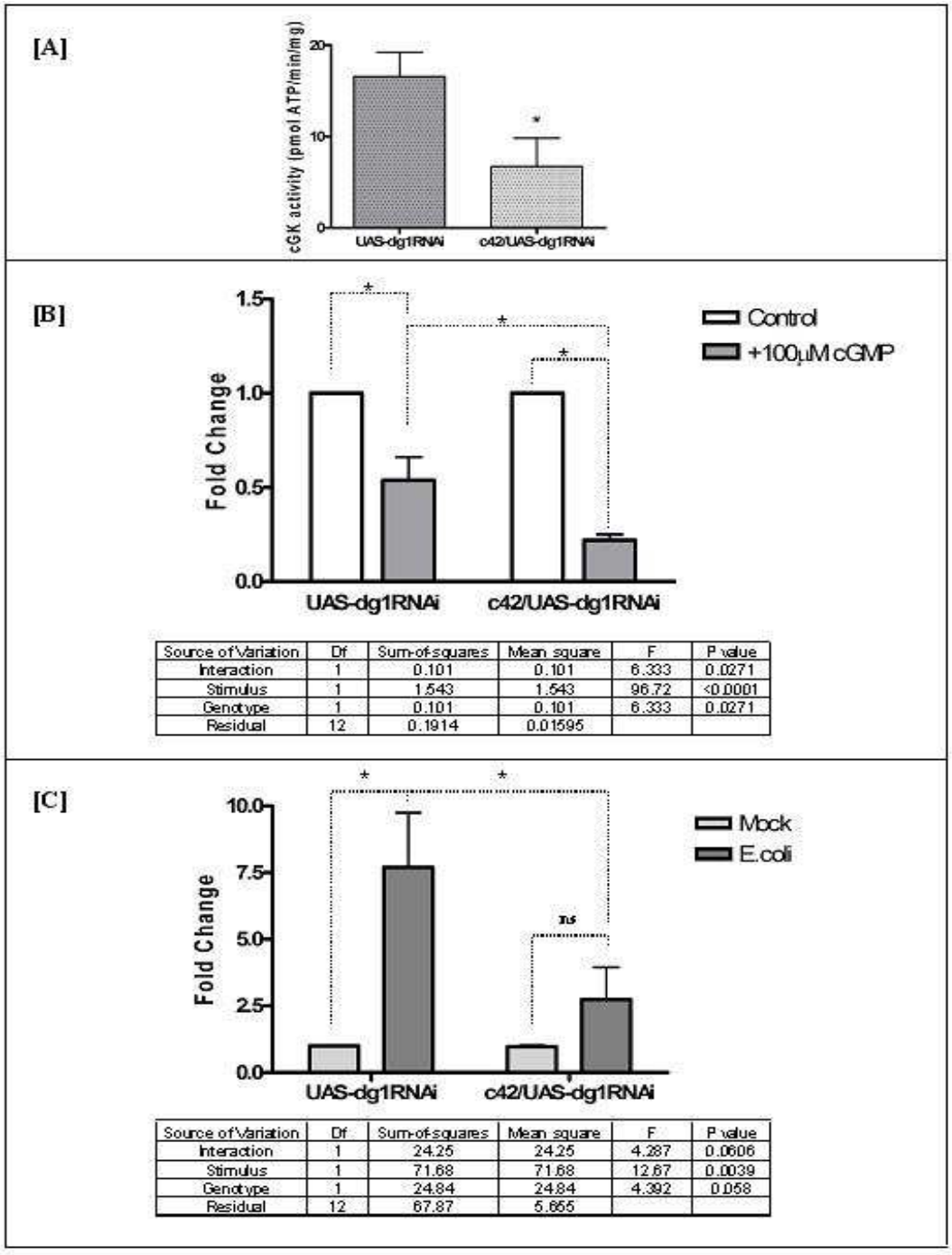
Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	41.99	41.99	35.81	<0.0001
Stimulus	1	32.72	32.72	27.91	0.0002
Genotype	1	78.85	78.85	67.24	<0.0001
Residual	12	14.07	1.173		

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	1.894	1.894	0.4692	0.5064
Stimulus	1	197.3	197.3	48.87	<0.0001
Genotype	1	19.5	19.5	4.832	0.0483
Residual	12	48.43	4.036		

Figure 4.1 – Targeted overexpression of *dg1* to the principal cells of the tubule results in increased diptericin expression. Expression of diptericin in response to specific stimuli was assessed by Q-PCR in excised tubules of adult flies in which *dg1* expression was modulated in tubule principal cells using the GAL4/UAS binary system. Resulting data were normalised against expression of the standard, *rp49*, and expressed as a fold change of parental control expression where control =1 (N = 4, $\pm$ SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis was carried out using Bonferroni tests, whereby significant data are indicated by * ($P<0.05$). [A] Diptericin expression in the tubule in response to cGMP when *dg1* is overexpressed. Analysis by two-way ANOVA reveals a significant interaction between genotype and stimulus (See table). Interestingly, *post hoc* analysis shows a significant difference in diptericin expression in the tubules of c42/UAS-*dg1* flies compared to parental flies in response to cGMP. [B] Diptericin expression in the tubule in response to *E.coli* when *dg1* is overexpressed. Analysis by two-way ANOVA reveals no significant interaction between genotype and stimulus. However, the effect of stimulus alone and genotype alone is considered significant. Although diptericin expression levels are higher in the tubules of c42/UAS-*dg1* flies compared to parental flies in response to *E.coli*, *post hoc* analysis did not reveal a significant difference.

the excised tubules of adult flies either over-expressing *dg1* or flies where *dg1* expression was significantly reduced in the tubules via RNAi. To do this, parental lines containing either UAS-*dg1* or UAS-*dg1*RNAi transgenes were crossed to c42 driver flies. Tubules were excised from 7-day old adult flies of both the UAS-transgene parental lines (as a control) and the c42/UAS-transgene progeny. Excised tubules were then incubated for 3 h in either sterile Schneider's medium (control) or sterile Schneider's medium containing 100 μM cGMP. cDNA was then generated from these samples and diptericin expression quantified by Q-PCR. Similarly, in order to test the effect of DG1 on diptericin expression in the tubule after infection, the same fly lines were inoculated via bacterial injection of *E.coli*, a Gram-negative bacteria known to induce the Imd pathway (Lemaitre et al. 1995a). In order to control for possible changes in diptericin expression as a result of injury from injection, a number of flies from each fly line were also mock-injected using a sterile needle. Tubules were then excised 3 h post infection and diptericin expression quantified by Q-PCR as described above.

Interestingly, it can be seen from the results that DG1 acts as a positive regulator of diptericin expression in the tubule. As Figure 4.1A shows, targeted overexpression of *dg1* results in an approximately 2-fold increase in diptericin expression in the tubules of c42/UAS-*dg1* flies compared to parental controls, even in the absence of stimulation. Unsurprisingly, when tubules of UAS-*dg1* parental flies are stimulated with 100 μM cGMP, diptericin expression is significantly reduced in the tubule, which is similar to the response of wild-type flies demonstrated in the previous chapter. However in contrast, diptericin expression is significantly increased in the tubules of c42/UAS-*dg1* flies in response to cGMP stimulation. These data therefore indicate that the changes in diptericin expression seen in the tubules of c42/UAS-*dg1* flies are a result of activation of DG1 by cGMP. When flies are infected with *E.coli* (Figure 4.1B), diptericin expression levels are significantly higher in the tubules of both the UAS-*dg1* parental and the c42/UAS-*dg1* flies. However, it can be seen that diptericin expression is induced to a higher degree in the tubules of flies where *dg1* is overexpressed than those where *dg1* expression has not been modulated, although, according to statistical analysis, expression levels between genotypes are not significantly different. Therefore, although it is indicated from Figure 4.1B that DG1 acts as a positive regulator



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	0.101	0.101	6.333	0.0271
Stimulus	1	1.543	1.543	96.72	<0.0001
Genotype	1	0.101	0.101	6.333	0.0271
Residual	12	0.1914	0.01595		

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	24.25	24.25	4.287	0.0606
Stimulus	1	71.68	71.68	12.67	0.0039
Genotype	1	24.84	24.84	4.392	0.058
Residual	12	67.87	5.655		

Figure 4.2 – Targeted reduction of *dg1* expression in the principal cells of the tubule results in reduced diptericin expression. Expression of diptericin in response to specific stimuli was assessed by Q-PCR in excised tubules of adult flies as described previously (N = 4, $\pm$ SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis carried out using Bonferroni tests, whereby significant data are indicated by * ($P<0.05$) or, where relevant, ns (not significant). [A] cGK activity in the tubules when *dg1* expression is knocked-down (N = 6, $\pm$ SEM). cGK activity is significantly reduced in the tubules of c42/UAS-*dg1*RNAi flies compared to parental controls. [B] Diptericin expression in the tubule in response to cGMP after *dg1*-knockdown in the tubule. Analysis by two-way ANOVA reveals a significant interaction between genotype and stimulus (See table). Additionally, *post hoc* analysis shows a significant difference in diptericin expression in the tubules of c42/UAS-*dg1*RNAi flies compared to parental flies in response to cGMP. [C] Diptericin expression in the tubule in response to *E.coli* when *dg1* expression is reduced in the tubule. Analysis by two-way ANOVA reveals no significant interaction between genotype and stimulus. Interestingly, *post hoc* analysis shows that tubules with reduced *dg1* expression are unable to significantly induce diptericin expression in response to *E.coli*, unlike controls.

of diptericin expression in the tubule in response to *E.coli*, further investigation is required to determine the significance of this effect.

Therefore, in order to confirm the positive effect of DG1, studies were carried out to monitor diptericin expression in tubules where *dg1* expression was knocked-down by RNAi. Experiments were carried as above, using progeny with the c42/UAS-*dg1*RNAi genotype. This was compared with tubules from 7-day old parental line UAS-*dg1*RNAi and c42/UAS-*dg1*RNAi transgenic flies.

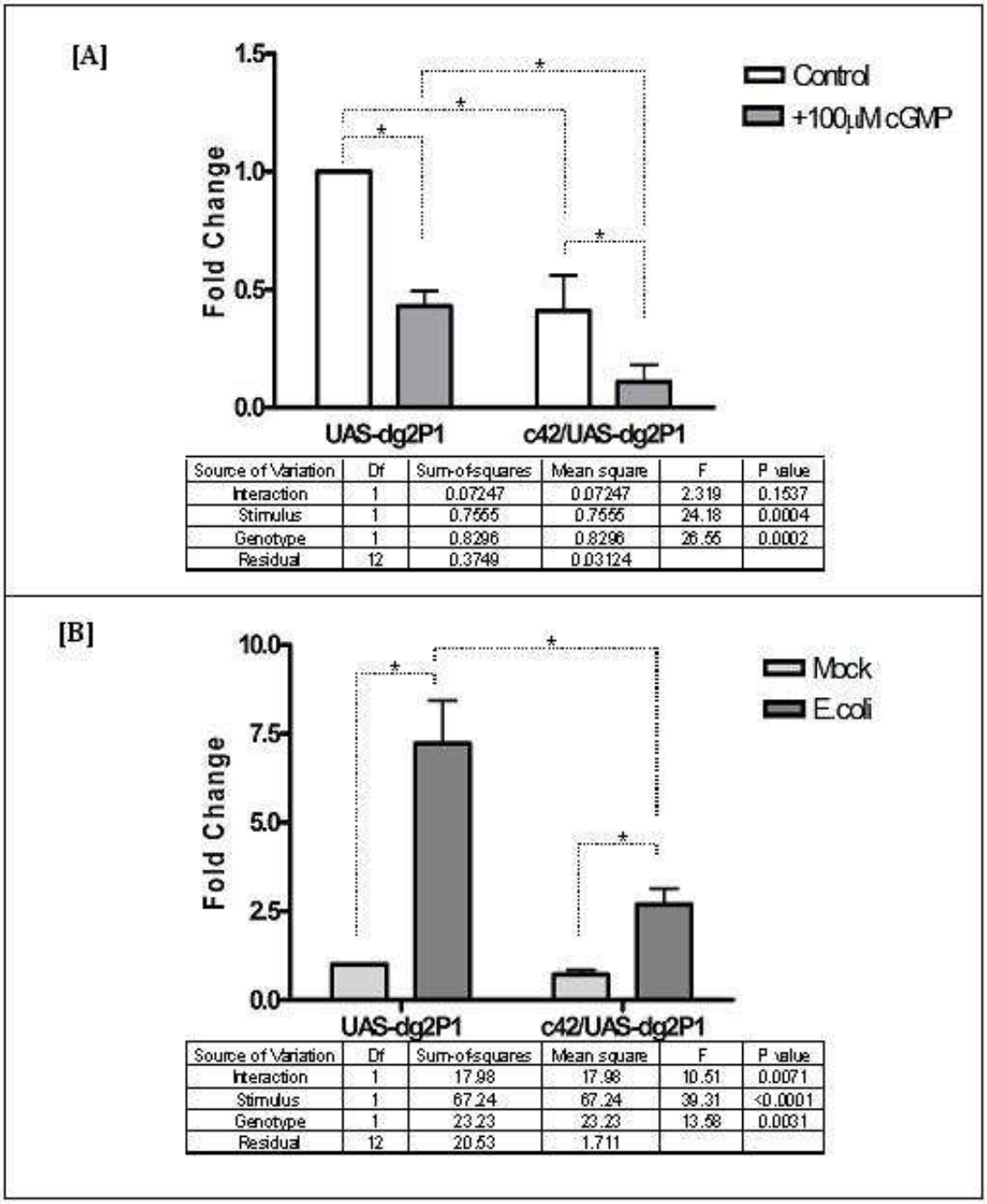
Figure 4.2A shows that assay of cGK activity in the tubules of UAS-*dg1*RNAi and c42/UAS-*dg1*RNAi flies results in a significant reduction in cGK activity in the tubules of flies where *dg1* expression is knocked down. These data therefore confirm that *dg1* expression has been knocked down sufficiently enough to significantly effect cGK activity in the tubules of c42/UAS-*dg1*RNAi flies.

It can be seen from Figure 4.2B that, as with wild type flies, diptericin expression is reduced in the tubules of UAS-*dg1*RNAi parental controls in response to cGMP. However, in contrast to results seen in Figure 4.1A, diptericin expression is also significantly reduced in the tubules of c42/UAS-*dg1*RNAi flies in response to cGMP. Interestingly, results show that the reduction of diptericin expression seen in response to cGMP in the tubules of the flies where *dg1* expression is knocked-down is significantly greater than in those of parental controls. Given that the negative effect of 100 μM cGMP on diptericin expression normally seen in the tubules of both wild-type flies and parental controls is significantly enhanced in the absence of DG1, these data therefore further suggest that DG1 plays a role in positively regulating diptericin expression in the tubule. In support of this data, it can be seen from Figure 4.2C that tubules from c42/UAS-*dg1*RNAi flies are unable to significantly induce diptericin expression in response to infection with *E.coli*, unlike parental controls. These data would therefore suggest that DG1 is required by the tubule in order to sufficiently induce diptericin expression in response to infection with Gram-negative bacteria. Overall, the data presented in Figures 4.1 and 4.2 demonstrate a stimulatory role for DG1 in the regulation of diptericin expression in the tubule.

4.2.3 DG2P1 and DG2P2 are negative regulators of diptericin expression in the principal cells of the tubule

To date, a definitive role for the *dg2* gene has been established in a number of neuronal processes such as feeding behaviour, sensory responsiveness, and learning and memory (Osborne et al. 1997; Scheiner et al. 2004; Mery et al. 2007). However, as with *dg1*, *dg2* has also been established to play an important role in cGMP-mediated epithelial fluid transport in the Malpighian tubules (MacPherson et al. 2004a; 2004b). As mentioned previously, *dg2* is a complex gene that encodes ten major transcripts (http://flybase.bio.indiana.edu/cgi-bin/uniq.html?FBgn0000721%3Efbtr). However, to date, functional studies have only identified the two major transcripts of *dg2*, DG2P1 and DG2P2 to exhibit *bona fide* cGK activity (MacPherson et al. 2004b). Interestingly, these studies also revealed that these transcripts are differentially localised within the tubule, whereby DG2P1 is located on the apical membrane and DG2P2 is located on both the basolateral and apical membrane (MacPherson et al. 2004b). As mentioned above, DG2P1 and DG2P2 have both been implicated in the regulation of fluid secretion in the tubule. However, due to the presence of multiple isoforms of DG2 in the tubule, and the demonstration that the two major transcripts of DG2 are differentially localised within this tissue, it can be suggested that there may be multiple roles for DG2 in tubule function that have yet to be explored.

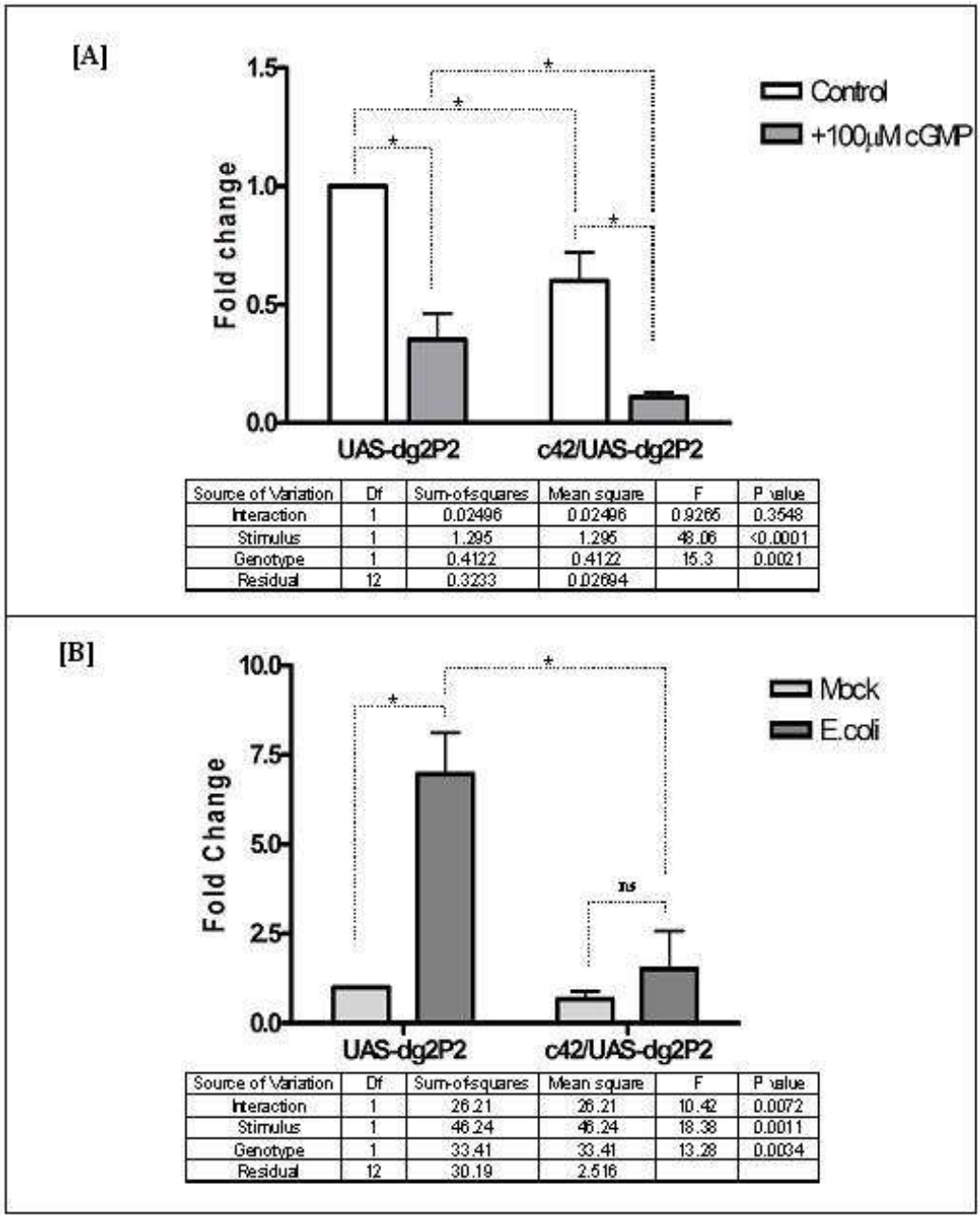
Therefore, studies were initiated in order to determine the possible effect of the two major isoforms of DG2, P1 and P2, on cGMP-mediated immune regulation. As with previous experiments, expression of diptericin was monitored in response to either 100 μM cGMP or infection with *E.coli* in the tubules of flies either over-expressing *dg2P1* or *dg2P2*, or flies where expression of the *dg2* gene has been knocked-down in the tubules via RNAi. Targeted expression of *dg2P1* and *dg2P2* in the tubule was achieved by crossing parental lines containing UAS-*dg2P1* or UAS-*dg2P2* transgenes to c42 GAL4 driver flies. Tubules were then excised from both UAS-transgene parental lines and the resultant c42/UAS-transgene progeny and stimulated as described in section 4.2.2. Similarly, each fly line was inoculated with *E.coli* by bacterial injection as described previously. Diptericin expression was then quantified for each sample obtained by Q-PCR.



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	0.07247	0.07247	2.319	0.1537
Stimulus	1	0.7555	0.7555	24.18	0.0004
Genotype	1	0.8296	0.8296	26.55	0.0002
Residual	12	0.3749	0.03124		

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	17.98	17.98	10.51	0.0071
Stimulus	1	67.24	67.24	39.31	<0.0001
Genotype	1	23.23	23.23	13.58	0.0031
Residual	12	20.53	1.711		

Figure 4.3 - Targeted overexpression of *dg2P1* to the principal cells of the tubule results in decreased diptericin expression. Expression of diptericin in response to specific stimuli was assessed by Q-PCR in excised tubules of adult flies whereby *dg2P1* expression was modulated in tubule principal cells using the GAL4/UAS binary system. Resulting data were normalised against expression of the standard, *rp49*, and expressed as a fold change of parental control expression where control =1 (N = 4, $\pm$ SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis was carried out using Bonferroni tests whereby significant data are indicated by * ($P<0.05$). [A] Diptericin expression in the tubules in response to cGMP when *dg2P1* is overexpressed. Analysis by two-way ANOVA reveals no significant interaction between genotype and stimulus (See table). However, the effects of stimulus alone and genotype alone are considered significant. *Post hoc* analysis shows a significant difference in diptericin expression in the tubules of c42/UAS-*dg2P1* flies compared to parental flies both in the absence and in the presence of cGMP. [B] Diptericin expression in the tubules in response to *E.coli* when *dg2P1* is overexpressed. Analysis by two-way ANOVA reveals a significant interaction between genotype and stimulus. Additionally, *post hoc* analysis shows that diptericin expression is significantly lower in the tubules of c42/UAS-*dg2P1* flies than in UAS-*dg2P1* parents in response to *E.coli*.



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	0.02496	0.02496	0.9265	0.3548
Stimulus	1	1.295	1.295	48.06	<0.0001
Genotype	1	0.4122	0.4122	15.3	0.0021
Residual	12	0.3233	0.02694		

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	26.21	26.21	10.42	0.0072
Stimulus	1	46.24	46.24	18.38	0.0011
Genotype	1	33.41	33.41	13.28	0.0034
Residual	12	30.19	2.516		

Figure 4.4 - Targeted overexpression of *dg2P2* to the principal cells of the tubule results in decreased diptericin expression. Expression of diptericin in response to specific stimuli was assessed by Q-PCR in excised tubules of adult flies whereby DG2P2 expression was modulated in tubule principal cells using the GAL4/UAS binary system. Resulting data were normalised against expression of the standard, *rp49,* and expressed as a fold change of parental control expression where control =1 (N = 4, $\pm$ SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis was carried out using Bonferroni tests whereby significant data are indicated by * ($P<0.05$) or , where relevant, ns (not significant). [A] Diptericin expression in the tubule in response to cGMP when *dg2P2* is overexpressed. Analysis by two-way ANOVA reveals no significant interaction between genotype and stimulus (See table). However, the effect of stimulus alone and genotype alone are considered significant. Additionally, *post hoc* analysis shows a significant difference in diptericin expression in the tubules of c42/UAS-*dg2P2* flies compared to parental flies both in the absence and in the presence of cGMP. [B] Diptericin expression in the tubule in response to *E.coli* when *dg2P2* is overexpressed. Analysis by two-way ANOVA reveals a significant interaction between genotype and stimulus. However, *post hoc* analysis shows that tubules of c42/UAS-*dg2P2* flies are unable to significantly induce diptericin expression in response to *E.coli.* Additionally, diptericin expression is significantly lower in the tubules of c42/UAS-*dg2P1* flies than in UAS-*dg2P1* parents in response to *E.coli.*

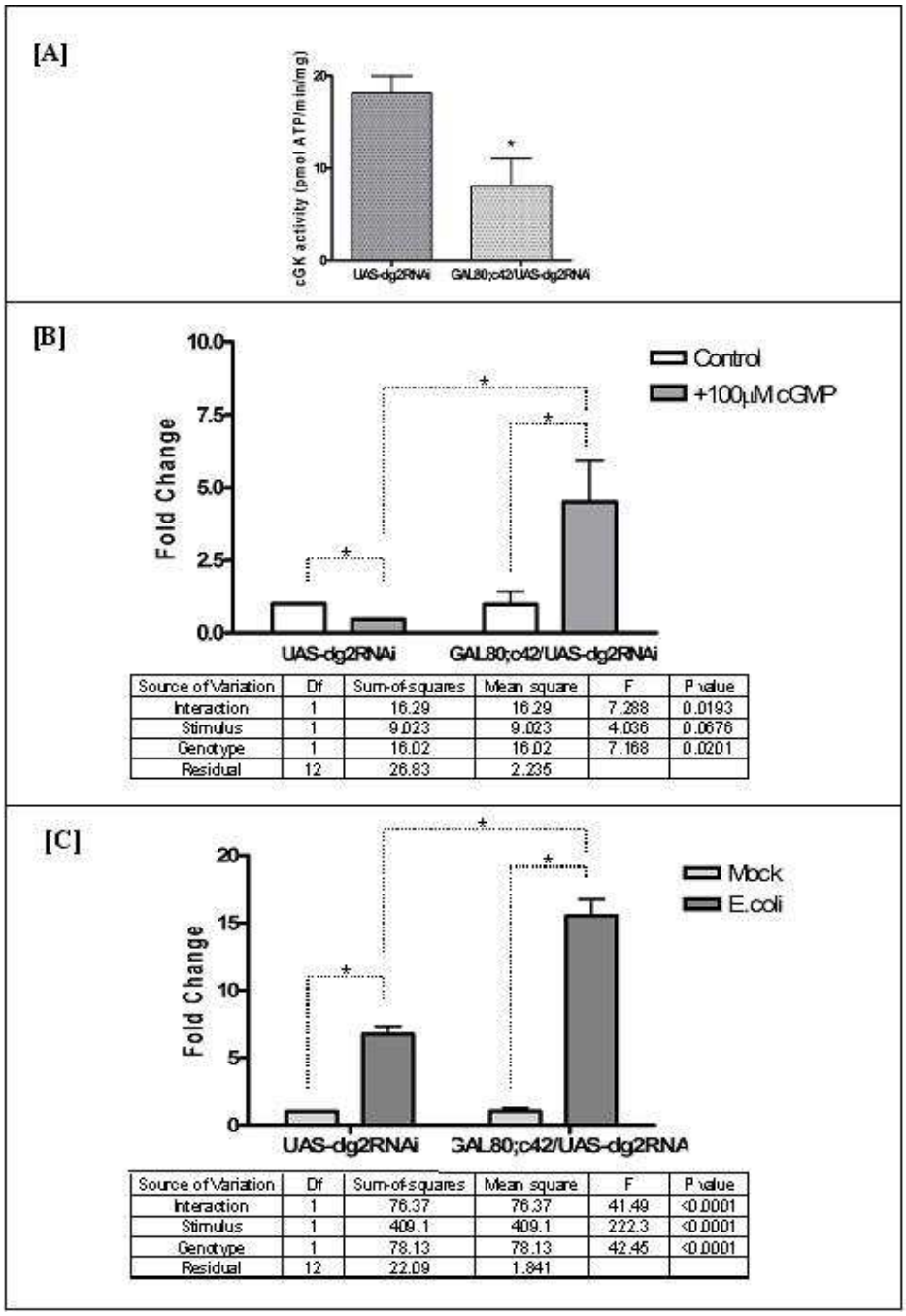
As Figure 4.3A shows, diptericin expression is significantly reduced in the tubules of flies overexpressing *dg2P1* even in the absence of exogenous cGMP. When stimulated with 100 μM cGMP, it can be seen that expression of diptericin is even further reduced in the tubules of c42/UAS-*dg2P1* flies. This reduction in expression follows the same trend as parental lines, however it can be seen that diptericin expression is significantly lower in the tubules of c42/UAS-*dg2P1* flies than in the tubules of parental flies in response to cGMP. These data therefore indicate that, in contrast to DG1, DG2P1 may play an inhibitory role in regulation of diptericin expression. In support of this data, it is demonstrated in Figure 4.3B that when flies are infected with *E.coli,* the tubules of c42/UAS-*dg2P1* flies are unable to induce diptericin expression to the same degree as the tubules of parental flies, further suggesting a role for DG2P1 as a negative regulator of diptericin expression in the tubule.

Similarly, it can be seen from Figure 4.4 that DG2P2 also plays an inhibitory role in regulation of diptericin expression. As with the tubules of flies over-expressing *dg2P1*, when *dg2P2* is over-expressed in the tubule, expression of diptericin is significantly lower than in the tubules of parental controls, even in the absence of stimulation with cGMP (Figure 4.4A). Equally, when tubules are stimulated with 100 μM cGMP, it can be seen that expression of diptericin is even further reduced in the tubules of c42/UAS-*dg2P2* flies. Again it can be seen that changes in diptericin expression in the tubules of both the parental control flies and the c42/UAS-*dg2P2* progeny are following the same trend in response to 100μM cGMP, however diptericin expression is significantly lower in c42/UAS-*dg2P2* flies, thus suggesting that activation of DG2P2 by cGMP acts to negatively regulate diptericin expression in the tubule. In support of these data, it can be seen from Figure 4.4B that the tubules of c42/UAS-*dg2P2* flies are unable to significantly induce diptericin expression in response to infection with *E.coli*. This is in contrast to the tubules of parental controls whereby an approximately 7-fold increase in diptericin expression is demonstrated in response to infection.

In order to confirm the inhibitory role of DG2P1 and DG2P2 on regulation of diptericin expression in the tubule, the effect of knock-down of *dg2* expression was investigated. This was achieved using a UAS-*dg2*RNAi transgenic line (generated by Dr JP Day, University of Glasgow). Unfortunately, when this line was crossed to c42, therefore

knocking down *dg2* expression in the principal cells of the tubule, survival of the progeny beyond pupal stage was minimal, suggesting a critical role for DG2 in the tubules during pupal development. In order to overcome this problem the GAL80 system was used (Lee and Luo 1999). GAL80 is a temperature-sensitive yeast repressor enzyme able to potently inhibit GAL4 activity by binding to its transcriptional activation domain. Fortunately, GAL80 is only active at temperatures under 30 °C, therefore GAL4 activity can be resumed by means of a simple heat-shock step. As such, by combining flies expressing GAL80 with an appropriate GAL4 driver line, it is possible to reversibly repress GAL4-induced expression of target UAS-transgenes in both developing and adult *Drosophila*. For this study, a homozygous stable fly line was generated by crossing flies expressing the GAL80 transgene under the control of a tubulin promoter ([w^*]; P{tubP-$GAL80^{ts}$}; TM2/TM6b - Bloomington Stock Centre) with the c42 GAL4 driver line (P-element insertion on the 3^{rd} chromosome). Following the generation of this stable line ([w^*]; P{tubP-$GAL80^{ts}$}; c42), inducible knock-down of *dg2* expression was achieved by crossing with UAS-*dg2*RNAi transgenic flies. In the progeny of this cross, GAL4 expression was inhibited until 24 h before dissection, at which time flies were incubated at 30°C in order to inactivate GAL80. It should be noted that UAS-*dg2*RNAi parental flies were also incubated at 30°C for 24 h in order to control for changes in expression as a result of temperature.

In order to firstly confirm the expression of the UAS-*dg2*RNAi transgene in the tubules of the GAL80;c42/UAS-*dg2*RNAi flies, cGK activity was assessed in both the UAS-*dg2*RNAi parental line and the GAL80;c42/UAS-*dg2*RNAi progeny. As Figure 4.5A shows, cGK activity is significantly reduced in the tubules of the GAL80;c42/UAS-*dg2*RNAi flies, thus confirming that *dg2* expression has been sufficiently reduced. Following this, experiments were carried out as described previously, whereby diptericin expression was quantified in the tubules of both UAS-*dg2*RNAi and GAL80;c42/UAS-*dg2*RNAi flies in response to either stimulation with cGMP or infection with *E.coli*. As expected, it can be seen from Figure 4.5B that in parental controls diptericin expression is significantly reduced in the tubules in response to 100 μM cGMP. However, in contrast to data shown in Figure 4.3A and 4.4A, it is demonstrated that knock-down of *dg2* expression in the tubules of adult flies results in a significant increase in diptericin expression in response to cGMP. These data therefore further support the indication from



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	16.29	16.29	7.288	0.0193
Stimulus	1	9.023	9.023	4.036	0.0676
Genotype	1	16.02	16.02	7.168	0.0201
Residual	12	26.83	2.235		

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	76.37	76.37	41.49	<0.0001
Stimulus	1	409.1	409.1	222.3	<0.0001
Genotype	1	78.13	78.13	42.45	<0.0001
Residual	12	22.09	1.841		

Figure 4.5 - Targeted knock-down of *dg2* expression by RNAi results in increased diptericin expression in the tubule. Expression of diptericin in response to specific stimuli was assessed by Q-PCR in excised tubules of adult flies as described previously (N = 4, $\pm$ SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis carried out using Bonferroni tests whereby significant data are indicated by * ($P<0.05$). [A] cGK activity in the tubules when *dg2* expression is knocked-down (N = 6, $\pm$ SEM). Shows that cGK activity is significantly reduced in the tubules of GAL80;c42/UAS-*dg2*RNAi flies compared to parental controls. [B] Diptericin expression in the tubule in response to cGMP after *dg2*-knockdown in the tubule. Analysis by two-way ANOVA reveals a significant interaction between genotype and stimulus (See table). *Post hoc* analysis shows a significant difference in diptericin expression in the tubules of GAL80;c42/UAS-*dg2*RNAi flies compared to parental flies in response to cGMP. [C] Diptericin expression in the tubule in response to *E.coli* when *dg2* expression is knocked-down in the tubule. Analysis by two-way ANOVA reveals a significant interaction between genotype and stimulus. Additionally, *post hoc* analysis shows that when *dg2* expression is knocked-down, diptericin expression is significantly higher in the tubules of GAL80;c42/UAS-*dg2*RNAi flies in response to *E.coli* than in parental flies.

previous results that DG2P1 and DG2P2 act as negative regulators of diptericin expression. Similarly, it can be seen from Figure 4.5C that in tubules where *dg2* expression has been knocked-down, diptericin expression is significantly higher than UAS-*dg2*RNAi parental controls in response to infection with *E.coli*. These data therefore confirm that the cGKs encoded by *dg2* act as suppressors of diptericin expression in the tubule.

4.3 Modulation of immune response by cGKs in the tubule is critical to fly survival in response to septic infection

4.3.1 Introduction

The data described in previous sections of this chapter clearly demonstrate that the *Drosophila* cGKs DG1, DG2P1 and DG2P2 exhibit differential effects on diptericin expression in the tubules of the adult fly. However, the relevance of these findings with regards to overall immunity of the whole animal has not been determined. Traditionally, the fat body has been considered the critical tissue with regards to systemic production of AMPs in response to infection (Silverman and Maniatis 2001). Therefore, it could be suggested that the tubule may only play an auxiliary role as an immune-sensing tissue in the adult fly, and might not impact on survival of the whole organism when under immune challenge. However, as mentioned previously, it has been indicated in a recent study that this is not the case (McGettigan et al. 2005). In this study, it was shown that targeted expression of *dNOS* to tubule principal cells results in increased diptericin expression in the tubules, which in turn was demonstrated to enhance overall survival of adult flies in response to infection with *E.coli*. These data therefore indicate that the tubule comprises an important immune system, with a significant role to play in maintaining fly survival in response to infection.

Studies were therefore initiated in order to determine the overall impact of cGK-mediated modulation of diptericin expression in the tubule. In these studies, each of the transgenic cGK fly lines described previously were monitored for survival in response to septic infection with various bacteria. For each transgenic line, bacteria were introduced directly into the hemolymph of adult flies using a sterile needle dipped into a concentrated suspension of bacterial culture. Similarly, as with previous experiments, a number of flies

from each transgenic line were also mock injected in order to control for any negative impact on survival caused solely by injury. Following infection, each fly line was then monitored for survival and results plotted using a Kaplin-Meier survival curve (Graphpad Prism Version 4.0).

4.3.2 Modulation of immune response by cGKs in the tubule is critical to survival in response to septic infection with Gram-negative bacteria.

As mentioned previously, expression of diptericin is induced following the activation of the Imd signalling pathway. A number of studies have identified a fundamental role of the Imd pathway in response to invasion by Gram-negative bacteria (reviewed in (Lemaitre and Hoffmann 2007). Therefore, in order to test whether cGK-mediated immune regulation in the tubule is sufficient to effect survival of the whole animal, assays were performed to assess survival of cGK transgenic flies in response to septic infection with Gram-negative bacteria. Given that the previous data in this chapter has already revealed that diptericin expression is induced in the tubule in response to *E.coli*, and that this expression can be differentially modulated by DG1, DG2P1 and DG2P2, studies were first of all carried out in order to assess survival of cGK transgenic flies in response to infection with *E.coli*. Targeted expression of UAS-*dg1*, UAS-*dg1*RNAi, UAS-*dg2P1*, UAS-*dg2P2* and UAS-*dg2*RNAi to the principal cells of the tubule was achieved as described previously. In addition, in order to confirm that the modulatory effects of cGKs on immune regulation are tubule-specific, UAS-parental flies were also crossed to the fat body-specific GAL4 driver line c564 (kind gift from Professor S Kurata, University of Sendai, Japan). Finally, it should be noted that, under normal conditions *E.coli* is not a natural pathogen of *Drosophila*. Therefore, it was expected that infection with this bacteria would not confer any significant survival phenotypes in samples of wild-type or parental control flies.

Figure 4.6 shows survival in response to infection with *E.coli* in flies where *dg1* is either overexpressed or knocked-down in the tubule. Previous data has demonstrated that *dg1* acts as a positive regulator of diptericin expression in the tubule, therefore it was expected that overexpression of *dg1* in the tubule would enhance survival of flies in response to *E.coli*. As Figure 4.6A shows, there is no significant difference in survival of c42/UAS-*dg1* flies compared to either wild-type flies (OrR) or parental controls.

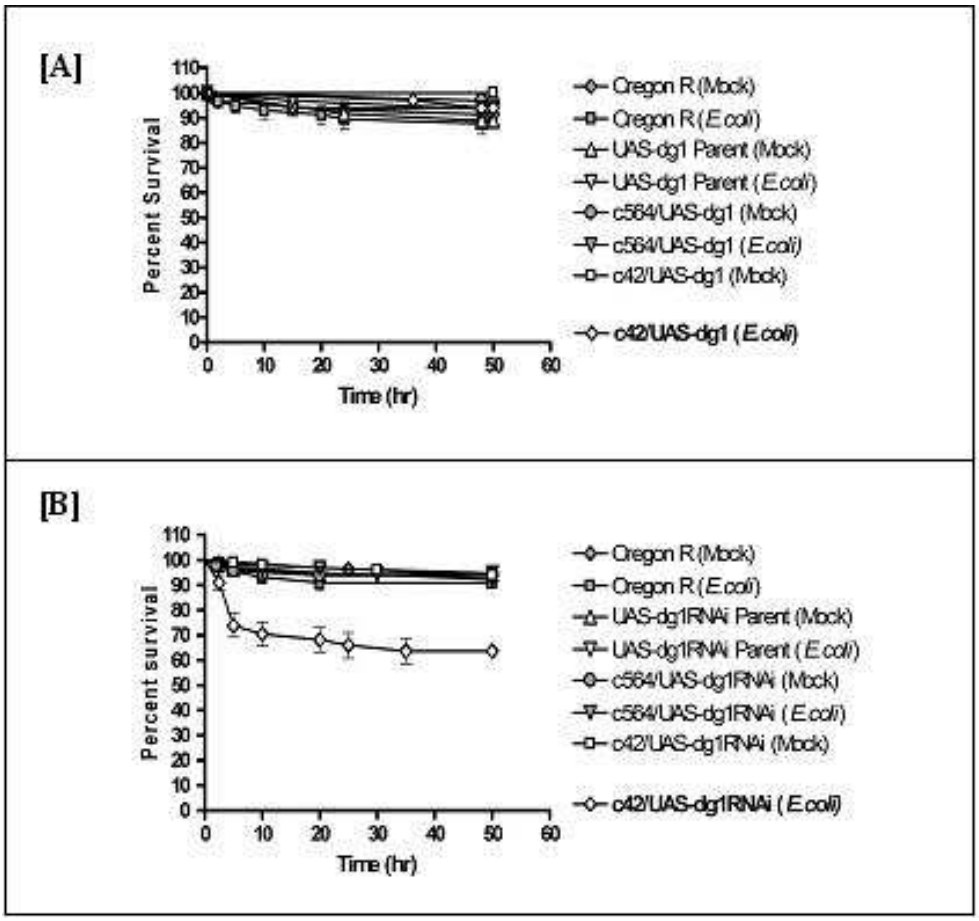


Figure 4.6 – Survival in response to *E.coli* when *dg1* is over-expressed or knocked-down in the tubules. Survival was monitored in 5-day old adult flies that were either infected with a concentrated suspension of *E.coli* via a thin needle, or were mock infected using a sterile needle. Survival of flies was then monitored at appropriate intervals for a 50 h period (N = 30). This protocol was repeated three times on each fly line. Results for each line were then pooled and expressed as a percentage of survival using a Kaplin-Meier survival curve ($^{\pm}$ SEM). [A] Survival in response to *E.coli* when *dg1* is overexpressed in either the tubule (c42) or the fat body (c564) [B] Survival in response to *E.coli* when *dg1* is knocked-down in either the tubule (c42) or the fat body (c564). Data shows a significant decrease in survival in response to *E.coli* when *dg1* is knocked-down in the tubule.

However, as *E.coli* is not naturally pathogenic to *Drosophila*, no definite conclusions can be drawn from this data. However, it can be seen from Figure 4.6B that when *dg1* expression is knocked-down in the tubule, a significant decrease in survival is observed, with approximately 30% of flies dying in the first 5 h after infection. These data therefore indicate, in support of previous data, that DG1 is required by the tubule to mount an appropriate immune response when challenged with *E.coli*. Importantly, it is also demonstrated from this data that sufficient activation of immune responses in the tubule appears to be critical to survival of the whole organism. Interestingly, it can be seen that

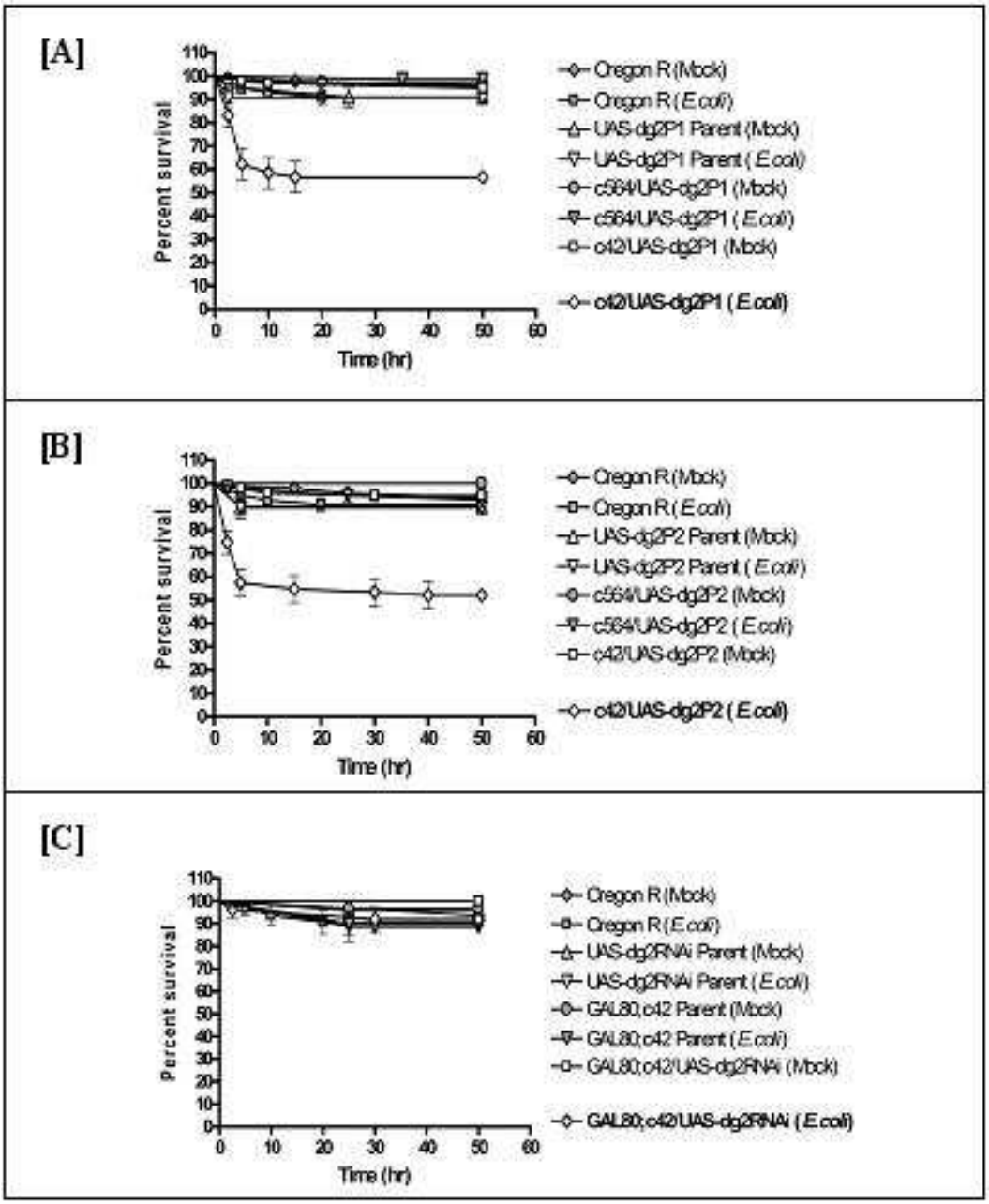


Figure 4.7 - Survival in response to *E.coli* when *dg2* is over-expressed or knocked-down in the tubules. Survival was monitored in 5-day old adult flies that were either infected with a concentrated suspension of *E.coli* via a thin needle, or were mock infected using a sterile needle. Survival of flies was then monitored at appropriate intervals for a 50 h period (N = 30). This protocol was repeated three times on each fly line. Results for each line were then pooled and expressed as a percentage of survival using a Kaplin-Meier survival curve ($^{\pm}$ SEM). [A] Survival in response to *E.coli* when *dg2P1* is overexpressed in either the tubule (c42) or the fat body (c564). Data shows a significant decrease in survival in response to *E.coli* when *dg2P1* is overexpressed in the tubule. [B] Survival in response to *E.coli* when *dg2P2* is overexpressed in either the tubule (c42) or the fat body (c564) Data shows a significant decrease in survival in response to *E.coli* when *dg2P2* is overexpressed in the tubule. [C] Survival in response to *E.coli* when DG2 is knocked-down in either the tubule (GAL80;c42)

DG1 appears to have no effect on survival when expression is modulated in the fat body, further suggesting that cGK-mediated immune regulation is tubule-specific. When survival is monitored in response to *E.coli* in flies overexpressing the two DG2 isoforms, it can be seen that targeted expression of *dg2P1* or *dg2P2* to the tubules results in significant survival phenotypes in each line. As Figures 4.7A and 4.7B show, in each case, infection with *E.coli* results in an approximately 40% decrease in survival when *dg2P1* or *dg2P2* are overexpressed in the tubule. Again, these data support previous results where it was demonstrated that DG2P1 and DG2P2 act as negative regulators of diptericin expression in the tubule. In contrast, it can be seen that when *dg2* expression is knocked-down in the tubule, there is no significant difference in fly survival in response to *E.coli* infection compared to controls (Figure 4.7C). Overall, this data further demonstrates the inhibitory role DG2P1 and DG2P2 play in maintaining appropriate levels of immune function in the tubules in response to infection with *E.coli.* As before, the important role of the tubules in overall fly immunity is also highlighted from the data. Interestingly, again it can be seen from Figures 4.7A and 4.7B that the modulatory effects of either DG2P1 or DG2P2 on immune function do not extend to the fat body. Therefore, again it can be seen that this effect appears to be specific to the tubule.

Overall, it can be seen from this data that cGK-mediated modulation of immune response in the tubule is necessary for the survival of the whole fly in response to *E.coli.*

As mentioned above, *E.coli* is not a natural pathogen to *Drosophila* under normal circumstances. Thus, it is difficult to confirm any enhancement to survival in response to infection in the c42/UAS-*dg1* or GAL80;c42/UAS-*dg2*RNAi fly lines. Therefore, studies were initiated in order to determine survival of transgenic cGK fly lines in response to *Pseudomonas aeruginosa*, an opportunistic and versatile Gram-negative bacterium that is highly pathogenic to wild-type *Drosophila*, whereby 100% fly mortality has been observed within 30 h of infection (D'Argenio et al. 2001; Apidianakis et al. 2005). Previous studies have shown that resistance to infection with these bacteria is both Imd and Toll-dependent, and that a wide variety of AMPs, especially those associated with the Imd pathway, are induced in response to infection. However, despite induction of systemic immune mechanisms in response to these bacteria, it has been shown that *P.aeruginosa* is able to overcome and suppress this induction (Lau et al. 2003; Apidianakis et al. 2005). Therefore, it was hypothesised that, in flies where cGK activity

has been modulated in the tubules in order to either stimulate or inhibit AMP expression prior to infection, different rates of survival may be observed in response to infection with *P.aeruginosa*. Targeted expression or knock-down of cGKs was achieved as described previously and adult flies were inoculated as before using a sterile needle dipped into an appropriate suspension of *P.aeruginosa*.

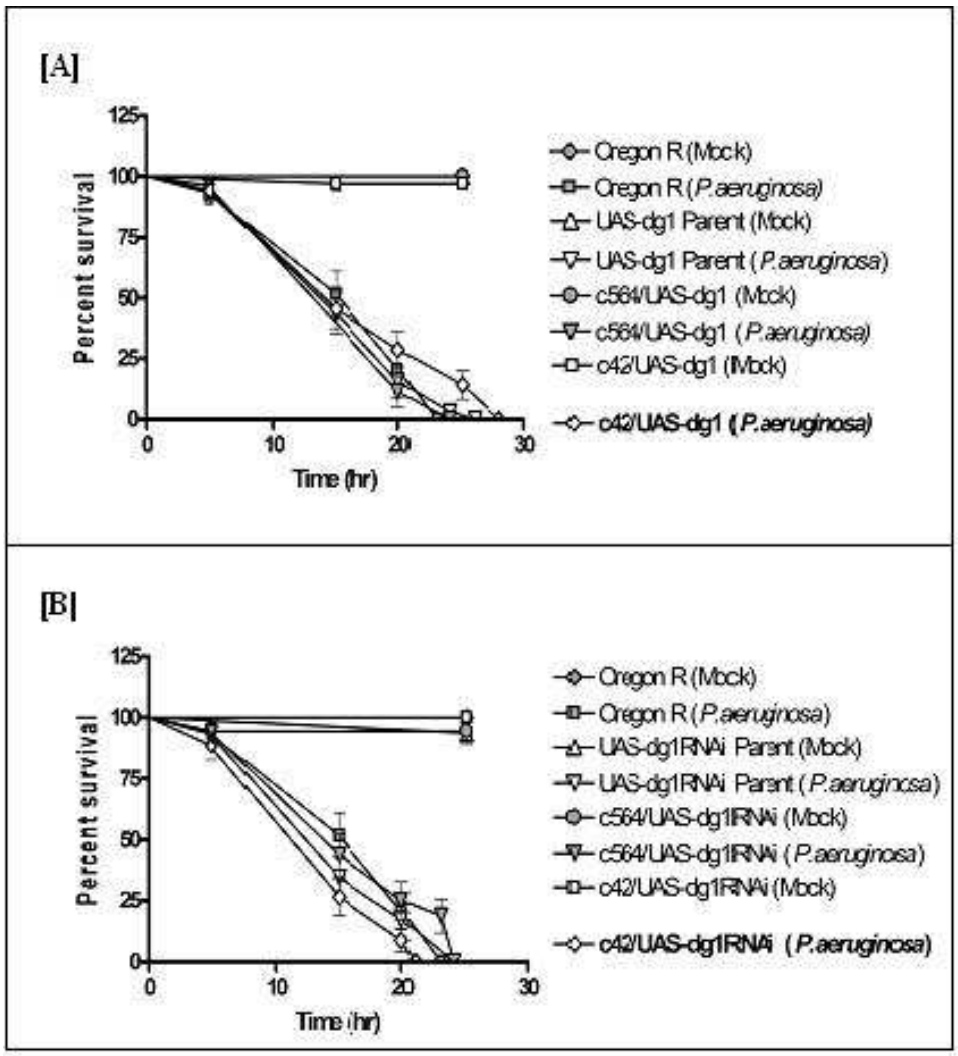


Figure 4.8 - Survival in response to *P.aeruginosa* when *dg1* is over-expressed or knocked-down in the tubules. Survival was monitored in 5-day old adult flies that were either infected with a concentrated suspension of *P.aeruginosa* via a thin needle, or were mock infected using a sterile needle. Survival of flies was then monitored at appropriate intervals for a 30 h period (N = 30). This protocol was repeated three times on each fly line. Results for each line were then pooled and expressed as a percentage of survival using a Kaplin-Meier survival curve ($^{\pm}$ SEM). [A] Survival in response to *P.aeruginosa* when *dg1* is overexpressed in either the tubule (c42) or the fat body (c564). Data shows a slight, but non-significant enhancement of survival in response to *P.aeruginosa* in c42/UAS-*dg1* flies [B] Survival in response to *P.aeruginosa* when *dg1* is knocked-down in either the tubule (c42) or the fat body (c564). Data shows a slight, but non-significant, decrease in survival in response to *P.aeruginosa* in c42/UAS-*dg1*RNAi flies.

It can be seen from the results shown in Figures 4.8 and 4.9 that, in agreement to previous studies, infection with *P.aeruginosa* results in 100% mortality within 30 h in all fly lines tested. However, it can also be seen that in flies where cGK activity has been modulated in the tubule, rates of survival are either enhanced or decreased in response to infection, although this effect is not significant. To explain further, it can be seen from Figure 4.8A that when *dg1* is overexpressed in the tubule, complete fly mortality does not occur until ~28 h post infection, unlike in all other fly lines whereby 100% mortality is observed at 24-25 h post infection. Similarly, in Figure 4.8B, it can be seen that when *dg1* is knocked-down in the tubule, rate of survival is decreased compared to all other fly lines whereby complete fly mortality is observed at ~21 h and 24-25 h post infection respectively. Although these data do not exhibit a significant change in survival rates in response to *P.aeruginosa* infection when *dg1* expression is modulated in the tubule, they do imply that DG1 may act in the tubule to enhance immune response and therefore survival of flies in response to infection with these bacteria. Additionally, as with infection by *E.coli*, modulation of DG1 in the fat body does not appear to mediate any effect on survival in response to *P.aeruginosa*. Similarly, it can be seen that when *dg2* is modulated in the tubules, a negative effect on fly survival is observed in response to infection with *P.aeruginosa* (Figure 4.9). As Figure 4.9A and 4.9B shows, when expression of *dg2P1* or *dg2P2* is increased in the tubule, complete fly mortality is seen at ~20 h post infection. Again it can be seen that 100 % mortality of all other lines tested is observed at 24-25 h after infection, suggesting that DG2P1 and DG2P2 confer an inhibitory effect on immune responses in the tubule in response to *P.aeruginosa*. When *dg2* is knocked-down in the tubule, a very slight enhancement of survival is observed compared to all other fly lines tested, although again this enhancement is not significant (Figure 4.9C). It can therefore be implied from the data shown in Figure 4.9 that again, although modulation of DG2 in the tubule does not confer a significant effect on survival of flies in response to *P.aeruginosa*, DG2P1 and DG2P2 may act to inhibit induction of immune responses in the tubule in response to infection with these bacteria.

Overall, the role of cGK-mediated immune regulation in the tubule in response to infection with *P.aeruginosa* can only be implied from the data shown in Figures 4.8 and 4.9. Although modulation of cGKs in the tubule has a clear effect on survival in the majority of cases, the effects observed are not deemed significantly different. Therefore further investigation is required in order to confirm these findings.

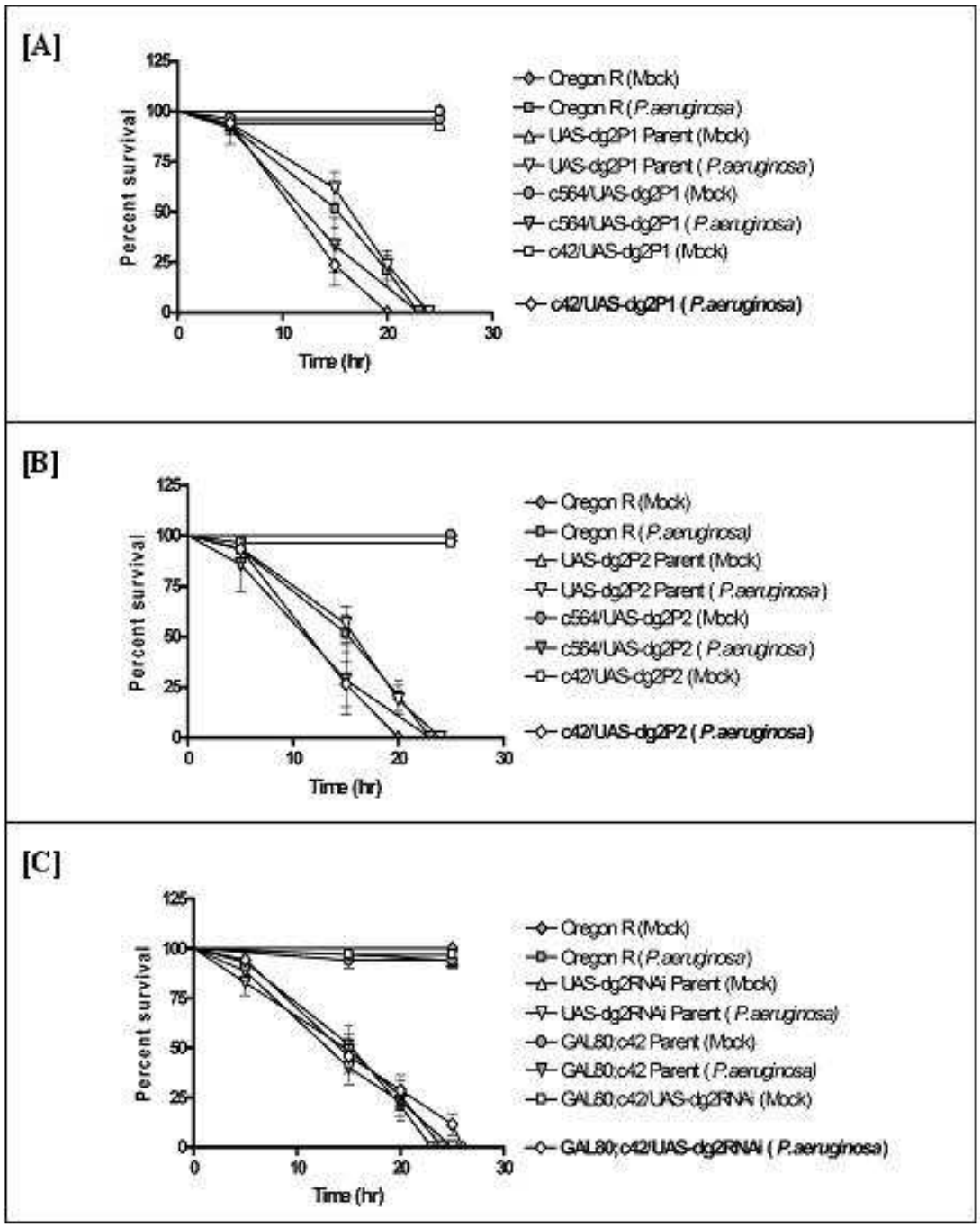


Figure 4.9 - Survival in response to *P.aeruginosa* when *dg2* is over-expressed or knocked-down in the tubules. Survival was monitored in 5-day old adult flies that were either infected with a concentrated suspension of *P.aeruginosa* via a thin needle, or were mock infected using a sterile needle. Survival of flies was then monitored at appropriate intervals for a 30 h period (N = 30). This protocol was repeated three times on each fly line. Results for each line were then pooled and expressed as a percentage of survival using a Kaplin-Meier survival curve ($^{\pm}$ SEM). [A] Survival in response to *P.aeruginosa* when *dg2P1* is overexpressed in either the tubule (c42) or the fat body (c564). Data shows a slight, but non-significant, decrease in survival in response to *P.aeruginosa* when *dg2P1* is overexpressed in the tubule. [B] Survival in response to *P.aeruginosa* when *dg2P2* is overexpressed in either the tubule (c42) or the fat body (c564) Data shows a slight, but non-significant decrease in survival when *dg2P2* is overexpressed in the tubule. [C] Survival in response to *P.aeruginosa* when *dg2* expression is knocked-down in the tubule. Data shows a very slight, but non-significant, increase in survival in GAL80;c42/UAS-*dg2*RNAi flies.

4.3.3 Modulation of immune response by cGKs in the tubule does not effect survival in response to septic infection with the Gram-positive bacteria *Bacillus subtilis*.

As mentioned previously, activation of the Imd pathway is deemed critical to the production of specific AMPs in response to infection with Gram-negative bacteria (Lemaitre et al. 1995a). In contrast, the other systemic immune pathway, the Toll pathway, has been demonstrated as fundamental to responses against infection with either Gram-positive bacteria or fungi. Therefore, studies were initiated in order to determine the effect of cGK-mediated AMP modulation in the tubule in response to Gram-positive bacteria. Since previous data has only demonstrated an effect of cGMP on Imd pathway-associated AMPs, it was hypothesised that cGKs would also only play a role in regulation of Imd pathway-associated AMP expression.

In order to carry out this experiment, flies were inoculated as before with the Gram-positive bacteria *Bacillus subtilis*. Previous studies have shown that, although harmless to humans, *B. subtilis* is naturally pathogenic to *Drosophila* (Tzou et al. 2002b). However, as with *P. aeruginosa*, observations that flies carrying mutations in both *imd* and *spz* (Spaetzle) are more susceptible to infection with *B. subtilis* than wild-type flies suggest that *Drosophila* is able to induce a limited defence against this pathogen. This defence appears to be dependent on the induction of the Toll pathway-dependent AMP defensin, as studies show that ubiquitous overexpression of this AMP prior to infection is enough to confer complete resistance against *B.subtilis* (Tzou et al. 2002). Therefore, as data presented in this thesis so far has not indicated a role for cGMP or cGKs in regulation of defensin expression, it is hypothesised that cGK-mediated immune regulation in the tubule will have no effect on survival in response to infection with *B.subtilis*.

As Figure 4.10A shows, when *dg1* is over-expressed in the tubules, no significant effect on survival in response to infection with *B. subtilis* is observed compared to controls. Equally, when *dg1* is knocked-down in the tubule (Figure 4.10B), there is no significant difference in survival between all fly lines tested in response to *B. subtilis*. Additionally, it can be seen that, as with all previous survival data, modulation of *dg1* expression in the fat body does not effect survival in response to infection. These data therefore

demonstrate that DG1 is not required for regulation of immune responses in either the tubule or the fat body in response to infection by *B. subtilis*.

Similarly, it can be seen from Figure 4.11 that modulation of *dg2* expression in the tubule also has no effect on fly survival in response to *B. subtilis*. As both Figures 4.11A and 4.11B show, there is no significant difference in rate of survival in any of the fly lines tested. Similarly, it can be seen from Figure 4.11C that knock-down of *dg2* in the tubule does not confer a significantly different survival phenotype in response to infection either. Again, there is also no significant effect when *dg2P1* or *dg2P2* are overexpressed

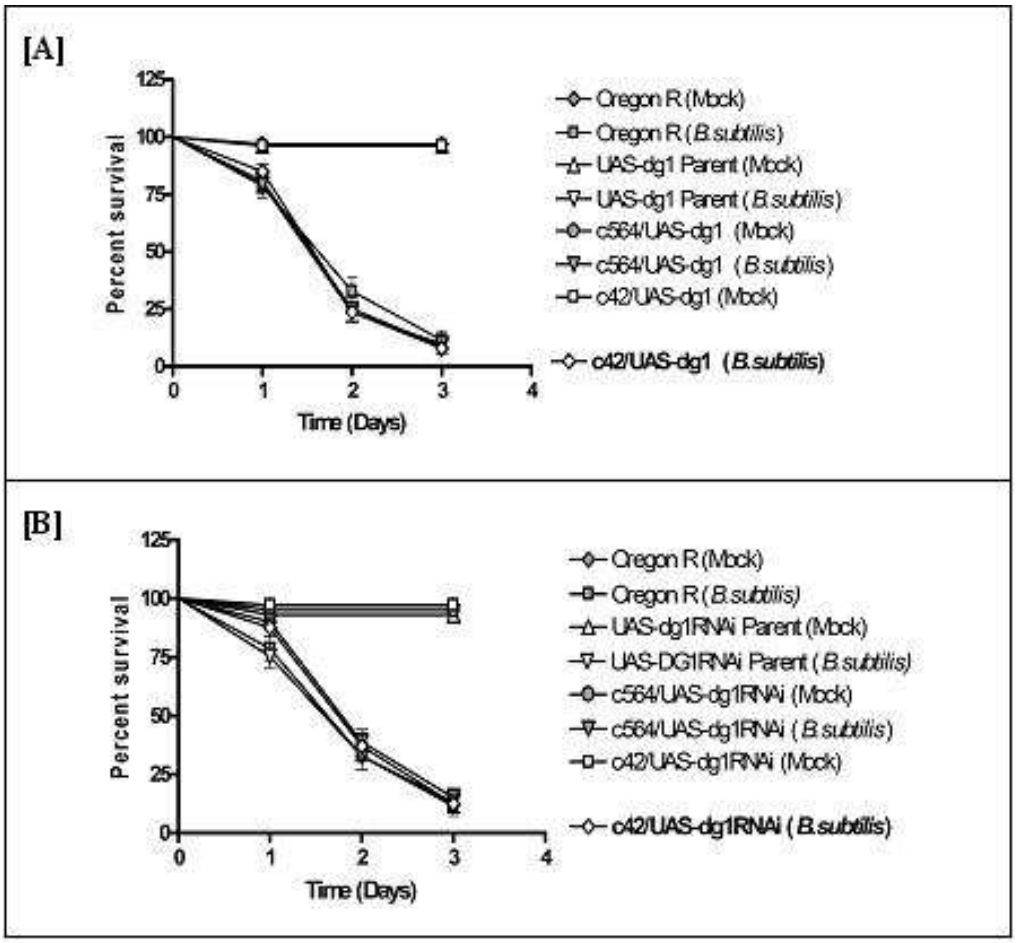


Figure 4.10 - Survival in response to *B. subtilis* when *dg1*1 is over-expressed or knocked-down in the tubules. Survival was monitored in 5-day old adult flies that were either infected with a concentrated suspension of *B. subtilis* via a thin needle, or were mock infected using a sterile needle. Survival of flies was then monitored at appropriate intervals for a 3 day period (N = 30). This protocol was repeated three times on each fly line. Results for each line were then pooled and expressed as a percentage of survival using a Kaplin-Meier survival curve ($^{\pm}$ SEM). [A] Survival in response to *B. subtilis* when *dg1* is overexpressed in either the tubule (c42) or the fat body (c564). [B] Survival in response to *B. subtilis* when *dg1* is knocked-down in either the tubule (c42) or the fat body (c564).

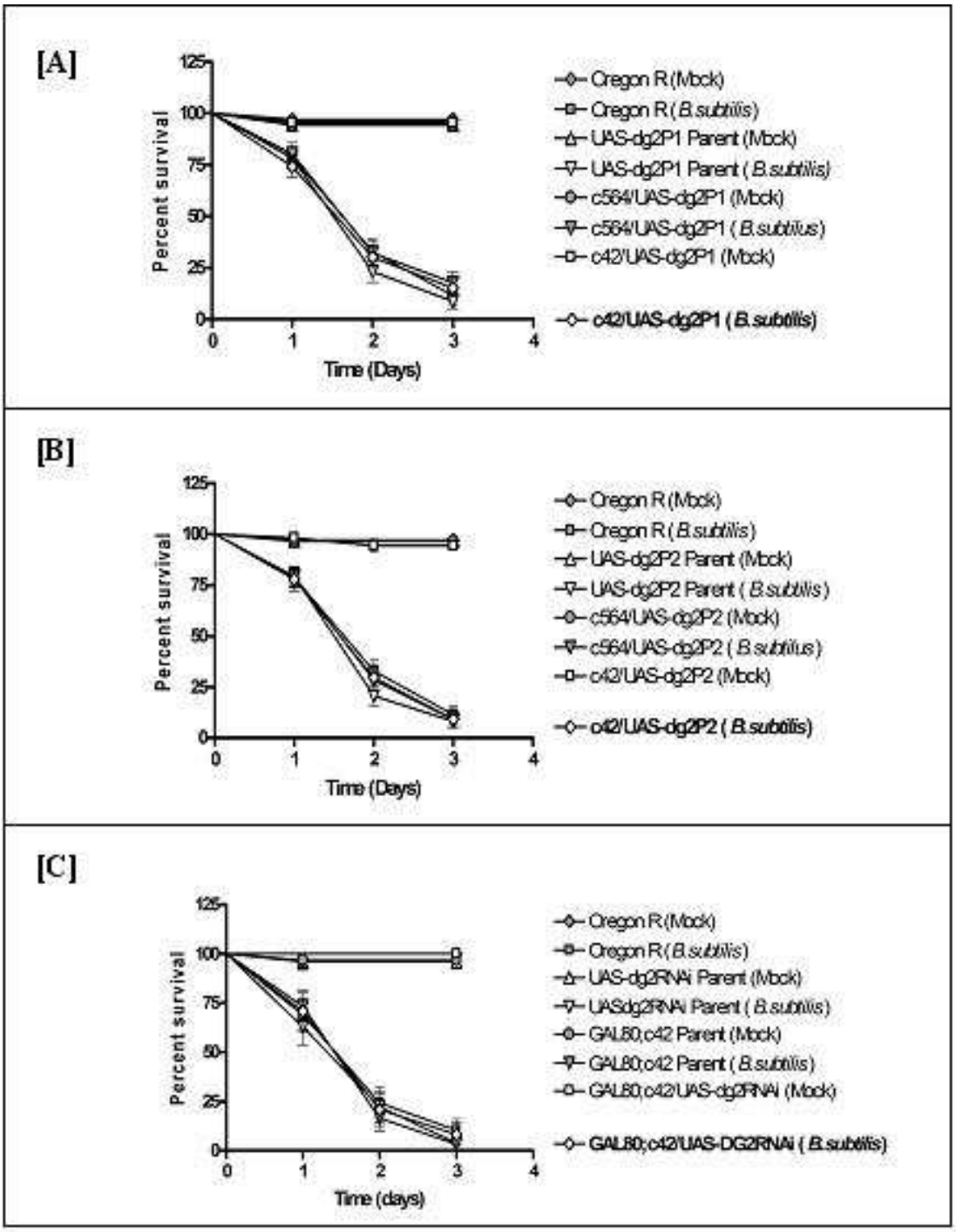


Figure 4.11 - Survival in response to *B. subtilis* when *dg2* is over-expressed or knocked-down in the tubules. Survival was monitored in 5-day old adult flies that were either infected with a concentrated suspension of *B. subtilis* via a thin needle, or were mock infected using a sterile needle. Survival of flies was then monitored at for a 3 day period (N = 30). This was repeated three times on each fly line. Results for each line were then pooled and expressed as a percentage of survival using a Kaplin-Meier survival curve ($^{\pm}$ SEM). [A] Survival in response to *B. subtilis* when *dg2P1* is overexpressed in either the tubule (c42) or the fat body (c564). [B] Survival in response to *B. subtilis* when *dg2P2* is overexpressed in either the tubule (c42) or the fat body (c564) [C] Survival in response to *B. subtilis* when *dg2* is knocked-down in the tubule.

in the fat body. As with data shown in Figure 4.10, it can therefore be concluded that DG2 does not play a role in regulation of immune response in the tubule or the fat body in response to infection with *B. subtilis*.

Overall, the data shown in this section indicates that cGK-mediated regulation of immune response in the tubule is not relevant to infection with *B. subtilis* and therefore, as expected, does not extend to regulation of defensin expression in the tubule.

4.4 Modulation of immune response by cGKs in the tubule is important in response to natural infection with *E.coli.*

4.4.1 Introduction

The data described previously in this chapter has demonstrated an important regulatory role for *Drosophila* cGKs in the tubules in response to septic infection with Gram-negative bacteria. However, in nature it is far more common for an insect to become infected via the ingestion of microbe-contaminated food, as opposed to infection as a result of wounding. Despite the central role of the systemic immune response following septic infection, systemic AMP production is not deemed critical to host survival in response to natural infection (Ferrandon et al. 1998; Liehl et al. 2006; Ryu et al. 2006). Instead, *Drosophila* are known to combat natural infection via the local induction of AMP synthesis in several epithelial tissues, including the tubules (Ferrandon et al. 1998; Tzou et al. 2000). Studies have shown that, following oral infection with the Gram-negative bacteria *E. carotovora*, the tubules are able to induce strong expression of diptericin, as well as moderate expression of both cecropin and the anti-fungal metchnikowin (Tzou et al. 2000). Additionally, it has been demonstrated that local induction of all AMPs in epithelial tissues, including the anti-fungal drosomycin and metchnikowin, is dependent on the activation of the Imd pathway (Ferrandon et al. 1998; Tzou et al. 2000; Onfelt Tingvall et al. 2001)

Given that data shown previously in this study has demonstrated a critical role for *Drosophila* cGKs in the regulation of diptericin expression in the tubules following septic infection with Gram-negative bacteria, studies were initiated in order to investigate the potential regulatory role for cGKs in the tubules following natural infection. In this

approach, UAS-cGK transgenic flies were crossed to the principle cell-specific GAL4 driver c42, as described previously, and the progeny assessed following natural infection with Gram-negative bacteria. As with previous experiments, these studies were carried out using *E.coli*, where adult flies of the appropriate genotype were fed for 24 h on filter paper soaked in 5 % sucrose solution contaminated with concentrated *E.coli*. Control flies were fed for 24 h on filter paper soaked in a sucrose-only solution. The impact of cGK modulation in the tubules following natural infection was then assessed in each transgenic fly line by analysis of bacterial proliferation in the gut. In addition, diptericin expression was monitored in the tubules of each transgenic fly line by Q-PCR.

4.4.2 Natural Infection with *E.coli* induces diptericin expression in the Malpighian tubules of the adult fly.

Previous studies have shown that following oral infection with *E.carotovora*, diptericin expression is strongly induced in the tubules and the midgut of the adult fly (Tzou et al. 2000). However, as all previous experiments in this chapter have used *E.coli* as an effective immune inducer, and, to date, the effects of natural infection with *E.coli* on epithelial AMP expression have not yet been reported, studies were initiated in order to determine whether *E.coli* elicits a similar response to that of *E. carotovora* after natural infection. To achieve this, adult flies expressing a fluorescent diptericin-GFP reporter gene were orally infected with *E.coli* as described above. Following infection, the entire gut, with tubules attached, was dissected from each fly and mounted in Phosphate Buffered Saline (PBS) for immediate viewing under fluorescence using the Zeiss 510 Meta confocal system.

As Figure 4.12 shows, diptericin expression is strongly induced in the tubules following oral infection with *E.coli*. Interestingly, unlike the diptericin response observed following infection with *E. carotovora* shown in previous studies, it can be seen that there is no visible expression of diptericin in any areas of the gut following infection with *E.coli*, despite the obvious exposure of this tissue to the invading micro-organism. This would suggest that the gut induces a different set of AMPs in response to *E.coli* than it does in response to *E. carotovora*. Therefore, it can be suggested that the tubules are the critical epithelial tissue with regards to local diptericin expression following natural infection with *E. coli*.

Overall, it is shown here for the first time, that natural infection with *E.coli* results in an epithelial immune response in the adult fly, and that the tubules play an important role in the induction of diptericin expression during this response.

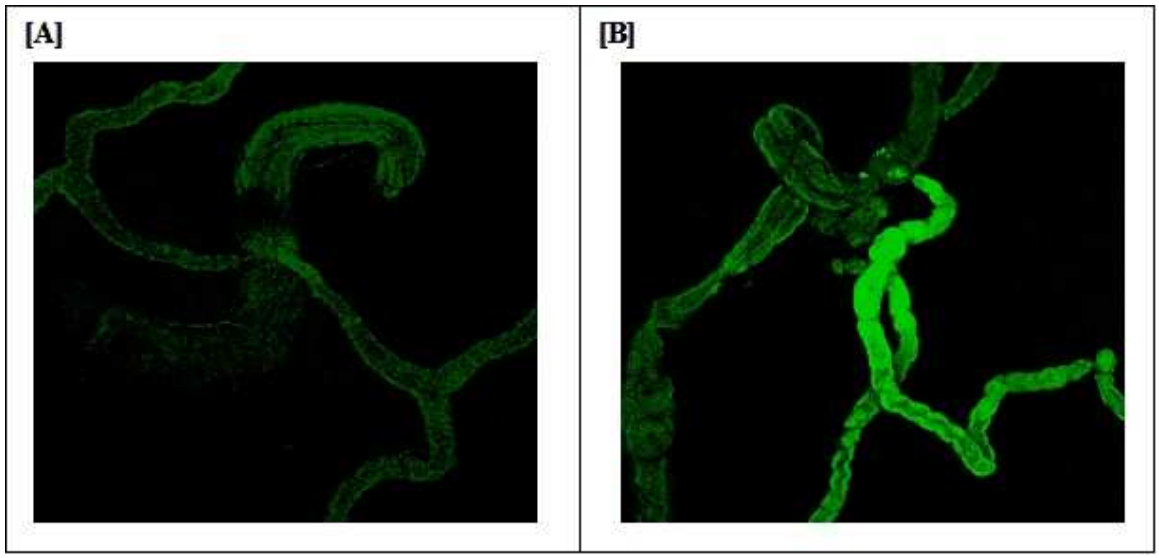


Figure 4.12 – Expression of diptericin-GFP in the Malpighian tubules after oral infection with *E.coli.* 7-day old adult flies expressing diptericin-GFP were monitored for diptericin expression 24 h after oral infection with *E.coli* using the Zeiss 510 Meta confocal system. [A] Control flies were fed on a 5 % sucrose solution for 24 h prior to dissection. Data shows no visible expression of diptericin in the tubules or the gut. [B] Infected flies were fed on a concentrated *E.coli* pellet re-suspended in 5 % sucrose solution for 24 h prior to dissection. Results show strong expression of diptericin in the tubule but no visible expression of diptericin in the gut.

4.4.3 Modulation of cGK expression in the tubule results in differential effects on bacterial clearance in the gut following natural infection with *E.coli.*

In order to assess the potential importance of cGKs in the tubule with regards to immune response following natural infection, studies were initiated in order to assess bacterial persistence in the midguts of flies whereby cGK expression was modulated in the tubule. In these experiments, UAS-*dg1*, UAS-*dg1*RNAi and UAS-*dg2P1* flies were crossed to the principle cell-specific GAL4 driver c42, and progeny naturally infected with ampicillin-resisitant *E.coli* as described above. Following infection, fly midguts were dissected and surface-sterilised in 70 % (v/v) ethanol before homogenisation in PBS. Homogenates were then diluted 1:100 and spread on LB-Agar plates containing 100µg/ml ampicillin. Each plate was then assessed for its number of colony forming units (CFUs).

As Figure 4.13 shows, modulation of cGK expression in the tubule has a significant effect on the ability of flies to clear bacteria from the gut following natural infection with *E.coli*. To explain further, it can be seen from Figure 4.13A that, compared to that of parental control flies, intestinal bacterial load is significantly lower in flies where *dg1* is overexpressed in the tubule. This would suggest that, as with septic infection, DG1 acts to stimulate an immune response in tubules in response to natural infection. In support of this data, it can be seen from Figure 4.13B that when *dg1* expression is knocked-down in the tubules, the ability of the flies to clear bacteria from the gut is significantly impaired. These data therefore demonstrate an important role for DG1 in the activation of immune response in the tubule following natural infection. Furthermore, these results also reveal an important role for the tubule itself with regards to bacterial elimination in the gut in response to oral infection with *E.coli*.

Similarly, it can be seen from Figure 4.13C that DG2P1 appears to play an inhibitory role in the tubule in response to natural infection. Data shows that in flies where *dg2P1* has been over-expressed in the tubule, intestinal bacterial load is significantly higher than that of parental controls. These data therefore further demonstrate an important role for the tubules in bacterial clearance following natural infection with *E.coli* and show that this effect is negatively regulated by DG2P1. Unfortunately, due to time limitations, these experiments were not carried out in order to assess the effect of either overexpression of *dg2P2* or knock-down of *dg2* expression by RNAi, therefore the inhibitory effect shown here can only currently be applied to the DG2P1 isoform of DG2. However, as previous data has demonstrated a similar role for both DG2P1 and DG2P2 with regards to regulation of immune function in the tubule, it can be hypothesised that DG2P2 may also play an important inhibitory role in the tubule in response to natural infection. Investigation into the role of DG2P2 in the tubule in response to natural infection therefore remains a subject for future work.

Overall, these results demonstrate, for the first time, that the tubules play an important role in eliminating invading bacteria in the gut following natural infection with *E.coli*. Additionally, it is shown here that, as with septic infection, cGKs play an important role in regulation of this effect.

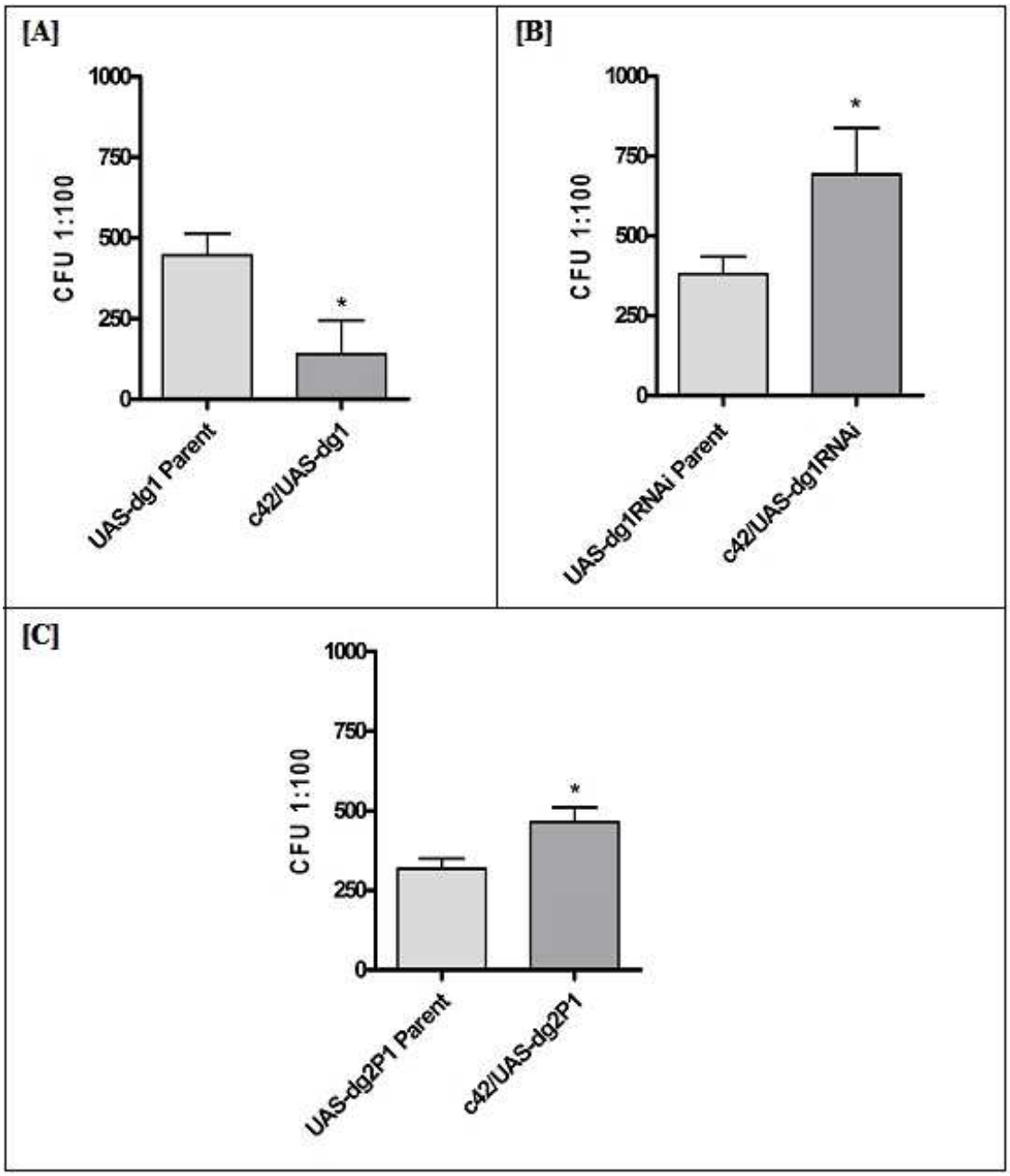


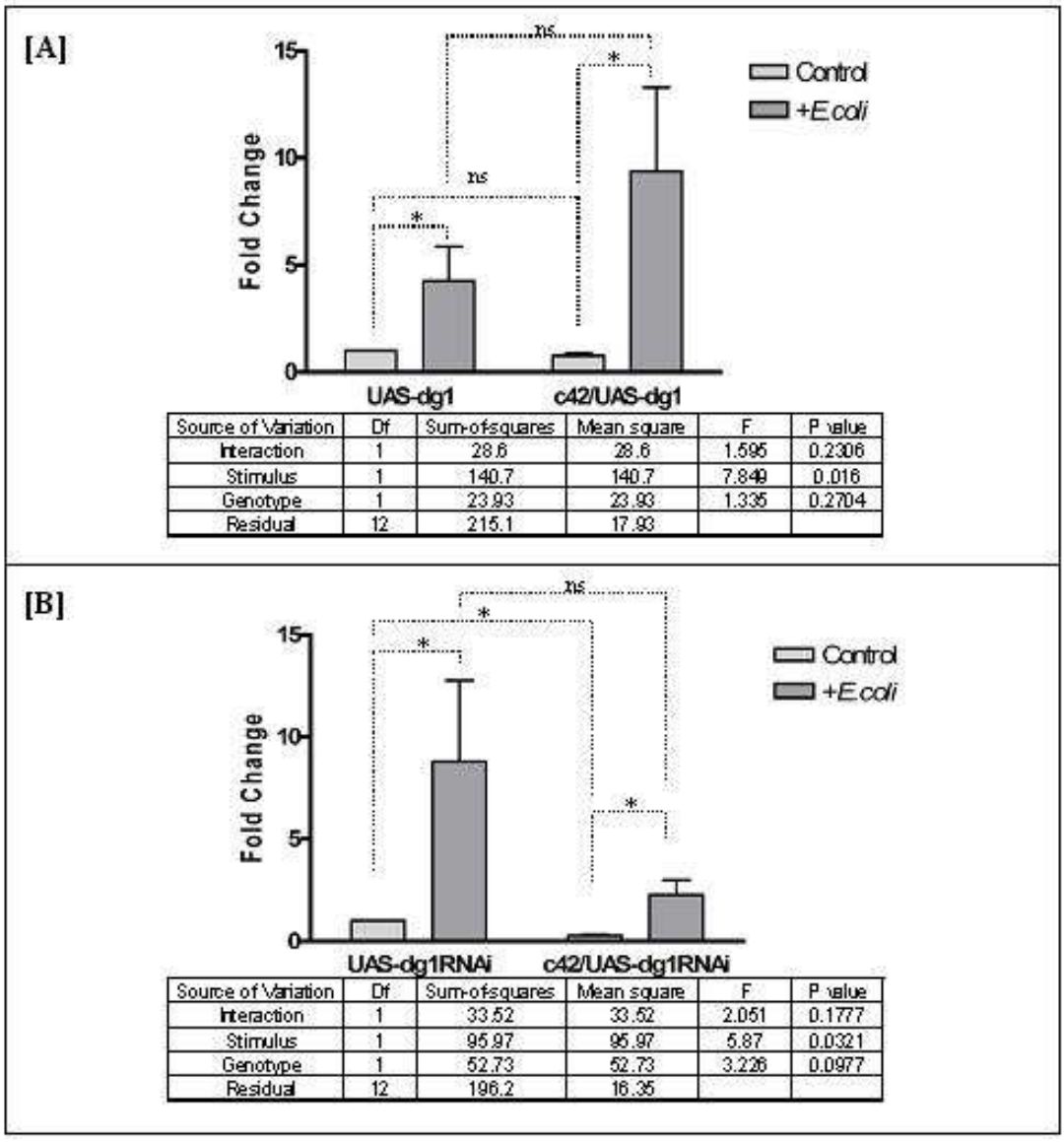
Figure 4.13 - Targeted overexpression or knock-down of cGKs to the principal cells of the tubule results in differential effects on bacterial clearance in the gut following natural infection with *E.coli*. Bacterial clearance was assessed in the midguts of 7-day old flies whereby cGK expression was modulated in the tubules after natural infection with ampicillin-resistant *E.coli*. Following infection, ten midguts were dissected per sample and surface-sterilized before homogenation in PBS. Homogenate was then diluted and spread on LB-ampicillin plates and resultant CFUs counted. Results are displayed as a mean of $N = 4$ ($\pm$ SEM). Significance of data was determined by Students *t*-tests and data significant from control indicated by * ($P < 0.05$). [A] Bacterial load in the midguts of flies where *dg1* is overexpressed in the tubule. [B] Bacterial load in the midguts of flies where *dg1* expression is knocked-down in the tubule. [C] Bacterial load in the midguts of flies where *dg2P1* is overexpressed in the tubule.

4.4.4 Modulation of cGKs in the tubule results in differential effects on diptericin expression following natural infection with *E.coli.*

In order to confirm that the results shown in Figure 4.13 are a result of cGK-mediated modulation of AMP production, experiments were carried out to assess diptericin expression in the tubules of *c42*/UAS-*dg1*, *c42*/UAS-*dg1*RNAi and *c42*/UAS-*dg2P1* flies following natural infection with *E.coli.* In these experiments, each of the fly lines above were infected with *E.coli* as described before. Following infection, the tubules of each fly line were excised and diptericin expression monitored by Q-PCR.

Figure 4.14 shows that, as expected, diptericin expression is significantly increased in the tubules of control flies in response to natural infection with *E.coli*. Interestingly, in support of the data shown in Figure 4.13, it can be seen that when *dg1* is overexpressed in the tubules, diptericin expression is further increased in response to *E.coli* in comparison to controls (Figure 4.14A). Unfortunately, due to variable results in the tubules of the infected flies, analysis has shown that this effect is not statistically significant. Therefore a stimulatory role for DG1 on diptericin expression in the tubule following natural infection can only be implied from this data, and further work is required in order to confirm this hypothesis. Equally, when *dg1* expression is knocked-down in the tubules, diptericin expression is reduced in response to *E.coli* in comparison to parental tubules (Figure 4.14B). These data therefore further suggest a role for DG1 as a positive regulator of diptericin expression in response to natural infection. However, as with the tubules of *dg1*-overexpressing flies, due to large variability in data, these results are not deemed statistically significant. Therefore, despite the indication from the data shown in both Figure 4.14A and Figure 4.14B that DG1 acts to positively regulate diptericin expression in the tubule in response to natural infection with *E.coli*, further work is required in order definitively confirm this role.

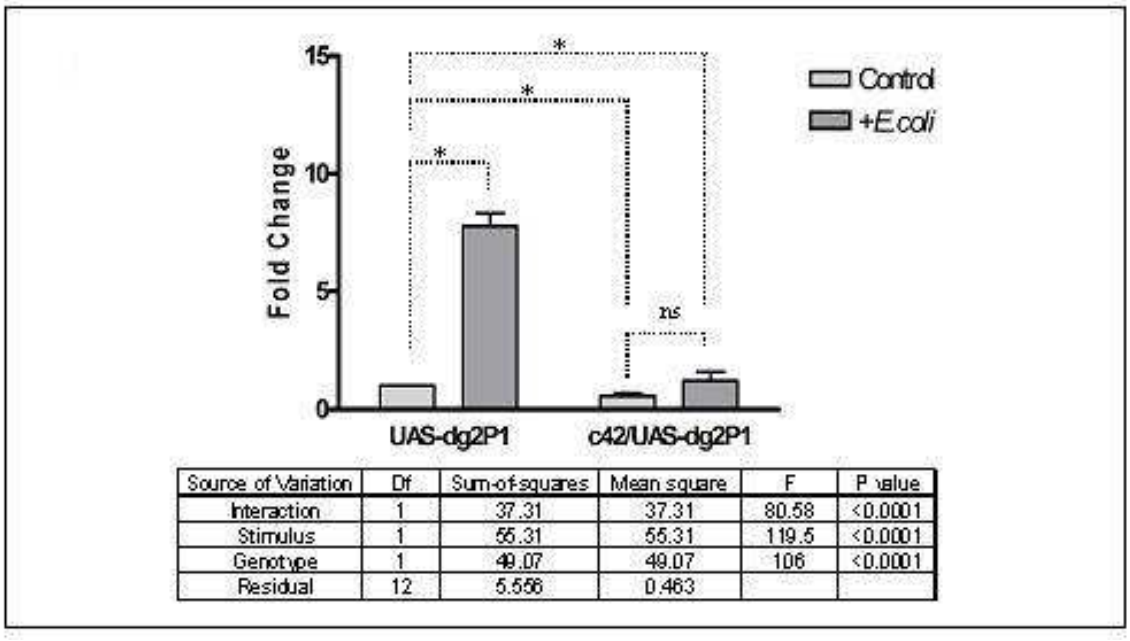
In contrast, it is demonstrated by the data shown in Figure 4.15 that overexpression of *dg2P1* in the tubules has a very significant effect on diptericin expression following natural infection with *E.coli*. Data shows that, unlike parental controls, *c42*/UAS-*dg2P1* flies are unable to significantly induce diptericin expression in the tubules following oral infection. These data therefore indicate an important inhibitory role for DG2P1 in the tubule in response to natural infection with *E.coli.* As before, due to time limitations, it



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	28.6	28.6	1.595	0.2306
Stimulus	1	140.7	140.7	7.849	0.016
Genotype	1	23.93	23.93	1.335	0.2704
Residual	12	215.1	17.93		

Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	33.52	33.52	2.051	0.1777
Stimulus	1	95.97	95.97	5.87	0.0321
Genotype	1	52.73	52.73	3.226	0.0977
Residual	12	196.2	16.35		

Figure 4.14 – Targeted overexpression or knock-down of *dg1* to the principal cells of the tubule results in differential effects on diptericin expression following natural infection with *E.coli.* Expression of diptericin was assessed by Q-PCR in response to natural infection with *E.coli* in excised tubules of adult flies in which cGK expression was modulated in tubule principal cells. Resulting data were normalised against expression of the standard, *rp49*, and expressed as a fold change of parental control expression where control =1 (N = 4, $\pm$ SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis was carried out using Bonferroni tests, whereby significant data are indicated by * ($P<0.05$) or ns (not significant) as appropriate. [A] Diptericin expression in the tubules in response to natural infection when *dg1* is overexpressed. Analysis by two-way ANOVA reveals no significant interaction between genotype and stimulus. The effect of stimulus alone is considered significant, but the effect of genotype alone is not considered significant. *Post hoc* analysis shows a significant difference in diptericin expression in the tubules of both parental flies and progeny in response to *E.coli*. Diptericin expression is higher in the tubules of *c42/*UAS-*dg1* flies compared to parents, though not significantly so [B] Diptericin expression in the tubules in response to natural infection when *dg1* is knocked-down in the tubule. Analysis by two-way ANOVA reveals no significant interaction between genotype and stimulus. The effect of stimulus alone is considered significant, however genotype alone is not considered significant. *Post hoc* analysis shows a significant difference in diptericin expression in the tubules of both parental flies and progeny in response to *E.coli*. However, diptericin expression is lower in the tubules of *c42/*UAS-*dg1*RNAi flies compared to parents, though not significantly so.



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	37.31	37.31	80.58	<0.0001
Stimulus	1	55.31	55.31	119.5	<0.0001
Genotype	1	49.07	49.07	106	<0.0001
Residual	12	5.556	0.463		

Figure 4.15 - Targeted overexpression of *dg2P1* to the principal cells of the tubule results in a negative effect on diptericin expression following natural infection with *E.coli*. Expression of diptericin was assessed by Q-PCR in response to natural infection with *E.coli* in excised tubules of adult flies in which *dg2P1* expression was modulated in tubule principal cells. Resulting data were normalised against expression of the standard, *rp49*, and expressed as a fold change of parental control expression where control =1 (N = 4, $\pm$ SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis was carried out using Bonferroni tests, whereby significant data are indicated by * ($P<0.05$), or ns (not significant) as appropriate. TWO-way ANOVA reveals an extremely significant interaction between stimulus and genotype. Additionally, the effect of stimulus and genotype alone are considered significant. *Post hoc* analysis shows that, unlike parents, the tubules of *c42*/UAS-*dg2P1* flies are unable to significantly induce diptericin expression in response to natural infection with *E.coli*.

was not possible to carry out these experiments in order to investigate the effect of *dg2P2* overexpression in the tubule or knock-down of *dg2* by RNAi. However, as before, it can be hypothesised that DG2P2 may play a similar inhibitory role to that of DG2P1 on diptericin expression in the tubule in response to natural infection with *E.coli*.

Overall, the data shown here demonstrates differential effects of DG1 and DG2P1 on diptericin expression in the tubule in response to natural infection with *E.coli*. Given that previous data has demonstrated that cGK modulation in the tubule has a significant effect on bacterial clearance in the gut following infection, it can be suggested from the data shown here that this effect is due to cGK modulation of diptericin expression. Unfortunately, in the case of DG1, the data obtained is not deemed statistically significant. Therefore, further work is required in order to definitively determine the importance of DG1 with regards to diptericin regulation in response to natural infection.

4.5 Discussion

In this chapter, a novel role has been described for the cognate *Drosophila* cGKs, DG1 and DG2 in the tubules of *Drosophila*. cGKs are known to function as part of the cGMP signalling pathway and, in the previous chapter, it was demonstrated that cGMP is able to either stimulate or inhibit expression of Imd pathway-associated AMPs in a dose-dependent manner. Data shown here demonstrates that the differential effects of cGMP on AMP expression in the tubules are mediated via activation of cGKs, whereby DG1 is shown to stimulate diptericin expression and the two main isoforms of DG2, P1 and P2, are shown to inhibit diptericin expression. Importantly, it has been demonstrated that the distinct effects exhibited by DG1 and DG2 are sufficient to confer differential survival phenotypes in the whole organism in response to septic infection with Gram-negative bacteria but not infection with Gram-positive bacteria. Additionally, it is shown here that the differential effects mediated by DG1 and DG2 on diptericin expression in the tubule play an important role in maintaining the ability of the fly to clear bacteria from the gut following natural infection with *E.coli*.

To date, due to a high sequence homology in the both the cGMP-binding and kinase domains of DG1 and DG2, *Drosophila* cGKs have been implicated to share related function (Kalderton and Rubin, 1989). Indeed, studies have shown that each of these cGKs share similar expression patterns and both have been implicated to play a role in neuronal function, as well as act as positive regulators of fluid secretion in the tubule (Kalderon and Rubin 1989; Foster et al. 1996; MacPherson et al. 2004a; MacPherson et al. 2004b). However, for the first time, it is shown here that DG1 and DG2 exhibit distinct function in the tubule, whereby DG1 is shown to stimulate diptericin expression and DG2 is shown to inhibit it. It can be suggested that the contrasting roles of DG1 and DG2 on Imd pathway regulation are a result of activation by different sources of cGMP. As described in the previous chapter, cGMP signalling is known to be compartmentalised within each cell in order to facilitate the regulation of a number of simultaneous physiological processes. Therefore, given that DG1, DG2P1 and DG2P2 are localised differently within the tubule, it is perhaps not surprising that DG1 and DG2 may play different roles in tubule function.

Interestingly, the data shown in this chapter supports the hypothesis presented in the previous chapter. In chapter 3, it was suggested that the stimulatory effect on Imd pathway regulation by cGMP seen in the tubule may be a result of activation of the cGMP pathway by NO. As mentioned previously, NO has been established in a number of studies as a positive regulator of the Imd pathway. It is a diffusible molecule known to activate cGMP signalling via the cytosolic enzyme sGC. Interestingly, DG1 has also been demonstrated to be localised to the cytosol in the tubule, implying that it may reside in close proximity to the cGMP generated by activation of sGC (MacPherson et al. 2004b). Indeed, *dg1* overexpression in tubules has previously been linked to the sensing of cytosolic cGMP (MacPherson et al. 2004b). It can therefore be suggested that NO-dependent regulation of the Imd pathway may be mediated by DG1.

Similarly, if the negative effect of cGMP on Imd pathway regulation is mediated by NO-independent means, i.e. through activation of a receptor guanylate cyclase, it is likely that the cGMP generated would mediate its effects through effector molecules in close proximity to the plasma membrane such as DG2P1 or DG2P2. Unfortunately to date, it is not known whether DG2P1 or DG2P2 reside in close proximity to any of the rGCs known to be expressed in the tubule, as expression studies have not yet been carried out. However, it can be implied that DG2-mediated regulation of immune response in the tubule may occur via activation of an rGC.

Importantly, cGK regulation of immune response in the tubules has been shown here to have a significant impact on whole fly survival in response to infection. It is demonstrated here for the first time that modulation of cGKs in the tubule significantly effects survival in response to septic infection with *E.coli*. Unfortunately, when the same fly lines were infected with another Gram-negative bacteria *P.aeruginosa*, despite the fact that cGK modulation in the tubule exhibited a clear but small effect on survival, the levels of survival observed were not significantly different. The reason for this is not known, however previous studies have demonstrated that *P.aeruginosa* is a very potent pathogen, known to overcome *Drosophila's* immune responses by suppressing induction of AMP expression (Lau et al. 2003; Apidianakis et al. 2005). It is suggested therefore that although cGK modulation of AMP expression in the tubules may have had some stimulatory or inhibitory effects on immune response immediately upon infection, resulting in the small differences in fly survival seen, these effects may have been

superseded after infection by the pathogenic action of the bacteria. Therefore, although the effect of cGK modulation in the tubule is confirmed to impact survival of flies in response to *E.coli*, it can only be implied from data that tubule cGKs may have a role to play in the regulation of immune mechanisms in response to *P.aeruginosa*. Interestingly, in support of data from the previous chapter, it is demonstrated here that cGK modulation in the fat body has no effect on survival in response to septic infection with Gram-negative bacteria. Additionally, as expected, when assessing the effect of cGK modulation in the tubule in response to infection with Gram-positive bacteria, no significant survival phenotypes were observed in flies infected with *B.subtilis*. These data therefore confirm previous data, which has shown that the cGMP signalling pathway is important in Imd pathway regulation, and not Toll pathway regulation.

Perhaps unsurprisingly, these studies have also demonstrated an important role for the tubule in response to natural infection. Previous studies have already reported the local induction of AMPs in the epithelia following natural infection with Gram-negative bacteria (Ferrandon et al. 1998; Tzou et al. 2000; Onfelt-Tingvall. 2001; Liehl et al. 2006). However, it is shown here for the first time, that activation of immune responses in the tubule have a significant effect on the ability of the fly to clear bacteria from the gut following natural infection with *E.coli*. Importantly, data has indicated that as with septic infection, this effect is regulated by the differential action of DG1 and DG2 on diptericin expression in the tubule. Unfortunately, statistical analysis has deemed the effect of DG1 on diptericin expression following natural infection as not significant due to the large variability of expression in the tubules of infected flies. The reason for this effect is unknown, however it can be suggested that the variability of these results may simply be due to some inconsistency in the feeding of the flies. Certainly, the same degree of variability of diptericin expression is not seen in the tubules of flies fed on the sucrose-only solution. Furthermore, a clear effect of DG1 modulation in the tubule is observed in experiments assessing bacterial clearance in the gut, thus indicating a significant role for DG1 in regulation of immune response in the tubule. Therefore, although it is implied from the data obtained in this study that DG1-mediated stimulation of diptericin expression in the tubules is important following natural infection, further confirmation of the significance of this effect is required.

Overall, the data presented in this chapter not only demonstrate a completely novel role for *Drosophila* cGKs in the regulation of Imd pathway-associated immune response in the tubule, but they also highlight the fact that the tubule appears to be a critical tissue in the induction of immune mechanisms in response to both septic and natural infection with Gram-negative bacteria. At this time, it is not known whether the tubule acts as a completely independent immune tissue, or whether it acts as a signalling tissue in order to alert other tissues, such as the fat body, to activate or suppress immune mechanisms. Certainly, a role for NO has already been suggested in mediating signalling between tissues (Bassett et al. 2000; Foley and O'Farrell. 2003; Silverman. 2003). However, the answer to this question is beyond the scope of this study and therefore remains a subject for future work.

Chapter 5

Epistatic analysis of cGK-mediated Imd pathway regulation

5.1 Summary

In the previous chapter, the *Drosophila* cGKs, DG1 and DG2, were demonstrated to play a novel role in the regulation of Imd pathway-associated immune response within the tubule of the adult fly. Given that kinases are known to mediate their effects via direct phosphorylation of target proteins, it is hypothesised that DG1 and DG2 may therefore interact with components of the Imd pathway. Therefore in this chapter, studies were initiated in order to identify potential targets for DG1 and DG2 within the Imd pathway. This was carried out using a transgenic approach, whereby fly lines were generated in order to epistatically assess the effects of cGK modulation in the tubules of flies where components of the Imd pathway were either mutated or overexpressed. The tubules of transgenic flies were then either assessed for changes in diptericin expression by Q-PCR, or were monitored directly using an immuno-cytochemistry (ICC) approach. Data obtained demonstrates that DG1 acts downstream of Imd in the immune pathway. Furthermore, studies reveal that the dose-dependent modulation of diptericin expression by cGMP demonstrated in chapter 3 (Figure 3.4) is a result of regulation of Relish activation; whereby translocation of Relish into the nucleus is enhanced in response to low nanomolar concentrations of cGMP, and inhibited in response to higher micromolar concentrations. Additionally, data further demonstrates that these effects are mediated by DG1 and DG2. Results show that when both Relish and DG1 are overexpressed in the tubule, translocation of Relish into the nucleus is enhanced. Conversely, overexpression of Relish with either DG2P1 or DG2P2 in the tubule is demonstrated to inhibit translocation.

5.2 Introduction

Drosophila cGKs have been implicated in a number of neuronal processes such as foraging, learning and memory, and sensory responsiveness (Osborne et al. 1997; Scheiner et al. 2004; Mery et al. 2007). Additionally, cGKs have been demonstrated to play a stimulatory role in fluid secretion in the tubules . However, despite the established role of cGKs in *Drosophila*, to date there are no identified phosphorylation targets of these kinases. Conversely, a number of substrates for vertebrate cGKs have been identified, including Vasodilator-Stimulated Phosphoprotein (VASP) (Butt et al. 1994), the small GTPase RhoA (Gudi et al. 2002; Zhuang et al. 2004) and the transcription

factors TFII-I (Casteel et al. 2002) and cAMP response element-binding (CREB) (Gudi et al. 1996). Additionally, the NFκB transcription factors p49/52, p50 and p65 have been identified as phosphorylation targets for the vertebrate cGK, PKG, where studies have demonstrated that the transcriptional activity of p49/52, p50 and p65 is significantly enhanced as a result of direct phosphorylation by PKG (He and Weber 2003). Interestingly, p50 and p65 are known to dimerize to form the NFκB transcription factor involved in the mammalian Tumour Necrosis Factor α (TNFα) signalling pathway, which is known to share several homologous components with the *Drosophila* Imd pathway (reviewed in Li and Lin 2008). Consistent with the Imd pathway, the TNFα pathway is known to trigger activation of NFκB through a number of signalling molecules such as Receptor Interacting protein (RIP - Imd homologue), FADD, TRAF6 (which acts as an E3 ligase through its RING domain, much like *Drosophila* dIAP2) TAB2, TAK1, IKKα, IKKβ (ird5 homologue) and NEMO (Kenny homologue). Activation of NFκB in this pathway results in the regulation of a number of biological processes such as cell proliferation, differentiation, apoptosis and immune responses. To date, a number of studies have identified NO/cGMP/PKG as playing an important role in regulation of the TNFα pathway (Gertzberg et al. 2000; Kalra et al. 2000; Aizawa et al. 2003). For example, studies have demonstrated that NO acts to provoke TNFα/NFκB activation through a cGMP/PKG-dependent pathway in both mammalian heart tissues and T-lymphocytes (Kalra et al. 2000; He and Weber. 2003). Conversely, cGMP has been shown to significantly inhibit TNFα/NFκB activation in vascular smooth muscle cells (VSMCs) via both sGC and rGC (Aizawa et al. 2003). Significantly, in addition to the NFκB substrates for PKG, p50 and p65, it has also been demonstrated that PKG is able to activate the TNFα pathway via direct phosphorylation of IκB, the NFκB inhibitory protein (Kalra et al. 2000).

Given the established role of cGMP/PKG in the regulation of the TNFα pathway, and the identification that this regulation occurs via direct phosphorylation of two TNFα pathway components by PKG, perhaps *Drosophila* cGKs play a similar role in the direct regulation of the Imd pathway. Indeed, it can be suggested from the results obtained in mammalian studies that a putative target for DG1 and/or DG2 may be the NFκB transcription factor Relish, which consists not only of an N-terminal Rel-homology domain (RHD) but also a C-terminal IκB inhibitory domain (Dushay et al. 1996).

However, due to the opposing effects of DG1 and DG2 on Imd pathway regulation, it is likely that these cognate kinases mediate their effects via the phosphorylation of distinct substrates. Therefore, studies were initiated in order to determine the potential target(s) of DG1 and DG2 in the Imd pathway. This was carried out using a transgenic approach, where the effects of cGK-mediated modulation of the Imd pathway were assessed epistatically.

5.3 Generation of transgenic lines for epistatic analysis

In order to carry out epistatic analysis of cGK-regulation of the Imd pathway within the tubule, several fly lines were generated which express the tubule principal cell-specific driver, c42, in a transgenic Imd pathway background (Figure 5.1 - original *imd*^{1}, UAS-*imd* and *Key*^{1} (*Kenny)* transgenic fly strains, kind gift of Professor S Kurata, University of Tohoku, Japan; original UAS-*relish* transgenic fly strain, kind gift of Professor D Hultmark, University of Umea, Sweden). This was achieved using the crossing scheme shown in Figure 5.1, whereby balanced Imd pathway transgenic strains were crossed to a balanced c42 transgenic line. The resultant progeny were then selected to contain one copy of the Imd pathway transgene, over the 2nd chromosome *curly* (*CyO*) balancer, and one copy of c42, over either the *stubble* (*Sb*) or *tubby* (*Tb*) 3rd chromosome balancers. Selected flies were then crossed to homozygous UAS-cGK transgenic flies as appropriate and progeny selected to contain one copy of each transgene. Following selection, the tubules of transgenic flies were then either assessed for changes in diptericin expression by Q-PCR, or were monitored directly using an ICC approach.

Unfortunately, due to time limitations, and low yield of progeny of the correct genotype from some of the crosses, data was not obtained from crosses involving the *w*$^{(-)}$;*cn-bw(Key*^{1}*)/CyO*;*c42/TM2Tb* and *w*$^{(-)}$;*UAS-imd/CyO*;*c42/TM3Sb* transgenic fly lines. Therefore, the results shown here represent preliminary data from epistatic analysis.

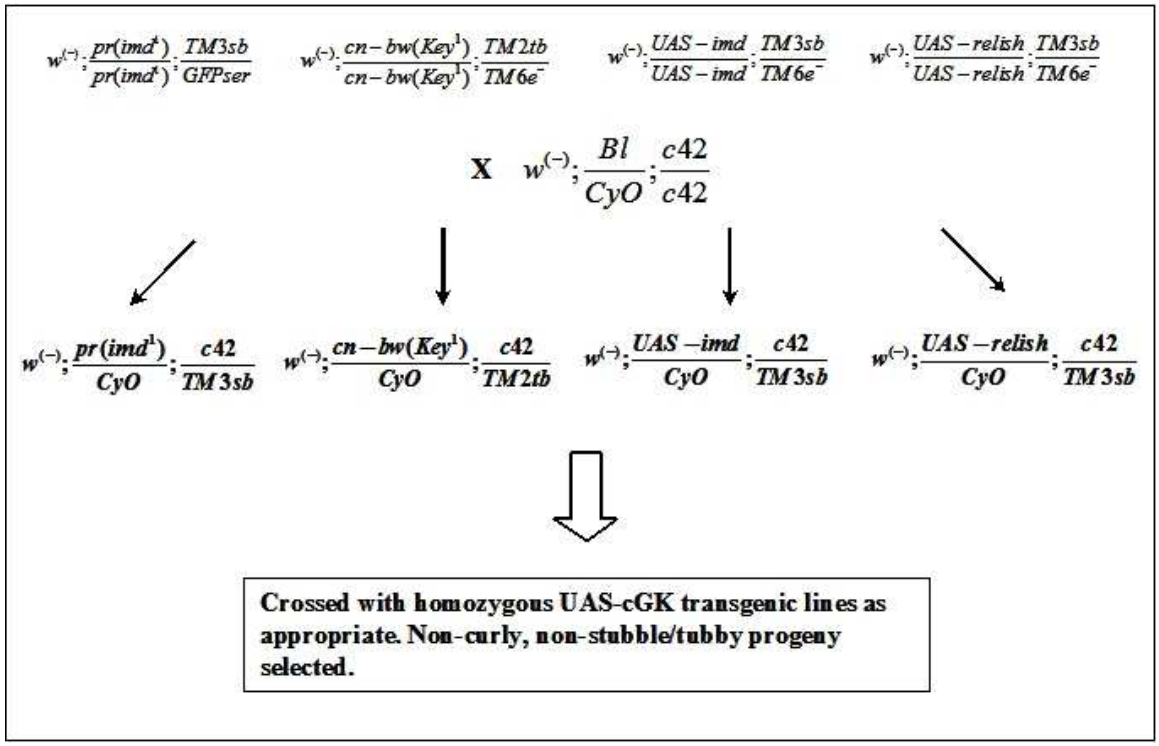


Figure 5.1 – Crossing scheme depicting generation of transgenic c42 driver flies in an Imd pathway mutant background. Balanced Imd pathway transgenic flies were crossed to a balanced c42 driver line. In order to maintain a balanced parental stock, for each cross progeny were selected to contain one copy of each transgene over an appropriate balancer gene [*curly (CyO)* on the 2nd chromosome and *stubble (Sb)* or *tubby (Tb)* on the 3rd chromosome]. Parental Imd-transgene/c42 flies were then crossed to homozygous UAS-cGK flies as appropriate and tubules of the progeny of the desired phenotype assessed for diptericin expression by Q-PCR or monitored directly by immunocytochemistry (ICC).

5.4 Peptidoglycan (PGN) activation of the Imd pathway in the *Drosophila* Malpighian tubule

Traditionally, in experiments requiring activation of the Imd pathway, crude preparations of lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, were used as an immune stimulant. However in recent years, a number of studies have been published regarding the efficiency of LPS for activating an immune response in *Drosophila* tissues (Leulier et al. 2003; Werner et al. 2003; Kaneko et al. 2004). In these studies, it has been demonstrated that LPS is a very weak activator of the Imd pathway and that DAP-type PGN(-), derived from Gram-negative bacteria, is a significantly stronger activator of the Imd pathway.

Therefore, for a number of the epistatic experiments carried out in this chapter, PGN(-) (derived from *E.coli* strain 0.111:B4 - Invivogen) was used as an Imd pathway activator. The use of PGN(-) as an immune stimulant in the *Drosophila* Malpighian tubule has not been published previously. Therefore, the effect of PGN(-) on activation of AMP expression in the tubules was first of all validated using Oregon R wild-type flies. In this experiment, tubules were excised and incubated in sterile Schneider's medium for 3 h both in the absence (as a control) or presence of 5 µg/ml PGN(-). Diptericin expression was then quantified by Q-PCR. As Figure 5.2 shows, diptericin expression is significantly increased in PGN(-)-stimulated tubules, thus confirming the potency of PGN(-) as an Imd pathway activator in the tubule.

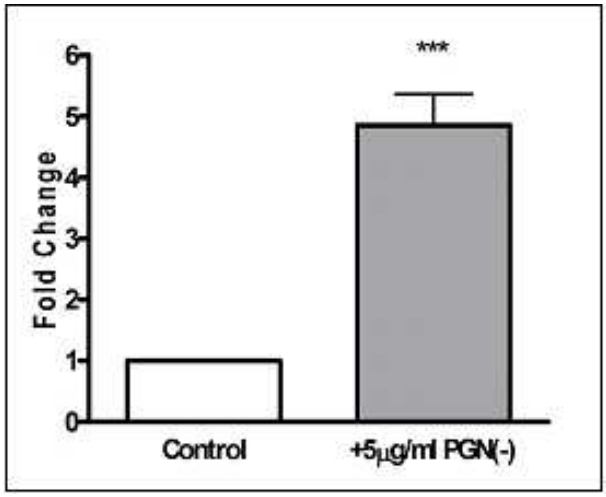
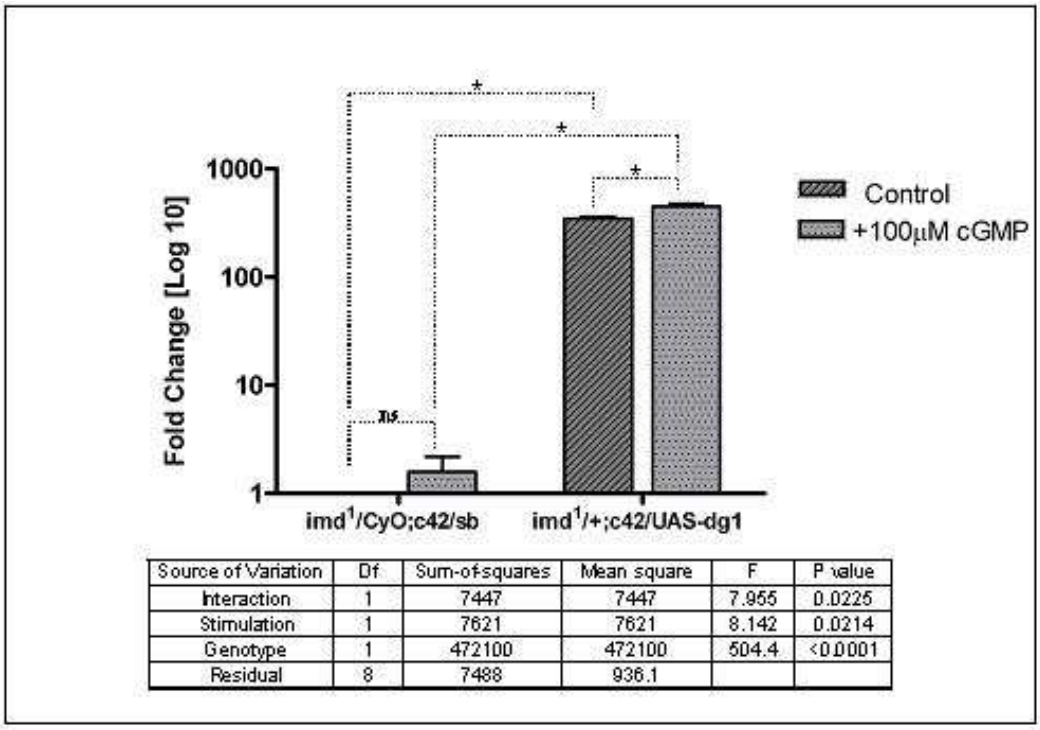


Figure 5.2 – PGN(-) stimulation of diptericin expression in the *Drosophila* Malpighian tubule. Expression of diptericin was assessed by Q-PCR in the excised tubules of Oregon R flies following stimulation with 5 µg/ml PGN(-). Resulting data were normalised against expression of the standard, *rp49*, and expressed as a fold change of control expression where control =1 (N = 3, ± SEM). Data significant from control are indicated by *** (P = <0.0001) (as analysed by Students *t*-test)

5.5 DG1 modulation of the Imd pathway in the tubule occurs downstream of Imd

As shown in the previous chapter, DG1 has been demonstrated to act as a positive regulator of Imd pathway activation. Therefore, for epistatic analysis of DG1-mediated regulation of Imd pathway, initial experiments were carried out to assess the effect of

overexpression of DG1 in the tubules of flies deficient in Imd. For this experiment, DG1 expression was targeted to the principal cells of the tubules by crossing homozygous UAS-*dg1* transgenic flies to the *w*$^{(-)}$;*pr(imd*^{1}*)/CyO;c42/TM3Sb imd*-deficient tubule driver line. Progeny were then selected to contain one copy of each transgene. As with previous experiments, tubules were then excised from 7-day old adult flies of both the *imd*^{1}*/CyO;c42/Sb* parental lines (as a control) and the *imd*^{1}*/+;c42/*UAS-*dg1* progeny. Excised tubules were then incubated for 3 h in sterile Schneider's medium alone (control) or sterile Schneider's medium containing 100 µM cGMP. Following incubation, RNA was extracted from each sample and diptericin expression quantified by Q-PCR (Figure 5.3).



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	7447	7447	7.955	0.0225
Stimulation	1	7621	7621	8.142	0.0214
Genotype	1	472100	472100	504.4	<0.0001
Residual	8	7488	936.1		

Figure 5.3 - Targeted overexpression of DG1 to the principal cells of the tubule in *imd*-deficient flies. Expression of diptericin was assessed by Q-PCR in excised tubules of *imd*-deficient adult flies following targeted overexpression of DG1 in tubule principal cells using the GAL4/UAS binary system. Resulting data were normalised against expression of the standard, *rp49,* and expressed as a Log 10 of fold change of parental control expression where control =1 (N = 3, ± SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis was carried out using Bonferroni tests, whereby significant data are indicated by * ($P<0.05$) or ns (not significant) where appropriate. Analysis by two-way ANOVA reveals a significant interaction between genotype and stimulus. The effect of stimulus and genotype alone are also considered significant (See table). Additionally, *post hoc* analysis shows a significant difference in diptericin expression in the tubules of *imd*^{1}*/+;c42/*UAS-*dg1* flies compared to *imd*^{1}*/CyO;c42/sb* parental flies in both controls and in cGMP-stimulated tubules. Furthermore, it can be seen that there is no significant effect on diptericin levels in the tubules of parental flies in response to cGMP, whereas diptericin expression is significantly increased in response to cGMP in the tubules of *imd*^{1}*/+;c42/*UAS-*dg1*flies.

It can be seen from the results in Figure 5.3 that, due to the presence of the *imd*^{1}-transgene, diptericin expression is extremely low in the tubules of the *imd*^{1}*/CyO;c42/Sb* parental controls. However, when *dg1* expression is targeted to the tubule in the *imd*-deficient flies, expression of diptericin is drastically increased (approximately 350-fold) compared to parental controls. Furthermore, when tubules from *imd*^{1}*/+;c42/*UAS-*dg1* flies are stimulated with exogenous cGMP, diptericin expression levels are further increased, confirming that the data observed are a result of activation of DG1 by cGMP. These findings are in support of data shown in chapter 4 (Figure 4.1), where it is demonstrated that overexpression of *dg1* in the tubule not only results in an up-regulation of diptericin expression in the tubule, but that this effect is enhanced in response to 100 µM cGMP. Interestingly, when the tubules of *imd*^{1}*/CyO;c42/Sb* parental flies are incubated with cGMP, there is an increase in diptericin expression, though this effect is not deemed significant (as determined by Bonferroni post-tests). Previous data has demonstrated an inhibitory effect of 100 µM cGMP on AMP expression in the tubule in both wild-type and parental flies (Figures 3.3, 4.1 – 4.5). However, it is probable that initial expression levels of diptericin are so low in the *imd*^{1} mutant flies that incubation with cGMP would have no significant effect on expression. Alternatively, it could be suggested that the inhibitory effect on AMP expression normally seen in response to high concentrations of cGMP is mediated upstream of Imd. Since these inhibitory effects are mediated via DG2, it can therefore be implied from this parental data that DG2-mediated regulation of the Imd pathway may occur upstream of Imd. However, further investigation is required in order to confirm this hypothesis.

Overall, it can be seen from results that overexpression of DG1 in the tubule is sufficient to significantly rescue activation of diptericin expression in the tubules of *imd*-deficient flies. These data therefore confirm that DG1-mediated regulation of the Imd pathway occurs downstream of Imd.

5.6 cGMP pathway regulation of the Imd pathway is a result of modulation of Relish activation

5.6.1 Introduction

As mentioned previously, stimulation of the Imd pathway in *Drosophila* ultimately results in the activation of the NFκB transcription factor, Relish. Relish is a 110 kDa protein, homologous to mammalian p105, comprising an N-terminal Rel-homology domain (RHD) and a C-terminal IκB-like inhibitory domain (Dushay et al. 1996). Studies have shown that upon activation full-length Relish is rapidly cleaved by the caspase-8 homologue DREDD to form two stable fragments; REL-68, containing the RHD, which translocates into the nucleus immediately upon cleavage, and REL-49, which contains the IκB inhibitory domain and is retained in the cytoplasm (Stoven et al. 2000; Stoven et al. 2003). Given that previous studies have identified mammalian NFκB as a phosphorylation target for PKG (He and Weber 2003), and that initial epistatic analysis has confirmed a role for DG1 downstream of Imd, studies were initiated in order to investigate Relish as a potential target for the action of cGMP/cGKs.

In recent years, a number of studies have used either fluorescent reporters or ICC methods to visualise translocation of REL-68 into the nucleus in both *Drosophila* cell lines and *ex vivo* in the fly (Stoven et al. 2000; Stoven et al. 2003; Bettencourt et al. 2004; Foley and O'Farrell 2004). The ability to visualise Relish activation in a cellular context has provided a valuable tool for defining upstream regulators of the Imd pathway. Therefore for this study, activation of Relish was fluorescently monitored in the tubules in order to assess the effects of cGMP/cGK-mediated regulation of the Imd pathway. In this approach, $w^{(-)}$;UAS-*relish/CyO*;*c42/Sb* flies (described in Section 5.3) were either monitored directly for Relish activation in response to stimulation with either cGMP or PGN(-), or were crossed to homozygous UAS-cGK transgenic flies and the activation of Relish assessed in the resultant progeny. To do this, the tubules of each transgenic line were excised and the localisation of Relish in each set of tubules was then determined by ICC. It should be noted that the original UAS-*relish* transgenic fly line used to generate the $w^{(-)}$;UAS-*relish/CyO*;*c42/Sb* parental flies has been described previously and can be characterised by its expression of a Relish fusion protein comprising the full-length Relish protein downstream of a N-terminal hexahistidine tag. Therefore, in these

experiments Relish activation was fluorescently detected using an anti-tetra-HIS primary antibody (QIAGEN) followed by a FITC-labelled secondary antibody (Jackson). Additionally, tubules were stained with 4, 6-diamidino-2-phenylindole (DAPI), a fluorescent dye known to bind double-stranded DNA, thus allowing visualisation of cell nuclei. Samples were then viewed using the Zeiss 510 Meta confocal system.

5.6.2 Nuclear translocation of Relish in the tubule is modulated by cGMP in response to immune challenge

Prior to carrying out experiments to determine the effect of cGK modulation on Relish activation, initial studies were carried out in order to assess the effect of stimulation with cGMP and/or PGN(-) on Relish activation in the tubules of the $w^{(-)}$;UAS-*relish/CyO*;*c42/TM3Sb* parental flies. To do this, tubules were excised and incubated for 3 h in either sterile Schneider's medium (as a control), or sterile Schneider's medium containing either 100 µM cGMP and/or 5 µg/ml PGN(-). Levels of Relish activation were then detected by ICC as described above. It should be noted that ICC experiments were also carried out on the tubules of $w^{(-)}$;UAS-*relish*;*TM3Sb/TM6e*$^{-}$ parental flies to act as a negative control for antibody staining.

As Figure 5.4A shows, in the absence of stimuli Relish localisation in the tubule is mainly basolateral (yellow arrows). Interestingly, low levels of Relish can also be detected in the nucleus (white arrows), despite the absence of an immune stimulant. This is in agreement with previous studies however, where it has been demonstrated that Relish is constitutively active at a basal level (Stoven et al. 2000; Bettencourt et al. 2004). Conversely, it can be seen that when an immune response is stimulated in the tubules via incubation with PGN(-), complete translocation of Relish into the nucleus is observed (Figure 5.4B). This data therefore further confirms the effectiveness of PGN(-) as an Imd pathway-activator in the tubule. When tubules are stimulated with 100 µM cGMP, a concentration shown previously to inhibit diptericin expression in the tubules, it can be seen that localisation of Relish remains predominately basolateral (Figure 5.4C). As with controls, only minimal levels of nuclear Relish can be detected in cGMP-stimulated tubules, though it appears that levels of nuclear localisation of Relish are lower in cGMP-stimulated tubules compared to control tubules. Interestingly, it can be seen from Figure

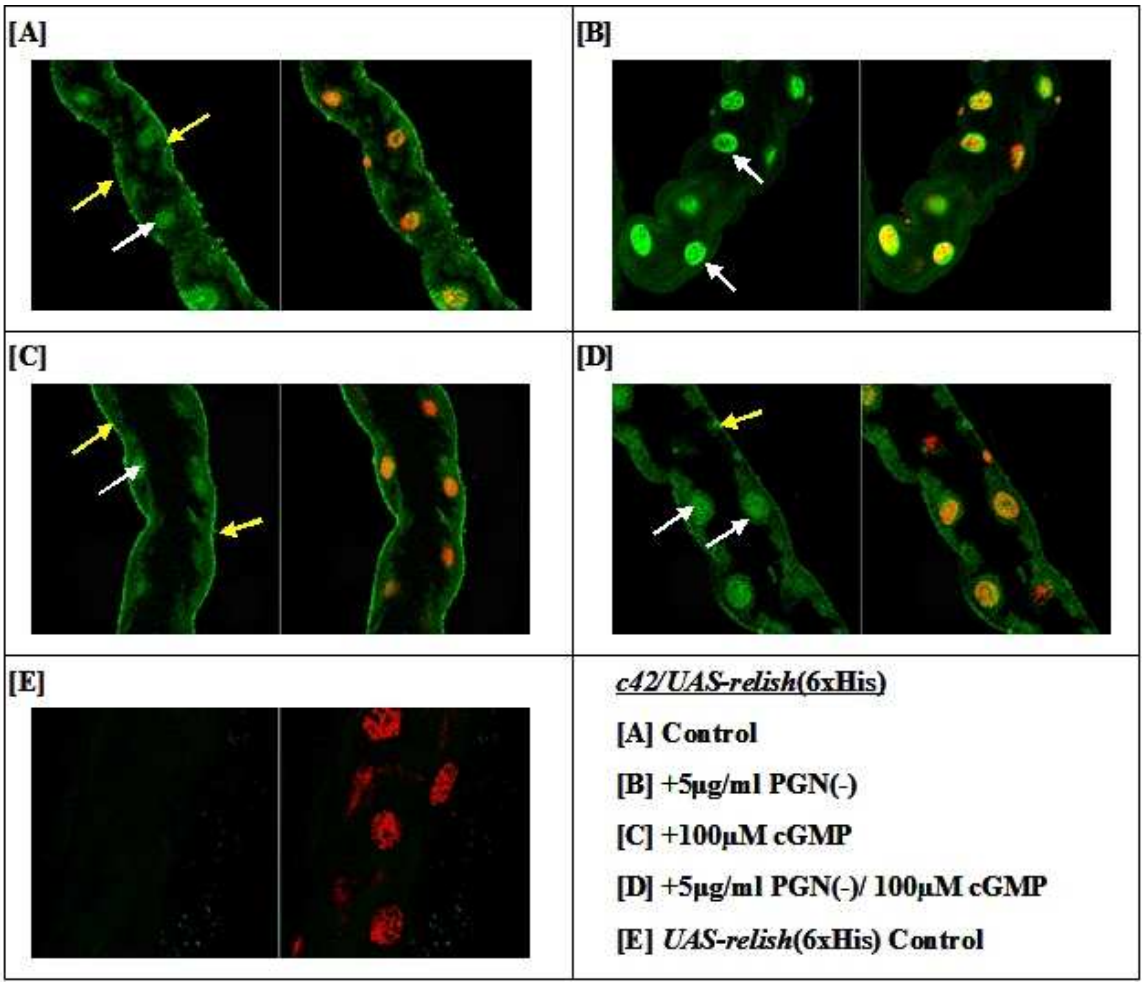


Figure 5.4 – Nuclear translocation of Relish in the tubules. Relish localisation was monitored in the tubules of c42/UAS-*relish*(6xHis) flies using ICC. [A] In the absence of stimuli Relish is mainly localised to the basolateral membrane with basal levels of localisation to the nucleus, indicating a basal level of relish activation. [B] When an immune response is activated in the tubule using PGN(-), there is complete translocation of Relish to the nucleus, with no basolateral localisation. [C] Relish localisation in response to 100 µM cGMP. As with controls, Relish is mainly localised to the basolateral membrane, with minimal localisation to the nucleus [D] Immune activation with PGN(-) in the presence of 100 µM cGMP results in the inhibition of nuclear translocation. [E] UAS-*relish* parental control. Left hand panel – FITC (Green); right hand panel – FITC and DAPI (Red) merged (staining in the nuclei may appear red/yellow or yellow/green depending on levels of Relish translocation). White arrows – nuclear staining, yellow arrows – basolateral staining

5.4D that incubation of the tubules with both 5 µg/ml PGN(-) and 100 µM cGMP results in mainly basolateral localisation of Relish, with only slightly higher levels of Relish translocation into the nucleus than in control tubules, despite the presence of an immune stimulant. This data therefore confirms that the negative effect of 100 µM cGMP on AMP expression shown in previous results is a result of regulation of Relish activation, and subsequent regulation of AMP transcription.

5.6.3 Nuclear translocation of Relish is modulated by cGMP in a dose-dependent manner.

In chapter 3, it was demonstrated that the effect of cGMP on AMP expression in the tubule is dependent on concentration; whereby low, nanomolar concentrations of cGMP were shown to enhance diptericin expression, and high, micromolar concentrations were shown to inhibit diptericin expression. Therefore, in order to investigate this effect with regards to Relish activation, studies were initiated using the $w^{(-)}$;UAS-*relish/CyO*;*c42/TM3Sb* transgenic flies described previously. In these experiments, tubules were excised and incubated for 3 h in sterile Schneider's medium in the presence of varying concentrations of cGMP. Levels of Relish activation were then detected by ICC as described above.

As Figure 5.5 shows, the dose-dependent effect of cGMP on diptericin expression described previously is a result of cGMP-mediated modulation of Relish activation within the tubule. To explain further, it can be seen from control tubules (Figure 5.5A) that, as before, Relish is localised mainly basolaterally with only basal levels of nuclear Relish observed. Conversely, it is demonstrated that when tubules are stimulated with 1 nM cGMP (Figure 5.5B*i*), complete translocation of Relish to the nucleus occurs, thus indicating complete activation of the protein. Similarly, as Figures 5.5B*ii* and 5.5B*iii* show, when tubules are stimulated with either 10 nM or 100 nM cGMP respectively, almost complete nuclear translocation occurs, with only minimal levels of inactivated Relish observed basolaterally.

Equally, it can be seen from Figure 5.5C that incubation of the tubules with micromolar concentrations of cGMP appears to have an inhibitory effect on Relish activation. When tubules are incubated with 1 µM cGMP (Figure 5.5C*i*), Relish localisation appears to be very similar to that of control tubules, whereby Relish appears to be mainly basolateral with only low levels of nuclear translocation. Similarly, when tubules are incubated with either 10 µM or 100 µM cGMP (Figures 5.5C*ii* and 5.5C*iii* respectively), Relish localisation is again mainly basolateral. However, it can be seen from the results that as cGMP concentration is increased, levels of nuclear Relish are decreased. Indeed, it is demonstrated in Figure 5.5C*iii* that, as before, only minimal levels of nuclear Relish can

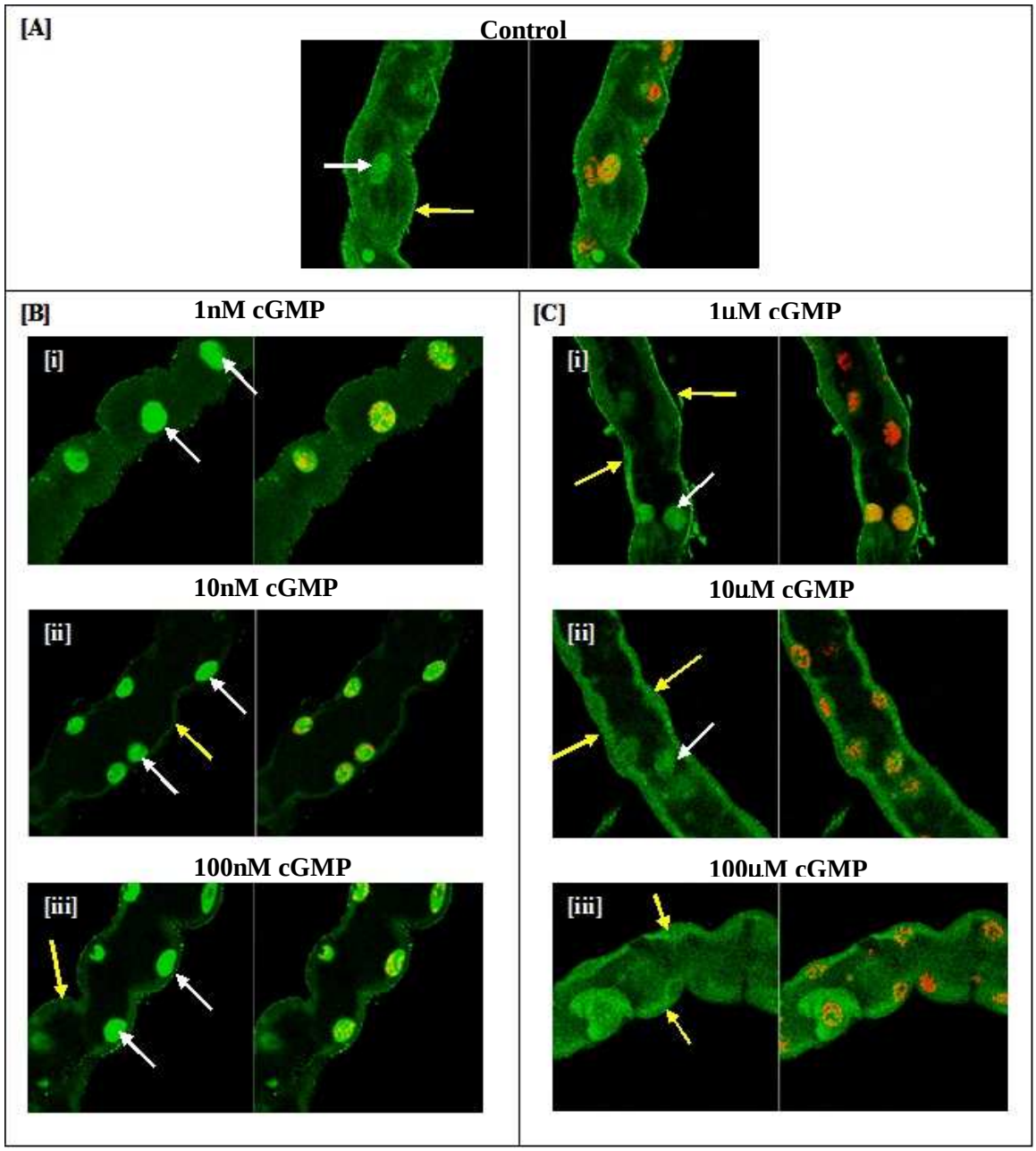


Figure 5.5 - Nuclear translocation of Relish is modulated by cGMP in the tubules. Relish localisation was monitored in the tubules of c42/UAS-*relish*(6xHis) flies in response to various concentrations of cGMP. [A] Control - In the absence of challenge Relish is mainly localised to the basolateral membrane with slight localisation to the nucleus, indicating a basal level of activation. [B] Relish localisation in response to low nanomolar concentrations of cGMP. Stimulation with 1 nM cGMP [i] results in complete translocation of Relish into the nucleus. Stimulation with 10 nM cGMP [ii] or 100 nM cGMP [iii] results in almost complete nuclear translocation of Relish, however some basolateral staining is still observed. [C] Relish localisation in response to high micromolar concentrations of cGMP. As with controls, stimulation with either 1 µM cGMP [i] or 10 µM cGMP [ii] results in high levels of basolateral staining, with low levels of localisation to the nucleus, however basal levels of nuclear translocation in response to 10 µM appears to be slightly decreased compared to controls. Stimulation with 100 µM cGMP [iii] results in almost completely basolateral staining, with minimal nuclear localisation. Left hand panel – FITC (Green); right hand panel – FITC and DAPI (Red) merged (staining in the nuclei may appear red/yellow or yellow/green depending on levels of Relish translocation). White arrows – nuclear staining, yellow arrows – basolateral staining

be observed in the tubules compared to controls in response to 100 µM cGMP, suggesting an inhibitory effect of cGMP at this concentration.

Overall, these data show for the first time, that dose-dependent regulation of the Imd pathway by cGMP is a result of modulation of Relish activation.

5.6.4 DG1 modulation of the Imd pathway activates Relish translocation in the tubule

As demonstrated previously, stimulation of the Imd pathway by cGMP is mediated by DG1. Therefore, given that the results shown in Figure 5.4B demonstrate that cGMP-mediated stimulation of the Imd pathway occurs via modulation of Relish activation, it is probable that this effect occurs via activation of DG1. Studies were therefore initiated in order to assess Relish activation in the tubules of flies whereby *dg1* was either overexpressed or knocked-down via RNAi. To do this, homozygous transgenic flies containing either UAS-*dg1* or UAS-*dg1*RNAi transgenes were crossed to $w^{(-)}$;UAS-*relish/CyO*;*c42/TM3Sb* flies and resultant progeny were selected to contain one copy of each transgene. Tubules were then excised and incubated for 3 h in either sterile Schneider's medium (as a control), or sterile Schneider's medium containing either 100 µM cGMP and/or 5 µg/ml PGN(-). Relish activation was then monitored in the tubules of selected flies by ICC as described previously.

As Figure 5.6A shows, it can be seen that targeted expression of *dg1* to the tubule principle cells results in enhanced levels of Relish activation, even in the absence of an immune stimulus. It is demonstrated that, in contrast to UAS-*relish/CyO*;*c42/Sb* parental controls (Figure 5.3A), targeted overexpression of *dg1* to the tubules results in almost total activation of Relish, with only minimal basolateral staining detected (Figure 5.6A*i*). Interestingly, it can be seen that enhanced Relish activation also occurs in the tubules of flies over-expressing *dg1* in response to 100 µM cGMP (Figure 5.6A*iii*). These results support previous data (Figure 4.1) which shows that diptericin expression is increased in the tubules in response to 100 µM cGMP when *dg1* is overexpressed. As expected, when the tubules are stimulated with exogenous PGN(-), complete translocation of Relish is observed (Figure 5.6A*ii*). However, in contrast to results obtained from the tubules of the UAS-*relish/CyO*;*c42/Sb* parental flies (Figure 5.3D), it can be seen that complete

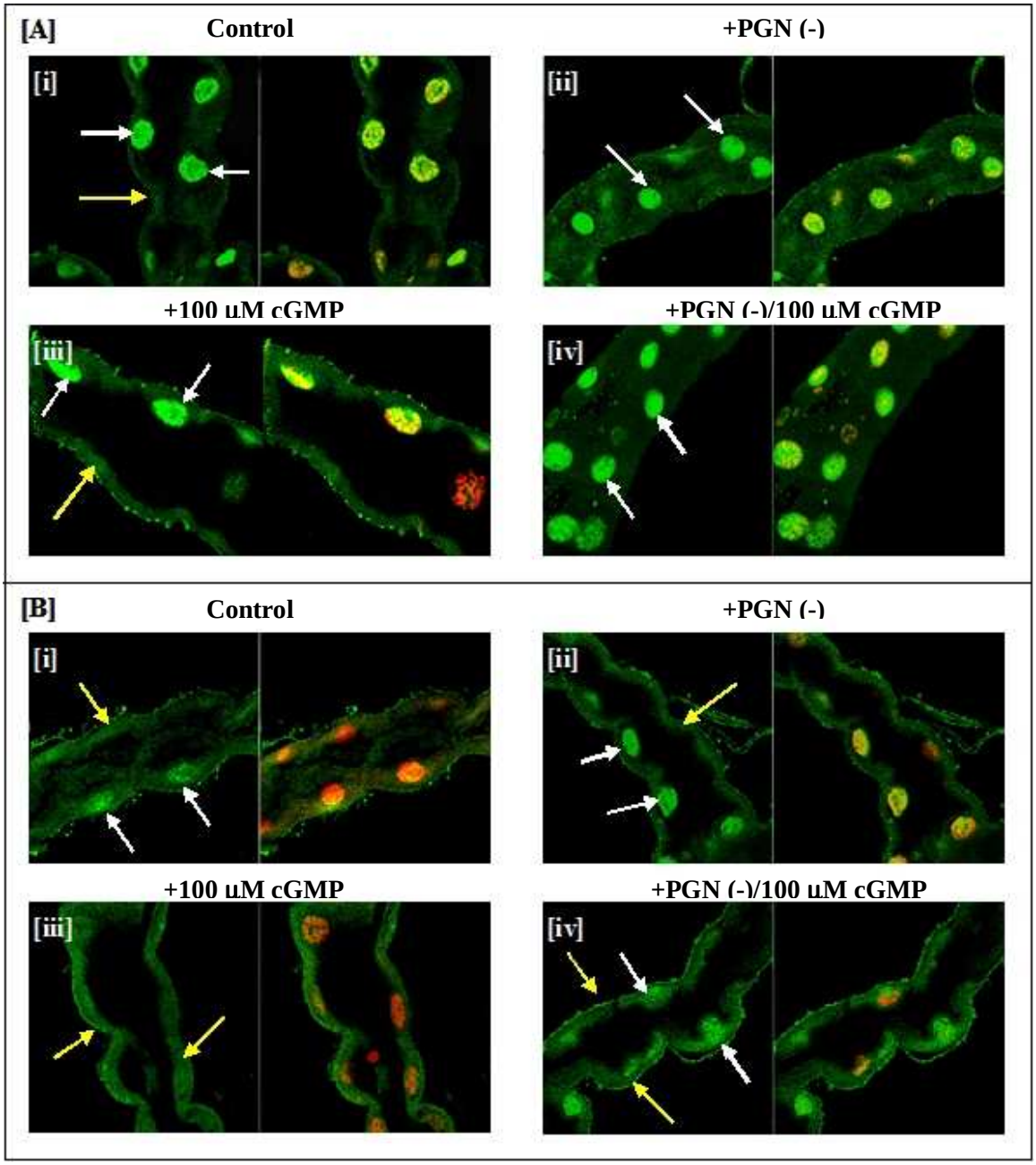


Figure 5.6 - Nuclear translocation of Relish is stimulated by DG1 in the tubules. Relish localisation was monitored in the tubules of flies whereby *dg1* expression was modulated using the GAL4/UAS binary system. Targeted overexpression or knock-down of *dg1* was achieved by crossing c42/UAS-*relish*(6xHis) flies to UAS-*dg1* or UAS-*dg1*RNAi flies respectively. [A] Relish localisation in the tubule when *dg1* is overexpressed [i] Control – Shows high levels of nuclear translocation of Relish with minimal basolateral staining [ii] +5 µg/ml PGN(-) – results in complete nuclear translocation of Relish [iii] +100 µM cGMP – shows almost complete translocation of Relish, with a low level of basolateral staining [iv] +100 µM cGMP/5 µg/ml PGN(-) – results in complete nuclear translocation of Relish. [B] Relish localisation in the tubule when *dg1* expression is knocked-down [i] Control – As with parental controls (See Figure 5.4A), shows mainly basolateral staining with low levels of nuclear localisation [ii] +5 µg/ml PGN(-) – results in partial nuclear translocation of Relish with low levels of basolateral staining [iii] +100 µM cGMP - results in complete basolateral localisation [iv] +100 µM cGMP/5 µg/ml PGN(-) – results in low levels of nuclear translocation of Relish with high levels of basolateral localisation. Left hand panel – FITC (Green); right hand panel – FITC and DAPI (Red) merged (staining in the nuclei may appear red/yellow or yellow/green depending on levels of Relish translocation). White arrows – nuclear staining, yellow arrows – basolateral staining

translocation of Relish is also observed in response to PGN(-) in the presence of 100 µM cGMP (Figure 5.6A*iv*).

In support of this data, it is demonstrated that when *dg1* expression is knocked-down in the tubule principal cells, Relish activation is inhibited. As Figure 5.6B shows, it can be seen that the tubules of *dg1*-deficient flies are unable to induce Relish activation to the same degree as the tubules of UAS-*relish*/+;*c42/Sb* parental flies (Figure 5.3B) in response to stimulation with PGN(-) (Figure 5.6B*ii*). Results show that, unlike in parental tubules where Relish is localised completely in the nucleus after PGN(-) stimulation, only partial nuclear translocation of Relish can be observed in the tubules of *dg1*-deficient flies, with low levels of basolateral staining also detected. These results therefore indicate an inhibitory effect on the Imd pathway in the tubules when *dg1* expression is depleted. Indeed, this effect is further enhanced when tubules are stimulated with 100 µM cGMP. It can be seen from Figure 5.6B*iii* that in the presence of cGMP alone, Relish localisation is completely basolateral, with no nuclear staining detected. This suggests that the negative effect on Relish activation normally seen in the tubules of parental flies in response to 100 µM cGMP is enhanced in the absence of DG1, resulting in significant inhibition of basal levels of Relish activation. In support of this, it can be seen that when the tubules of *dg1*-deficient flies are stimulated by PGN(-) in the presence of 100 µM cGMP, Relish localisation remains mainly basolateral with only a low level of nuclear translocation detected.

Overall, the data shown in Figure 5.6 demonstrates a completely novel role for DG1 in the tubule as a positive regulator of Relish activation.

5.6.5 DG2 modulation of the Imd pathway inhibits Relish translocation in the tubule

As shown in the previous chapter, the two main isoforms of DG2, DG2P1 and DG2P2, have been demonstrated to act as negative regulators of diptericin expression. Therefore, given that the results shown in Figure 5.4C indicate that cGMP-mediated inhibition of the Imd pathway is a result of modulation of Relish activation, it is probable that this effect occurs via activation of DG2P1 and DG2P2. Therefore, studies were initiated to assess

Relish activation in the tubules of flies where expression of either *dg2P1* or *dg2P2* was modulated. To do this, homozygous transgenic flies containing either UAS-*dg2P1* or UAS-*dg2P2* transgenes were crossed to $w^{(-)}$;UAS-*relish/CyO*;*c42/TM3Sb* flies and, as with previous experiments, resultant progeny were selected to contain one copy of each transgene. Tubules were then excised and incubated with either PGN(-) and/or cGMP and Relish activation monitored by ICC.

It can be seen from Figure 5.7 that targeted expression of either *dg2P1* or *dg2P2* to the tubule results in inhibition of Relish activation. As with UAS-*relish*/+;*c42*/S*b* parental controls, it can be seen that in the control tubules of flies overexpressing either *dg2P1* or *dg2P2* (Figures 5.7A*i* and 5.7B*i* respectively), localisation of Relish is mainly basolateral with only minimal levels of nuclear localisation detected. It cannot be concluded from these figures, however, whether or not overexpression of *dg2P1* or *dg2P2* alone is enough to inhibit Relish activation in the tubule. Conversely, a clear inhibition of Relish activation can be seen in the tubules of both *dg2P1* and *dg2P2* overexpressing flies in response to PGN(-) (Figures 5.7A*ii* and 5.7B*ii* respectively). Unlike the tubules of parental flies, whereby complete translocation of Relish to the nucleus is observed in response to stimulation with PGN(-) (Figure 5.3B), it can be seen that in the tubules of the *dg2P1* and *dg2P2* overexpressing flies, a great deal of Relish is retained in the cytoplasm, with only partial translocation of Relish into the nucleus observed. Similarly, it is shown that this inhibitory effect is enhanced in the presence of cGMP. As Figure 5.7A*iii* shows, when the tubules of *dg2P1*-overexpressing flies are stimulated with 100 µM cGMP, localisation of Relish is almost completely basolateral, with only minimal levels of nuclear Relish observed. This effect is further enhanced in the tubules of *dg2P2*-overexpressing flies, where it can be seen that stimulation with 100 µM cGMP results in completely basolateral localisation of Relish (Figure 5.7B*iii*). Importantly, when the tubules of either *dg2P1* or *dg2P2* overexpressing flies are stimulated with PGN(-) in the presence of 100 µM cGMP (Figures 5.7A*iv* and 5.7B*iv* respectively), Relish activation does not appear to be enhanced above a basal level, despite PGN(-) stimulation.

Unfortunately, experiments assessing the impact of knock-down of *dg2* expression on Relish activation in the tubule could not be carried out. As mentioned in the previous chapter, when UAS-*dg2*RNAi flies are crossed to c42, only minimal survival of the

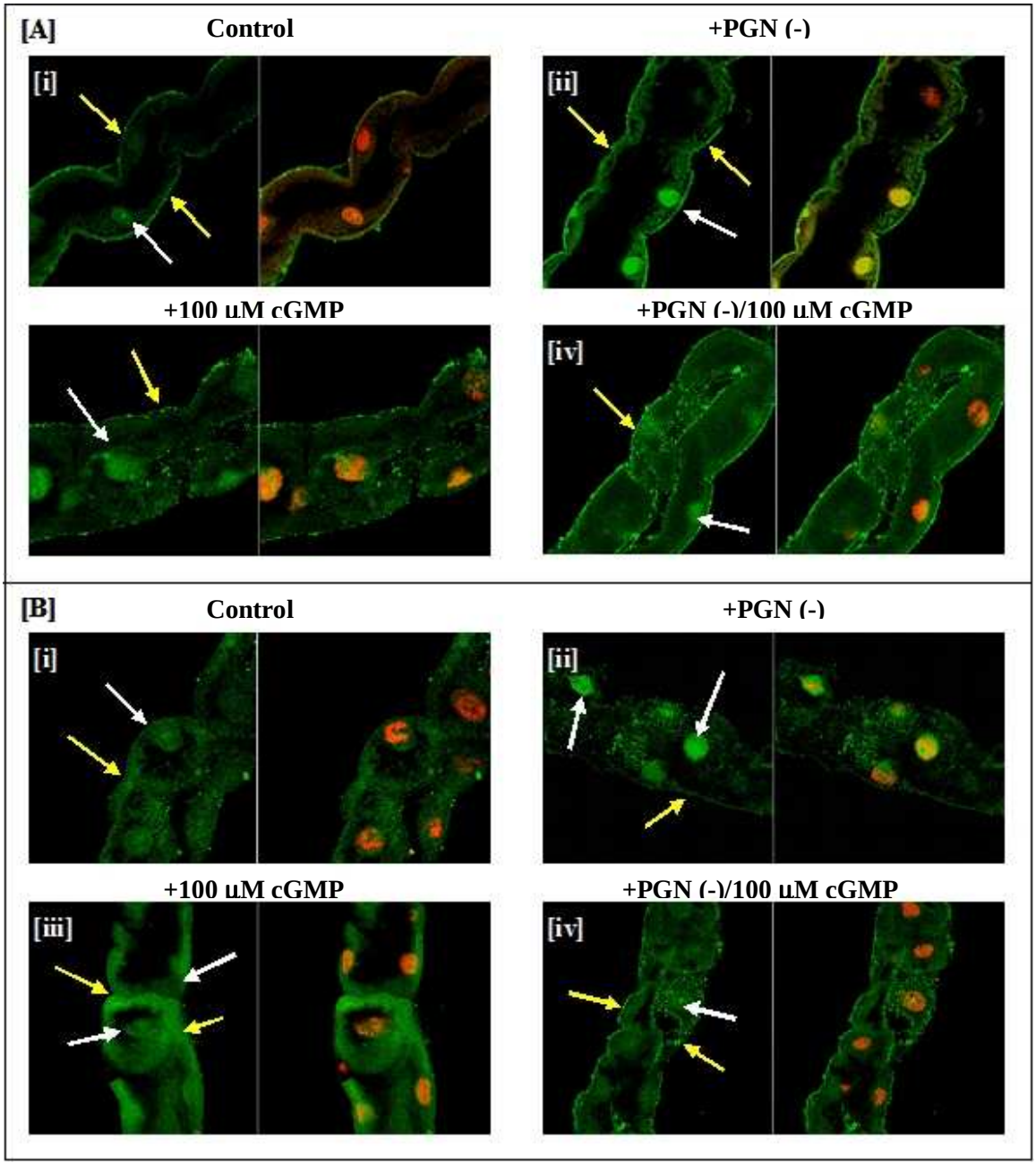


Figure 5.7 - Nuclear translocation of Relish is inhibited by DG2 in the tubules. Relish localisation was monitored in the tubules of flies in which DG2 expression was modulated using the GAL4/UAS binary system. Targeted overexpression of *dg2P1* and *dg2P2* was achieved by crossing c42/UAS-*relish*(6xHis) flies to UAS-*dg2P1* or UAS-*dg2P2* flies respectively. [A] Relish localisation in the tubule when *dg2P1* is overexpressed [i] Control – shows minimal levels of nuclear translocation of Relish with high levels of basolateral localisation [ii] +5 µg/ml PGN(-) – results in partial nuclear translocation of Relish with low levels of basolateral localisation [iii] +100 µM cGMP – shows minimal levels of nuclear translocation of Relish with high levels of basolateral localisation [iv] +100 µM cGMP/5 µg/ml PGN(-) – results in minimal nuclear translocation of Relish with high levels of basolateral localisation. [B] Relish localisation in the tubule when *dg2P2* is over-expressed [i] Control – shows mainly basolateral staining with minimal levels of nuclear localisation [ii] +5 µg/ml PGN(-) – results in partial nuclear translocation of Relish with low levels of basolateral localisation [iii] +100 µM cGMP - results in almost complete basolateral localisation with minimal nuclear localisation [iv] +100 µM cGMP/5 µg/ml PGN(-) – results in very low levels of nuclear translocation of Relish with high levels of basolateral localisation. Left hand panel – FITC (Green); right hand panel – FITC and DAPI (Red) merged (staining in the nuclei may appear red/yellow or yellow/green depending on levels of Relish translocation). White arrows – nuclear staining, yellow arrows – basolateral staining

progeny is observed beyond pupal stage. Therefore it was not possible to investigate the effect of *dg2* knock-down using the *w*$^{(-)}$;UAS-*relish*/*CyO*;*c42*/S*b* transgenic line generated for this study. Unfortunately, the use of the inducible GAL80;c42 transgenic line generated to facilitate UAS-*dg2*RNAi expression in the previous chapter was also not possible, as the generation of a transgenic line that expressed both UAS-*dg2*RNAi and UAS-*relish* alongside both GAL80 and c42 could not be achieved due to the chromosomal location of each of the transgenes. However, it is hypothesised that had it been possible to investigate the effect of *dg2* knock-down on Relish activation in the tubule, results would show stimulation of Relish in the absence of DG2.

Overall, a clear inhibition on Relish activation can be seen in the tubule of flies overexpressing either *dg2P1* or *dg2P2* in the tubule (Figure 5.7). Therefore the data shown here demonstrates a novel role for both DG2P1 and DG2P2 in the tubule as negative regulators of Relish activation.

5.7 Discussion

In the previous chapter, the *Drosophila* cGKs, DG1 and DG2, were demonstrated for the first time to play an important role in regulation of immune response in the tubules of the adult fly. In chapter 4, it was reported that these cognate kinases mediate differential effects on the production of the anti-microbial peptide diptericin, and that these effects are sufficient to impact on whole fly survival in response to bacterial challenge. As mentioned previously, induction of diptericin production is known to occur as a direct result of activation of the Imd pathway. Therefore, it can be hypothesised that cGK modulation of diptericin expression occurs either through interaction with one or more components of the Imd pathway, or by transcriptional regulation of diptericin expression itself. In this chapter, it is demonstrated for the first time that activation of the NFκB transcription factor, Relish, is dose-dependently modulated by cGMP and that these effects are mediated differentially by DG1 and DG2.

Initial epistatic analysis has shown that overexpression of DG1 is sufficient to significantly rescue activation of diptericin expression in the tubules of *imd*-deficient flies. This data has therefore confirmed the regulatory role of DG1 downstream of Imd. Interestingly, analysis of diptericin expression in the tubules of parental *imd*-deficient

flies has shown that, unlike in wild type tubules, where incubation with 100 µM cGMP has an inhibitory effect on diptericin expression, tubules from *imd*-deficient flies show no significant change in diptericin expression in response to 100 µM cGMP. This effect is most likely explained by the fact that initial expression levels of diptericin are already extremely low in the tubules of the imd^1 mutant flies, and therefore incubation with cGMP would have no significant effect on expression. Alternatively, this data may have revealed a potential serendipitous upstream role for DG2 in Imd pathway modulation. The typical inhibitory effect of 100µM cGMP on diptericin expression in the tubules is known to be mediated by DG2. As this effect is not observed in the tubules of *imd*-deficient flies, it can therefore be suggested that DG2 may act upstream of Imd. However, this can only be implied from the data obtained. Further epistatic analysis is required in order to conclusively determine a potential target for DG2 within the Imd pathway.

Importantly, it is demonstrated here that dose-dependent modulation of AMP expression by cGMP/cGKs is a result of activation/inhibition of Relish. Unfortunately, extensive epistatic analysis could not be carried out, thus it is not known whether the effects of these kinases are a consequence of upstream regulation of the Imd pathway, or are occurring through direct interaction with Relish itself. Therefore, further analysis is required in order to determine the exact target of action of each of these kinases. Furthermore, it cannot be concluded from this data whether DG1 or DG2 are mediating their effects on the Imd pathway directly or indirectly; i.e. – whether the action of these kinases is a result of direct phosphorylation of components of the Imd pathway, or via phosphorylation of an as of yet unidentified substrate. However, given that previous studies have identified NFκB as a phosphorylation target for mammalian PKG, it can be hypothesised that one, or both, of these kinases may be acting to modulate Relish activation through direct phosphorylation of Relish itself.

Overall, this study has identified that the novel, differential regulatory roles of DG1 and DG2 on AMP expression are a result of either stimulation or inhibition of Relish activation respectively, however further investigation is required in order to identify definitive candidates as substrate(s) for either DG1 or DG2 in this regulation.

Chapter 6

The role of other cGMP pathway components in Imd pathway regulation

6.1 Summary

As mentioned previously, cGMP is known to exist in localised pools, generated in close proximity to particular upstream activators, i.e. soluble or receptor guanylate cyclases (Davies and Day 2006). As such, the effect of each distinct cGMP signal within the cell is then determined by its proximity and affinity to particular downstream effectors such as cGKs, PDEs and CNG channels (Beavo and Brunton 2002; Piggott et al. 2006). This study has demonstrated that, in the tubule, cGMP mediates differential regulation of the Imd pathway, via the activation of the cGKs, DG1 and DG2. Due to their differing localisation within the tubule, it has been hypothesised that DG1 and DG2 are activated via different 'sources' of cGMP within the cell, thus facilitating the distinct action of these otherwise cognate kinases. Similarly, the activation of the kinases themselves is known to be finely regulated via the hydrolysis of cGMP to 5′GMP by specific PDEs. Therefore, using a combination of transgenic, pharmacological and Q-PCR approaches, preliminary studies were carried out in order to investigate the role of other cGMP pathway components in Imd pathway regulation in the tubule. Data obtained has demonstrated that the differential effects of cGMP on diptericin expression are mediated by activation of distinct guanylate cyclases, whereby diptericin expression is stimulated by sGC and inhibited by rGC. Similarly, a role for the dual-specificity phosphodiesterase, *Dm*PDE11, is described here, where it is demonstrated that knockdown of this enzyme in the tubule principal cells by RNAi results in inhibition of diptericin expression and reduced survival in response to septic infection with *E.coli*.

6.2 The differential effects of cGMP signalling on Imd pathway regulation are mediated via distinct guanylate cyclases

6.2.1 Introduction

To date, as with studies investigating cGK-related effects in *Drosophila*, investigation into the functional effects of *Drosophila* guanylate cyclases have mainly focussed on neuronal function. For example, isoforms of both sGC and rGC have been mapped to the head and nervous system (Yoshikawa et al. 1993; Liu et al. 1995; McNeil et al. 1995; Shah and Hyde 1995; Langlais et al. 2004; Morton et al. 2005). Similarly, a number of studies have identified GCs to play a role in such processes as foraging, axonal guidance, synaptic transmission and neuromuscular junction vesicle release (Wildemann and Bicker

1999; Ayoob et al. 2004; Riedl et al. 2005; Morton et al. 2008). In addition, a role for sGC has been described with regards to visual system development, where studies have shown that sGC is highly expressed in the retina and optic lobes and acts to regulate growth cone behaviour (Yoshikawa et al. 1993; Shah and Hyde 1995; Gibbs et al. 2001).

Rather unsurprisingly, given the important role of cGMP signalling in tubule function, studies have also demonstrated that sGC and a number of rGC isoforms, particularly Gyc76C, are enriched in the Malpighian tubule (Wang et al. 2004; Davies 2006; *www.flyatlas.org*). In addition, an important role for both sGC and rGC in the regulation of epithelial fluid secretion in the tubule has also been demonstrated (Davies et al. 1997; Kerr et al. 2004). However to date, aside from a role in fluid secretion, the specific function of each of these GCs with regards to the downstream physiological effects of cGMP signalling in the tubule has not yet been defined. Given that a number of GCs are expressed in the tubule, it can be suggested that the numerous physiological effects regulated by cGMP signalling in this tissue may be mediated via the activation of specific guanylate cyclases, which act to generate distinct localised cGMP signals within each cell. Indeed in this study, it has been hypothesised that the contrasting effects of cGMP signalling on Imd pathway regulation in the tubule are a result of the differential activation of cGKs by different 'sources' of cGMP. Therefore, in order to determine whether the contrasting roles of cGKs with regards to immune function are mediated by differential activation of GCs, preliminary studies were carried out, using a combination of transgenic, pharmacological and Q-PCR approaches, to investigate the role of guanylate cyclases in Imd pathway regulation.

6.2.2 Activation of soluble guanylate cyclase by NO mediates positive regulation of diptericin expression in the tubule

As described previously, soluble guanylate cyclases are activated through interaction with NO, a diffusible molecule that is produced by a family of enzymes known as nitric oxide synthases (NOS) (Stuehr 1999; Marletta and Spiering 2003). Recently, a number of studies have identified the *Drosophila* NOS, DNOS, as an important positive regulator of immune function (Foley and O'Farrell. 2003; McGettigan et al. 2005). In the first of these studies, it was shown that DNOS inhibition results in decreased survival of larvae in response to *E.caratovora*. Similarly, larvae that had been fed the NO donor S-Nitroso-

N-acetylpenicillamine (SNAP) were shown to exhibit elevated levels of diptericin expression (Foley and O'Farrell 2003). Interestingly, in the second of these studies, an important role for DNOS in tubule immune function has been described. In this study, the tubules were identified to exhibit increased NOS activity in response to immune challenge. Additionally, overexpression of *dNOS* in the tubule was demonstrated to increase tubule diptericin expression, which was sufficient to confer increased survival of the whole organism in response to septic infection with *E.coli* (McGettigan et al. 2005).

Considering that the main intracellular receptor for NO is sGC, it is probable that the effects of dNOS on immune regulation in the tubule are linked, via activation of sGC, to cGMP/cGK-mediated regulation of the Imd pathway, demonstrated earlier in this study. Furthermore, given that the role of dNOS has been demonstrated as stimulatory, it can be suggested that it may be mediating its effects, via sGC, through downstream activation of DG1. Indeed, if the differential effects of DG1 and DG2 on Imd pathway regulation in the tubule are mediated via the generation of distinct pools of cGMP within the cell, then the mainly cytosolic localisation of DG1 would suggest that activation of this kinase is mediated via a cytosolic source of cGMP, i.e. cGMP that has been generated via the activation of sGC by NO. Therefore in this study, preliminary experiments were initiated in order to determine whether NO-mediated immune regulation in the tubule is facilitated by activation of sGC and therefore subsequent activation of downstream cGMP signalling.

Prior to investigating the role of sGC in immune activation in the tubule, studies were first of all initiated in order to investigate the mechanism by which NO is mediating its effects on immune response. Previously in this study, it has been demonstrated that cGK-mediated regulation of diptericin expression is a result of modulation of Relish activation, whereby DG1 has been shown to enhance Relish activation and DG2 has been shown to inhibit it. Recent studies have demonstrated that, similar to DG1, NO acts to increase diptericin expression in the tubule (McGettigan et al. 2005). However, the mechanism by which NO achieves its effects has not yet been demonstrated. It can be suggested however, that if NO-mediated stimulation of diptericin in the tubule is a result of modulation of Relish activation, then perhaps NO may be mediating its effects via the cGMP signalling pathway. Therefore, in order to investigate this hypothesis, Relish activation was monitored in the tubules in response to the nitric oxide donor SNAP. To

do this, an ICC approach was used. Briefly, the tubules of $w^{(-)}$;UAS-*relish/CyO*;c42/*TM3sb* transgenic flies, described in chapter 5, were excised and incubated for 3 hr in either sterile Schneider's medium (as a control), or sterile Schneider's medium containing 1 mM of SNAP. Levels of Relish activation were then detected by ICC as described previously.

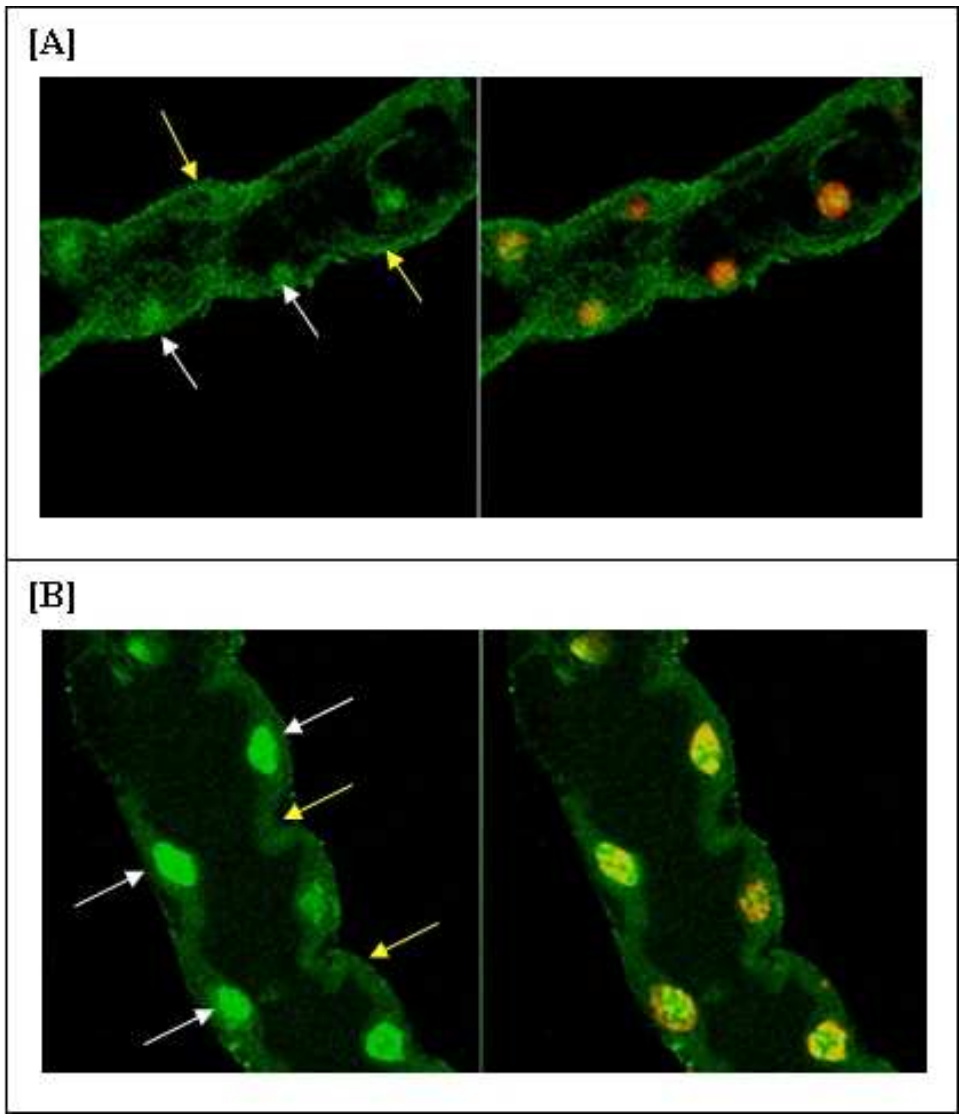


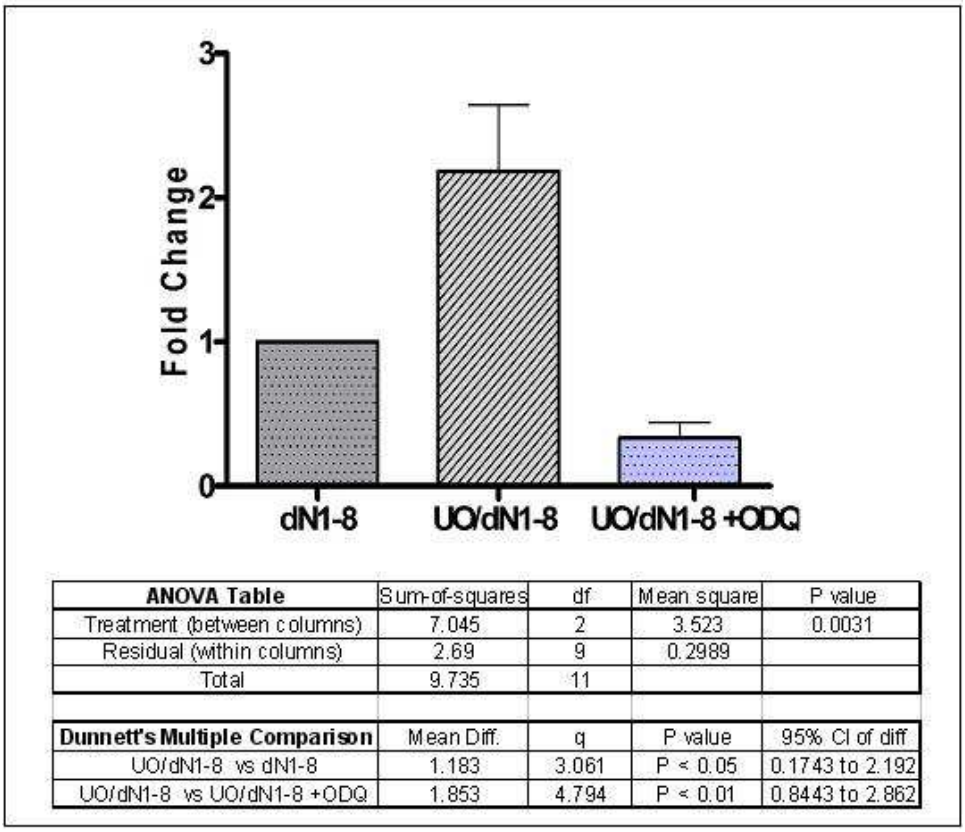
Figure 6.1 – Nuclear translocation of Relish in the tubules is stimulated by NO. Relish localisation was monitored in the tubules of c42/UAS-*relish*(6xHis) flies in response to the NO donor SNAP. [A] Control - In the absence of challenge Relish is mainly localised to the basolateral membrane with slight localisation to the nucleus, indicating a basal level of activation. [B] Incubation of the tubules with 1 mM SNAP results in almost complete nuclear translocation of Relish, with only minimal levels of basolateral staining observed. Left hand panel – FITC (Green); right hand panel – FITC and DAPI (Red) merged (staining in the nuclei may appear red/yellow or yellow/green depending on levels of Relish translocation). White arrows – nuclear staining, yellow arrows – basolateral staining

Figure 6.1 shows that, as with previous results, in the absence of stimuli Relish localisation is mainly basolateral, with only basal levels of activated Relish localised to the nucleus (Figure 6.1A). However, when the tubules are stimulated with SNAP, thus increasing intracellular NO concentration, almost complete translocation of Relish into the nucleus is observed (Figure 6.1B). These results therefore demonstrate that, similar to modulation of AMP production by cGMP signalling, NO-mediated stimulation of diptericin expression in the tubule is a result of increased Relish activation. It is therefore indicated by this data that NO is likely to mediate its effects on Imd pathway regulation through the cGMP signalling pathway, via activation of sGC.

In order to extend these studies, diptericin expression was monitored in the tubules of flies overexpressing *dNOS* in the presence of the sGC inhibitor 1H-(1,2,4) oxadiazolo-(4,3-a) quinaxalin-1-one (ODQ) (data generated by Dr Susan Wan, University of Glasgow). To do this, expression of *dNOS* was targeted to the tubules by crossing UAS-*dN1-8* (McGettigan et al. 2005), a transgenic line containing the *dNOS* gene under the control of a UAS promoter, to the principle cell-specific tubule GAL4 driver, UO (Terhzaz et al, in prep.). Tubules were then excised from 7-day old adult flies of both the UAS-*dN1-8* parental line (as a control) and the UO/UAS-*dN1-8* progeny. Excised tubules were then incubated for 1 h in either sterile Schneider's medium (control) or sterile Schneider's medium containing 1 μM ODQ. Diptericin expression was then monitored in each sample by Q-PCR.

As Figure 6.2 shows, in support of data shown in McGettigan et al (2005), when *dNOS* expression is targeted to the tubule, diptericin expression is significantly enhanced compared to parental controls. However, in contrast, when *dNOS* overexpressing tubules are incubated with the sGC inhibitor ODQ, this effect is reversed. Indeed, it can be seen that diptericin expression is not only significantly lower in ODQ-treated UO/UAS-*dN1-8* tubules compared to non-stimulated UO/UAS-*dN1-8* tubules, but is also lower compared to diptericin expression in the control tubules of parental lines. These data would therefore suggest that not only does sGC play a critical role in facilitating NO-mediated stimulation of diptericin expression, but also that NO/sGC/cGMP signalling may play an important role in maintaining basal levels of diptericin expression in the absence of immune challenge.

Overall, the data described here have demonstrated that the stimulatory effects of NO on diptericin expression in the tubule are mediated via regulation of Relish activation. Importantly, it has been shown here that this effect occurs as a result of downstream activation of the cGMP signalling pathway, via soluble guanylate cyclase.



ANOVA Table	Sum-of-squares	df	Mean square	P value
Treatment (between columns)	7.045	2	3.523	0.0031
Residual (within columns)	2.69	9	0.2989	
Total	9.735	11		

Dunnett's Multiple Comparison	Mean Diff.	q	P value	95% CI of diff
UO/dN1-8 vs dN1-8	1.183	3.061	P < 0.05	0.1743 to 2.192
UO/dN1-8 vs UO/dN1-8 +ODQ	1.853	4.794	P < 0.01	0.8443 to 2.862

Figure 6.2 - NO-mediated stimulation of diptericin expression in the tubules is a result of sGC activation. Expression of diptericin in response to the soluble guanylate cyclase inhibitor ODQ was assessed by Q-PCR in the excised tubules of *dNOS* overexpressing flies. Resulting data were normalised against expression of the standard, *rp49*, and expressed as a fold change of parental control expression where control =1 (N = 4, $^{+}$SEM). Significance of data was determined using One-way ANOVA (See table). Additionally, in order to compare significance between individual genotypes, *post hoc* analysis was carried out using Dunnett's multiple comparison tests (See table). Data shows that diptericin expression is significantly increase in the tubules of flies overexpressing *dNOS* compared to parental controls. However, when the tubules of these flies are incubated with 1 µM ODQ, diptericin expression is significantly lower, thus suggesting that NO-mediated effects on diptericin expression in the tubule are facilitated by sGC.

6.2.3 Ectopic expression of the rat rGC, GC-A, results in negative regulation of diptericin expression in the tubule

In addition to sGC, there are a number of rGCs expressed in the tubule. These include Gyc76C (Liu et al. 1995; McNeil et al. 1995; Ayoob et al. 2004), and the as of yet uncharacterised rGCs CG4224, CG9873 and CG5719. Of these, Gyc76C is particularly

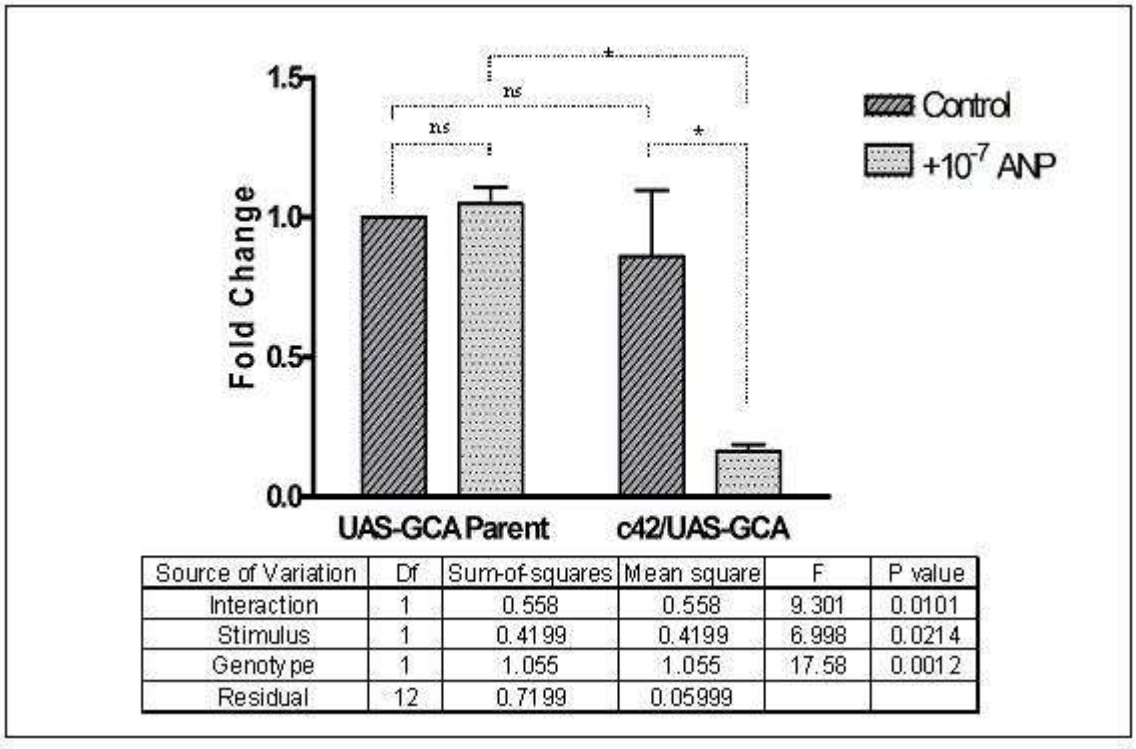
enriched in the tubule compared to other tissues, thus suggesting an important role in tubule function (Wang et al. 2004; *www.flyatlas.org*). In this study, it has been suggested that the differential effects of cGMP/cGKs on Imd pathway regulation in the tubule are a result of activation by different sources of cGMP, generated via distinct guanylate cyclases. As data shown earlier in this chapter has demonstated, cGMP/DG1-mediated positive regulation of the Imd pathway is facilitated by activation of sGC. Therefore, it can be hypothesised that cGMP/DG2-mediated inhibition of the Imd pathway may be facilitated via activation of an rGC. Indeed, given the localisation of DG2P1 and DG2P2 (MacPherson et al. 2004b), it can be suggested that they may be activated via a source of cGMP generated near the plasma membrane, i.e. in close proximity to a receptor guanylate cyclase.

Unfortunately, due to the lack of information regarding natural ligands for *Drosophila* rGCs, experimental manipulation of these enzymes is difficult to carry out. However, a recent study has demonstrated the successful activation of the cGMP pathway in the tubule via ectopic expression of a characterised mammalian rGC, GC-A (Kerr et al. 2004). GC-A is a well characterised rGC involved in natriuresis/diuresis in the kidneys of mammals and is activated by atrial natriuretic peptide (ANP) (Drewett and Garbers 1994). GC-A is also a homologue of Gyc76c, the *Drosophila* rGC found most abundantly in tubules (Wang et al. 2004). Therefore, in this study, in the absence of a suitable activating ligand for indigenous tubule rGCs, changes in diptericin expression were monitored in flies where GC-A is expressed ectopically in the tubule principal cells via the UAS/GAL4 system. To do this, flies containing a UAS-GCA transgene were crossed to the principle cell-specific GAL4 driver c42. Tubules were then excised from 7-day old adult flies of both the UAS-GCA parental line (as a control) and the c42/UAS-GCA progeny. Excised tubules were then incubated for 3 hr in either sterile Schneider's medium (control) or sterile Schneider's medium containing 10^{-7} M ANP. Diptericin expression was then monitored in each sample by Q-PCR. It should be noted that there is no natural receptor for ANP in *Drosophila*, therefore ensuring that any effect seen is due to specific activation of GC-A (Kerr et al. 2004).

It can be seen from the data shown in Figure 6.3 that, as expected, incubation of UAS-*GCA* parental flies with ANP has no effect on diptericin expression in the tubule. Similarly, when *GC-A* is expressed in the tubule in the absence of ligand, there is no

significant effect on diptericin expression. However, when the tubules of c42/UAS-*GCA* flies are incubated with exogenous ANP, diptericin expression is significantly inhibited. These data therefore indicate that, as with DG2P1 and DG2P2, activation of GC-A has a negative effect on Imd pathway regulation in the tubule. As such, given that GC-A is the mammalian homologue of *Drosophila* Gyc76C, it can be suggested that Gyc76C may function in a similar way in response to its natural ligand.

Overall, it is demonstrated by the data shown here that negative regulation of the Imd pathway in the tubule by cGMP/DG2 is likely to be mediated via activation of an rGC. Unfortunately, the identity of the specific *Drosophila* rGC involved in this effect remains elusive and is a subject for future work.



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	0.558	0.558	9.301	0.0101
Stimulus	1	0.4199	0.4199	6.998	0.0214
Genotype	1	1.055	1.055	17.58	0.0012
Residual	12	0.7199	0.05999		

Figure 6.3 - Targeted overexpression of the rat rGC, GC-A, to the principal cells of the tubule results in reduced diptericin expression in response to ANP. Expression of diptericin was assessed by Q-PCR in the excised tubules of flies overexpressing GC-A in response to its natural ligand, ANP. Resulting data were normalised against expression of the standard, *rp49*, and expressed as a fold change of parental control expression where control =1 (N = 4, $\pm$ SEM). Significance of data was determined by Two-way ANOVA and *post hoc* analysis was carried out using Bonferroni tests, whereby significant data is indicated by * ($P<0.05$) or ns (not significant) where appropriate. Analysis by two-way ANOVA reveals a significant interaction between genotype and stimulus. Addittionally, the effect of stimulus and genotype alone are also considered significant (see table). *Post hoc* analysis shows no significant difference in diptericin expression in the tubules of UAS-GCA parental flies in response to ANP. However, diptericin expression is extremely reduced in the tubules of GC-A overexpressing flies when incubated with ANP. Data therefore suggests that cGMP-mediated inhibition of diptericin expression is facilitated by an rGC.

6.3 A regulatory role for the dual-specificity PDE, DmPDE11, in Imd pathway immune function in the tubule

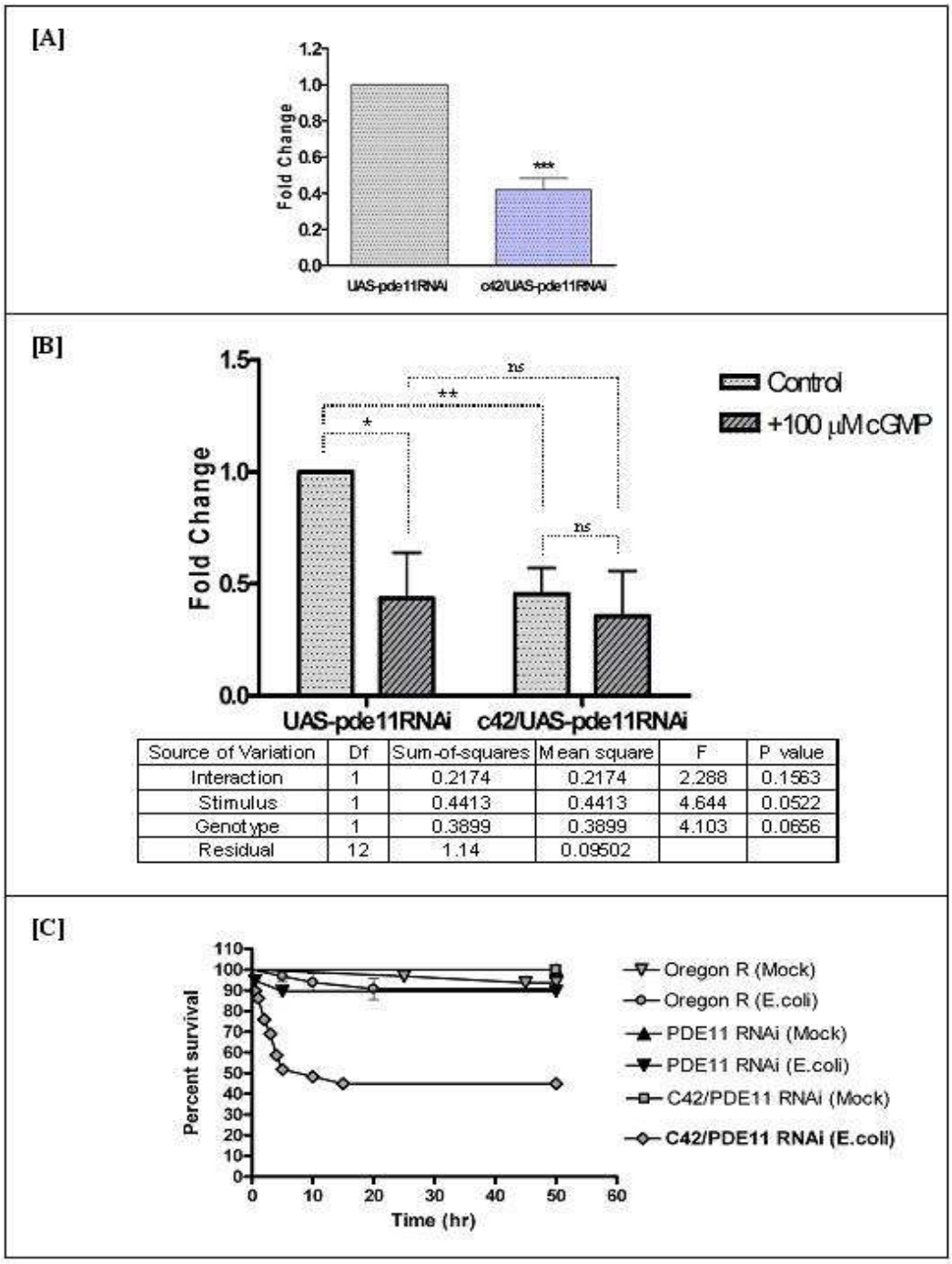
As mentioned previously, PDEs comprise a superfamily of metallophosphohydrolases that act as negative regulators of cGMP and cAMP signalling and are therefore pivotal in maintaining the role of cyclic nucleotides in cellular function (Omori and Kotera 2007). To date, studies investigating *Drosophila* PDEs have mainly focused on the cAMP-specific mammalian PDE4 homologue, *dunce*, which has been demonstrated to play a critical role in learning and memory (Davis and Dauwalder 1991). However, a recent study using the Malpighian tubule as a model system has identified a further five novel *Drosophila* PDEs (Day et al. 2005). Of these, the cGMP-specific *Dm*PDE6, the dual-specificity *Dm*PDE11 and the as yet to be characterised *Dm*PDE8 are highly enriched in the tubules compared to other tissues, suggesting an important role for each of these enzymes in tubule function (*www.flyatlas.org*). Surprisingly, despite its dual-specificity, *Dm*PDE11 has been demonstrated to exhibit the highest specificity for cGMP of all other *Drosophila* PDEs (K_m: $6 \pm 2\mu M$) (Day et al. 2005).

In this study, it has been suggested that the contrasting effects of cGMP/cGKs on Imd pathway regulation are a result of generation of specific pools of cGMP. Over the years, it has become clear that PDEs are pivotal to the regulation of cyclic nucleotide compartmentalisation within each cell, i.e. by controlling the duration, amplitude and localisation of each cyclic nucleotide signal (Bender and Beavo 2006; Omori and Kotera 2007). Therefore, it can be hypothesised that one or more *Drosophila* PDEs may have an important role to play in the regulation of cGMP/cGK-mediated Imd pathway modulation in the tubule. In order to investigate this, studies were initiated to assess both diptericin expression in the tubule and survival in response to *E.coli* in PDE transgenic flies. Due to its high specificity for cGMP, and its enrichment in the tubule, focus was placed on the dual-specificity PDE, *Dm*PDE11. Briefly, expression of *PDE11* was knocked-down in the tubule by crossing UAS-*pde11*RNAi transgenic flies (generated by Dr Jon Day, University of Glasgow) to the principle cell-specific GAL4 driver c42 (validation of *pde11* knockdown is shown in Figure 6.4A). Tubules of 7-day old adult flies were then dissected and incubated for 3 hr in sterile Schneider's medium alone (control) or Schneider's medium containing 100 µM cGMP. Diptericin expression was then monitored in each sample by Q-PCR. Additionally, in order to determine the survival of

the flies in response to immune challenge, adult flies were infected using a thin needle coated in a concentrated suspension of *E.coli*, as described previously. As before, in order to control for possible death as a result of injury, a number of flies from each line tested were mock-infected using a sterile needle. Survival of each fly line was then monitored over a number of hours and results plotted using a Kaplein-Meier survival curve (GraphPad Prism 4.0).

As figure 6.4B shows, when *pde11* expression is knocked down in the tubule, diptericin expression is significantly reduced compared to parental controls. Indeed, it can be seen that expression of diptericin in these flies is of a similar level to that of the tubules of UAS-*pde11*RNAi parental flies following stimulation with 100 µM cGMP. This would therefore suggest that knock-down of *pde11* expression in the tubules results in an *in vivo* increase of intracellular cGMP levels similar to that of the levels achieved in the tubules of wild-type or parental flies after *ex vivo* incubation with 100 µM cGMP. Interestingly, when the tubules of c42/UAS-*pde11*RNAi flies are incubated with 100 µM cGMP, a further decrease in diptericin expression is not observed. However, it can be suggested that the levels of intracellular cGMP reached within the tubule due to the knock-down of *pde11* expression are at the maximum level required to affect Imd pathway regulation. In support of this data, it can be seen from Figure 6.4C that knockdown of *pde11* expression in the tubule confers decreased survival of flies in response to *E.coli.* These data therefore demonstrate that regulation of cGMP signalling in the tubule by PDE11 is critical to survival of the whole fly when under immune attack.

Overall, the data described in Figure 6.4 demonstrates that cGMP-mediated modulation of the Imd pathway is finely regulated. Furthermore, it is shown here that the cGMP signal responsible for inhibition of diptericin expression in the tubule is regulated by PDE11.



Source of Variation	Df	Sum-of-squares	Mean square	F	P value
Interaction	1	0.2174	0.2174	2.288	0.1563
Stimulus	1	0.4413	0.4413	4.644	0.0522
Genotype	1	0.3899	0.3899	4.103	0.0656
Residual	12	1.14	0.09502		

Figure 6.4 - Targeted knock-down of *pde11* in the principal cells of the tubule results in reduced diptericin expression and decreased survival in response to *E.coli*. For Q-PCR experiments, expression was assessed in the excised tubules of 7-day old adult flies and analysed as described previously (N = 4, $\pm$ SEM). Where appropriate, significance of data was determined by Two-way ANOVA (see table) and *post hoc* analysis was carried out using Bonferroni tests, whereby significant data are indicated by * ($P<0.05$) or ns (not significant) where appropriate. [A] Q-PCR validation of the knock-down of *pde11* expression in the tubules of c42/UAS-*pde11*RNAi flies. [B] Diptericin expression in the tubule in response to cGMP after *pde11*-knockdown. Analysis by two-way ANOVA reveals that interaction between stimulus and genotype is not significant. Similarly, the effect of stimulus or genotype alone is not considered significant. However, *post hoc* analysis shows that diptericin expression is significantly lower in the tubules of c42/UAS-*pde11*RNAi flies compared to parents. Diptericin expression is not further reduced in response to cGMP, however, the maximum effective concentration of cGMP may have been reached by the knock-down alone. [C] Survival in response to *E.coli*. Data shows that survival of c42/UAS-*pde11*RNAi flies is significantly reduced compared to wild-type and parental lines when infected with *E.coli*.

6.4 Discussion

In previous chapters, an important role for the cGMP signalling pathway in the regulation of Imd pathway-related immune response has been described. However, thus far in this study, investigation into cGMP pathway-mediated regulation of the Imd pathway has focused on the role of the downstream cGMP effector proteins, DG1 and DG2. Therefore, in this chapter, the potential role of other cGMP pathway components in Imd pathway regulation was investigated. For the first time, it is shown here that NO-mediated stimulation of the Imd pathway occurs through activation of the cGMP signalling pathway, via sGC. Similarly it has been demonstrated in this chapter that cGMP-mediated inhibition of the Imd pathway in the tubule is facilitated by the activation of an rGC. Finally, an important regulatory role for the dual-specificity PDE, PDE11, has been described; where it was shown that knock-down of *pde11* expression in the tubule results in significantly decreased levels of diptericin expression.

It has been hypothesised throughout this study that the differential regulatory effects of cGMP/cGKs on the Imd pathway in the tubule are mediated through the generation of different sources of cGMP. As such, it has also been hypothesised that these different sources of cGMP are generated as a consequence of differential activation of guanylate cyclases; where, following activation by NO, sGC is thought to generate a cytosolic cGMP signal and rGCs are thought to mediate the generation of cGMP in close proximity to the plasma-membrane. In this study, these hypothesises have been confirmed. Data has shown that positive regulation of the Imd pathway in the tubule is mediated by NO/cGMP/DG1 via the activation of sGC. Similarly, through the ectopic expression of a mammalian rGC homologous to *Drosophila* Gyc76C, it has been demonstrated that cGMP/DG2 inhibition of the Imd pathway is mediated via activation of an rGC. Unfortunately, due to the lack of information regarding their activating ligands, the *Drosophila* rGC(s) that mediate this effect remain elusive. It can be hypothesised however that, as Gyc76C is the *Drosophila* homologue of mammalian GC-A and is so highly expressed in the tubule, it may be a good candidate for future investigation into the identity of the activating rGC in cGMP/DG2-mediated Imd pathway regulation.

Importantly, it has been shown here that the action of the cGMP signalling pathway on Imd pathway regulation in the tubule is finely regulated by PDEs. Data has shown that

when *pde11* expression is knocked-down in the tubule, inhibition of diptericin expression is observed. These data therefore indicate that PDE11 acts to negatively regulate cGMP/DG2 inhibition of the Imd pathway and therefore describes a role for this PDE for the first time. Unfortunately, the regulation of PDE11 itself is currently unknown. Previous studies have shown that PDEs can be regulated by diverse biochemical reactions including phosphorylation/dephosphorylation, allosteric binding of cGMP or cAMP, binding of Ca^{2+}/calmoldulin and various protein-protein interactions (Bender and Beavo 2006; Omori 2007). It can be hypothesised that, with regards to tubule immune response, PDE11 activation may be stimulated following immune challenge in order to repress the cGMP signal responsible for activating DG2, and thus repress inhibition of the Imd pathway. Unfortunately, the mechanism by which PDE11 activity is regulated in the tubule is beyond the scope of this study and therefore remains a subject for future work.

Overall, the data described in this chapter has demonstrated that the action of the cGMP signalling pathway on Imd pathway modulation in the tubule is elegantly regulated via the activation and inhibition of distinct sources of cGMP within each cell. As such, given the wealth of genetic tools available for manipulation of the cGMP pathway in this tissue, the tubule can be considered a powerful model for future investigation into Imd pathway regulation.

Chapter 7

Summary and future work

7.1 Summary

In recent years, due to the wealth of powerful and cost-effective genetic and genomic tools available, *Drosophila* has emerged as a potent model organism for integrative organismal studies. As such, our understanding of the fundamental mechanisms controlling the *Drosophila* innate immune response has progressed considerably over the years and, as a result, *Drosophila melanogaster* exhibits one of the best characterised host defence systems of all metazoans. Importantly, the *Drosophila* innate immune response has been demonstrated to share a number of similarities with the defence systems of other Diptera species, such as *Anopheles*, and also essential aspects of vertebrate innate immunity (Dimopoulos 2003; Rutschmann and Hoebe 2008). Therefore, *Drosophila* has emerged as a critical model organism for deciphering general innate immune mechanisms in both invertebrates and vertebrates.

Over the years, NO has been identified as an important regulator of the Imd pathway in *Drosophila*, however the mechanism by which this diffusible messenger acts has not yet been determined. Given that the main intracellular receptor for NO is sGC, it was hypothesised that NO may be mediating its effects via the cGMP signalling pathway. Therefore in this study, the role of cGMP signalling in regulation of the Imd pathway was investigated. This was carried out using the *Drosophila* Malpighian tubule as a model system. The suitability of the tubule as a model tissue for this study was two-fold: Firstly, previous studies have identified the tubule as an important immune-sensing tissue (Tzou et al. 2000; Kaneko et al. 2006), in which NO plays an important regulatory role (McGettigan et al. 2005). Secondly, the NO/cGMP signalling pathway has been demonstrated as critical to tubule function (Davies 2006; Davies 2007). In this chapter, the main findings and implications of this study are summarised. Future work is also suggested.

In this thesis, a novel role for the cGMP signalling pathway in the regulation of the Imd pathway in the Malpighian tubule of the adult fly has been described. Data has shown that cGMP is able to modulate the expression of Imd-pathway associated AMPs in the tubule in a dose-dependent manner; whereby low nanomolar concentrations of cGMP have been shown to stimulate diptericin expression and higher micromolar concentrations of cGMP have been shown to inhibit it.

These differential effects have been shown to be mediated via the activation of the cognate downstream effector molecules of cGMP, DG1 and DG2. Data has shown that diptericin expression is positively regulated by DG1 and negatively regulated by DG2. Importantly, these effects have been demonstrated to occur as a result of modulation of Relish activation, thus revealing for the first time, a novel regulatory role for *Drosophila* cGKs in Imd pathway regulation in the tubule. The exact mechanism by which DG1 and DG2 are mediating their effects on the Imd pathway was not determined in this study. Therefore, it is not known whether these kinases are acting on the Imd pathway directly, or indirectly by phosphorylation of an, as of yet, unidentified substrate(s). However, it has been hypothesised that, as previous studies have identified NFκB as a phosphorylation target for mammalian PKG (He and Weber 2003), DG1 and/or DG2 may be acting to modulate Relish activation via direct phosphorylation of Relish itself.

Significantly, it has been demonstrated in this study that cGK-mediated modulation of the Imd pathway in the tubule is sufficient to impact on the survival of the whole organism. Data has shown that targeted overexpression or knockdown of cGK expression in the tubules significantly effects survival of the adult fly in response to septic infection with the Gram-negative bacteria, *E.coli*. These findings therefore not only confirm the importance of cGKs in Imd pathway regulation in the tubule, but also highlight the critical role of this tissue in systemic Imd pathway induction in the adult fly. Traditionally, the fat body has been described as the critical tissue involved in systemic immune response in *Drosophila*. However, these data indicate that the tubule may contribute just as significantly to systemic production of Imd-pathway associated AMPs in the adult fly in response to immune challenge. These findings are not entirely surprising given the morphology and function of this epithelial tissue. As with the fat body, the tubules are spread throughout the body cavity and are likely to be one of the first tissues in contact with any invading organisms present in the haemolymph. Furthermore, given the main role of the tubule as an osmoregulatory and detoxifying tissue, where waste metabolites and toxins are cleared from the haemolymph at very high rates compared to the haemolymph volume (Dow et al. 1994a), it is likely that the tubule would be the first tissue exposed to key components derived from the bacterial coat of microbial invaders, such as PGN or LPS. Certainly, previous studies have described the ability of acutely-dissected tubules to autonomously bind and internalise exogenous LPS

(McGettigan et al. 2005). In addition, it has been demonstrated that constitutive secretion of AMPs by the tubule is sufficient to confer a significant killing effect on *E.coli*, with less than 50 % of bacteria remaining after the treatment of bacterial culture with the bathing media in which tubules have been incubated (McGettigan et al. 2005). Taken together, the data shown here and that described in previous studies therefore suggest that the role of the tubule may just as critical to the systemic production of AMPs in response to septic infection as that of the fat body.

At present, it cannot be concluded from this study whether the tubule acts as a completely independent immune tissue in response to septic infection, or if the tubule acts as a 'first-response' signalling tissue to alert other tissues, such as the fat body, to activate or suppress immune mechanisms appropriately. Certainly, a role for NO has already been suggested in mediating signalling between epithelial tissues and the fat body, possibly via hemocytes (Basset et al. 2000; Foley and O'Farrell 2003; Silverman 2003). Interestingly, it is shown here that regulation of the Imd pathway by cGMP does not appear to extend to the fat body, thus suggesting that the latter of these hypotheses may be correct. Overall, regardless of whether the tubule acts completely independently of other tissues, it has been demonstrated in this study that this tissue contributes significantly to the survival of the adult fly in response to septic infection with *E.coli*, and that cGK-mediated regulation of the Imd pathway in the tubule is critical to this mechanism.

Importantly, a role for the tubule in response to natural infection has also been described in this study. Data has shown that not only does the tubule play a critical role in inducing diptericin expression following natural infection with *E.coli*, but that this expression is regulated, as with septic infection, by the differential action of DG1 and DG2. Importantly, it has been demonstrated in this study that the diptericin produced in the tubule following natural infection is secreted into the gut and subsequently contributes to bacterial clearance in this tissue. These findings therefore describe a completely novel role for the both the tubule and cGKs in the elimination of bacterial invaders after natural infection with Gram-negative bacteria.

Finally, throughout this study, it has been suggested that the contrasting effects of the otherwise cognate cGKs, DG1 and DG2, are mediated via the generation of distinct sources of cGMP within the tubule. Certainly, the mainly cytosolic localisation of DG1

(MacPherson et al. 2004b) would suggest that it is activated via a cytosolic source of cGMP, such as that generated by the activation of sGC. Similarly, it was suggested that activation of DG2, localised near the plasma membrane of the tubule (MacPherson et al. 2004b), is mediated via a source of cGMP generated by an rGC. In this study, these hypotheses were confirmed. Data has shown that positive regulation of the Imd pathway by cGMP in the tubule is mediated by the activation of sGC by NO. Similarly, through the ectopic expression of the mammalian rGC, GC-A, negative regulation of the Imd pathway in the tubule by cGMP has been demonstrated to be mediated via the activation of rGC. Unfortunately, due to the lack of information on the activating ligands of rGCs in *Drosophila*, the identity of the *Drosophila* rGC mediating this effect is not confirmed. However, given that the *Drosophila* rGC, Gyc76C, is abundantly expressed in the tubules and is also a homologue of GC-A, it has been hypothesised that Gyc76C may be a good candidate for further investigation.

Interestingly, this study has also identified a regulatory element of the cGMP pathway itself with regards to Imd pathway modulation in the tubule. Data has shown that cGMP-mediated inhibition of the Imd pathway is regulated by the hydrolysis of cGMP by PDE11. PDE11 is a dual-specificity PDE that is abundantly expressed in the tubules and has shown a high affinity for cGMP compared to other tubule PDEs (Day et al. 2005). PDE11 has not previously been characterised, therefore a functional role for this enzyme has been described here for the first time.

Given the data obtained from this study, a model for the mechanism by which the cGMP signalling pathway is mediating its effects on the Imd pathway in the tubule is illustrated in Figure 7.1. In this model, it is proposed that the differential modulation of the Imd pathway by the cGMP signalling pathway in the tubule is finely regulated via the activation and inhibition of distinct sources of cGMP within each cell; whereby activation of sGC by NO results in a cytosolic source of cGMP and activation of rGC by an unknown ligand results in the generation of cGMP near the plasma membrane. Each of these cGMP sources are then demonstrated to act to mediate the activation of either DG1 or DG2 respectively, which then go on to mediate contrasting effects on the Imd pathway, and subsequently modulate AMP expression in the tubule. The exact mechanism of this action has not yet been determined, however it is known from data in this study that both

DG1 and DG2 act to modulate the activation of Relish, the NFκB transcription factor responsible for mediating the transcription of specific AMPs.

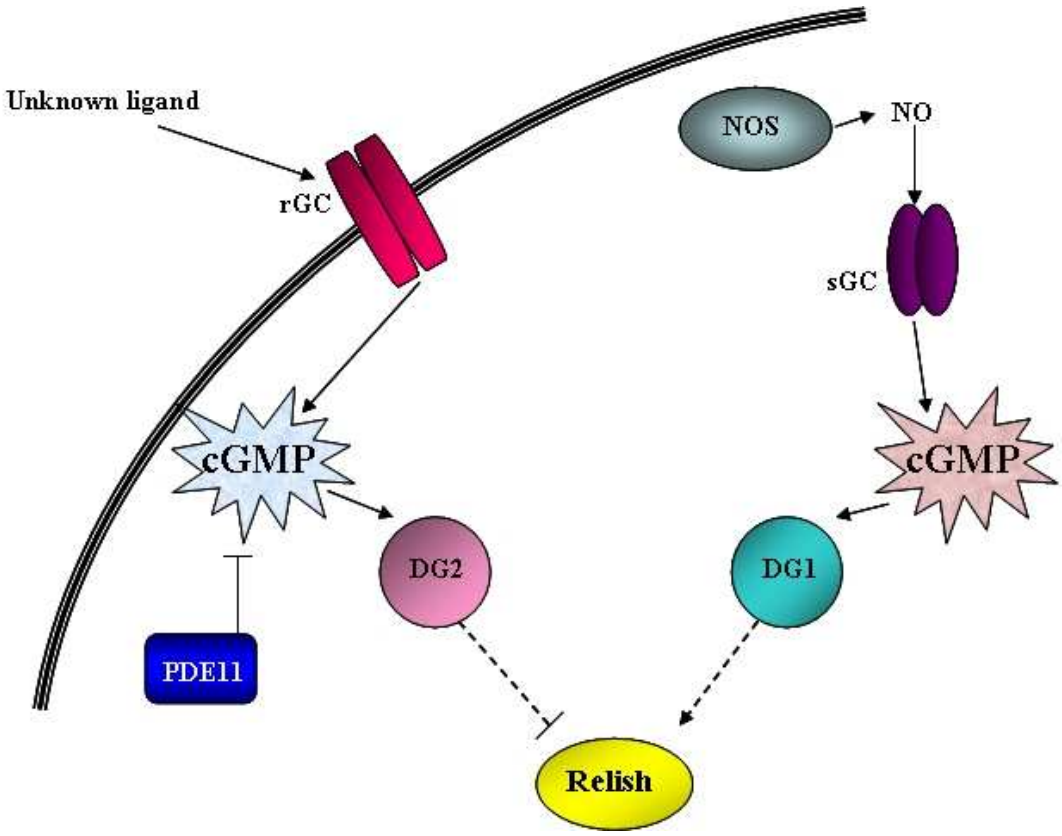


Figure 7.1 – Model for the mechanism of cGMP pathway-mediated regulation of the Imd pathway in the *Drosophila* Malpighian tubule. It is suggested that the differential effects of cGMP/cGKs on the Imd pathway are mediated by different sources of cGMP. Stimulation of the Imd pathway is thought to be regulated by the generation of cGMP in the cytosol, via activation of sGC, which leads to activation of DG1 and subsequent activation of Relish. Inhibition of the Imd pathway is suggested to be regulated by the generation of cGMP near the plasma membrane, via activation of an rGC, which leads to activation of DG2 and subsequent inhibition of Relish activation. Dashed lines indicate that the exact mechanism of action for this step has not yet been determined.

It should be noted that, to date, the upstream activator of DNOS, the enzyme responsible for the generation of NO, has not yet been identified in the tubule with regards to immune activation, although it is very probable that calcium plays an important role in the regulation of this response. Recent work has shown that calcineurin, a calcium-dependent phosphatase, mediates NO-induced AMP production in the fat body, possibly via the regulation of DNOS activation (Dijkers and O'Farrell 2007). Similarly, a number of studies have demonstrated an important role for the neuropeptide capa in the activation of

NO/cGMP signalling in tubule (Davies et al. 1995; Davies et al. 1997; Rosay et al. 1997; Kean et al. 2002). There are three capa peptides expressed in *Drosophila* (capa 1-3) (Predel and Wegener 2006). Of these, capa-1 and capa-2 have been shown to function via the capa receptor (Iversen et al. 2002) to increase $[Ca^{2+}]_i$ in the principle cells of the tubule through activation of either L-type voltage-dependent Ca^{2+} channels in the plasma membrane (Rosay et al. 1997; MacPherson et al. 2001) or through the intracellular production of inositol triphosphate (IP_3) (Pollock et al. 2003). This capa-induced raise in $[Ca^{2+}]_i$ has been shown to trigger the activation of DNOS and subsequently NO/cGMP signalling (Davies et al. 1995; Davies et al. 1997). Studies have shown that capa is instrumental to NO/cGMP-mediated fluid secretion (Davies et al. 1995; Davies et al. 1997; Kean et al. 2002) which is critical to tubule function. Therefore, it can be suggested that capa is a likely candidate as the upstream activator of DNOS in NO/cGMP-mediated Imd pathway regulation in the tubule, however this remains a subject for future work.

The implications from the findings in this thesis are extremely significant. As mentioned earlier, the *Drosophila* innate immune response has been demonstrated to be very similar to that of other Dipteran insects, as well as vertebrates such as humans (Dimopoulos 2003; Rutschmann and Hoebe 2008). With regards to other Diptera, information on the regulation of immunity in many of these insects can potentially have a huge impact on research into the development of suitable insecticides for those insects which are known agricultural pests or, more importantly, for those insects which act as vectors for infectious diseases such as malaria, yellow fever or Dengue fever, e.g. *Anopheles* or *Aedes aegypti*. Certainly, a number of studies are now using *Drosophila* as a comparative model organism in order to understand the mechanisms by which these disease vectors combat infection (Christophides et al. 2002; Dimopoulos 2003; Brandt et al. 2008).

With regards to the impact of these findings on what is currently known about human innate immunity, the data described here is also extremely significant. Despite the importance and sophistication of the acquired immune system in vertebrates, the innate immune system is still considered a critical component of host survival against many infectious agents. Indeed, studies have shown that many autoimmune and inflammatory diseases in humans, such as rheumatoid arthritis, multiple sclerosis, celiac disease, diabetes mellitus and lupus, are aggravated by alterations in the innate immune system (Lang et al. 2007). For example, studies have shown that diabetes mellitus is a major

cause of end-stage kidney disease in humans (Atkins 2005). Furthermore, it has been reported that activated innate immunity and inflammation, in particular activation of the TNFα signalling pathway, contributes significantly to the pathogenesis of this disease in the kidney (Navarro and Mora 2006). Given that the TNFα signalling pathway in mammals is highly homologous to the *Drosophila* Imd pathway, and that the Malpighian tubule represents the *Drosophila* equivalent of the mammalian kidney, the findings obtained in this thesis could therefore potentially prove valuable to research in this field, thus highlighting the suitability of *Drosophila* as a model organism for human disease.

Overall, not only has this study confirmed the role of the tubule as an important immune-sensing tissue in *Drosophila melanogaster,* but has also demonstrated, for the first time, completely novel roles for components of the cGMP signalling pathway, particularly DG1 and DG2, in the differential regulation of the Imd pathway in the tubule. The identification of cGMP signalling as an important regulator of immune response is therefore a significant advance in our understanding of the complexities of not only immune regulation in *Drosophila*, but also of the complexities of cGMP signalling, its compartmentalisation within each cell and subsequent regulation.

7.2 Future Work

Further epistatic analysis of cGK-mediated effects on the Imd pathway

As mentioned previously in the text, several transgenic fly lines were generated in order to determine where in the Imd pathway DG1 and DG2 were mediating their effects. Unfortunately, due to time limitations, not all of these lines were used. Therefore, future work would entail using these lines for further epistatic analysis of cGK-mediated regulation of the Imd pathway. For this approach, these lines would be crossed to UAS-cGK transgenic lines as appropriate and diptericin expression assessed by Q-PCR as before.

Phosphorylation targets for DG1 and DG2

Following the identification of potential targets for DG1 and DG2 through epistatic analysis, further work would be required to determine the mechanism by which these targets are modulated, i.e. whether they are directly phosphorylated by DG1 or DG2, or whether they are modulated by these kinases indirectly through phosphorylation of an

unknown substrate. For this approach, both immunoprecipitation (IP) and phosphorylation assays would be used. Initially, these experiments would be carried out using *Drosophila* S2 cells as a model system, whereby tagged components of the Imd pathway would be cloned and co-expressed with the appropriate cGK plasmid. Interaction between each cGK and its potential substrate could then be determined by IP of each cGK. Each IP fraction could then be analysed for potential cGK-interacting Imd pathway components by Western blot. Similarly, for phosphorylation assays, phosphorylation of potential targets of DG1 and DG2 would be investigated by incubating cell lysate, containing the co-expressed proteins, with the radioactive phosphate, γ-^{32}P. Imd pathway components of interest could then be isolated by IP, and any levels of phosphorylation detected by autoradiography. These approaches could then be carried out in the adult fly following the generation of the appropriate antibodies.

Identification of the rGC involved in Imd pathway inhibition in the tubule

As mentioned in the text, at present there is no information regarding the natural ligands of rGCs in *Drosophila*. Therefore, examination of the rGC involved in Imd pathway inhibition in the tubule could not be carried out pharmacologically. Therefore, for future work, a transgenic approach would have to be used. For this approach, initial studies would focus on the tubule-enriched rGC, Gyc76C, whereby expression of this enzyme would be modulated in the tubule using the GAL4/UAS system. Transgenic flies would then be monitored for survival following infection with various bacteria. Similarly, tubules would be excised from these transgenic flies following infection and levels of diptericin expression assessed by Q-PCR as described previously.

The role of other PDEs in Imd pathway regulation in the tubule

In this study, a role for PDE11 in the regulation of cGMP-mediated Imd pathway inhibition has been described. Therefore, further work could be carried out in order to investigate the potential role of other tubule PDEs in the regulation of cGMP-mediated Imd pathway modulation, possibly as potential regulators of the cGMP signal responsible for stimulation of the Imd pathway. For this approach, initial studies would focus on another cGMP-dependent tubule enriched PDE, PDE6. As with PDE11, investigation into the role of PDE6 in Imd pathway regulation would involve assessment of both survival of the whole organism in response to infection, and diptericin expression by Q-PCR in the tubules of PDE6 transgenic flies.

Initiation of tissue-tissue signalling by the tubule

As mentioned previously in this text, it is not currently known from this study whether the impact of AMP modulation in the tubule on fly survival is solely a result of altered AMP expression only in the tubule, or if, following regulation of AMP expression, the tubule acts as a signalling tissue to alert other tissues, such as the fat body, to induce or suppress their immune response mechanisms in the same way, thus having a greater impact on fly survival. Further work would therefore focus on assessing the levels of AMP expression following infection in other *Drosophila* tissues, such as the fat body, in flies where AMP expression has been modulated in the tubules by cGKs.

Visualisation of the cGMP signal in the tubule following infection

With the development of fluorescence resonance energy transfer (FRET) techniques, it has become possible to visualise real-time activation of a number processes within each cell, including the activation of the second messengers cAMP and cGMP (Lissandron et al. 2007; Russwurm et al. 2007). Therefore, a suggestion for further work would be to investigate the spatio-temporal dynamics of cGMP in the tubule in response to immune challenge by visual imaging of a FRET-based cGMP reporter. The data obtained from these experiments could then be used to further validate the model proposed in this study, where it is suggested that the differential effects of cGMP signalling on the Imd pathway are due to compartmentalisation of cGMP.

Appendices

Appendix 1: Media used in this study

Drosophila Media

Standard growth media per litre of water

10 g agar
15 g sucrose
30 g glucose
35 g dried yeast
15 g maize meal
10 g wheat germ
30 g treacle
10 g soya flour

Escherichia coli growth media

L-broth per litre of water

10 g Bacto-tryptone
5 g dried yeast
10 g NaCl

L-agar per litre of water

10 g Bacto-tryptone
5 g dried yeast
10 g NaCl
15 g Bacto-agar

Appendix 2: Primers used in this study

Table 7.1 – Primer sequences used in this study

Primer Name	Sequence (5' – 3')	Application
Dipt (Forward)	5' – TTG CCG TCG CCT TAC TTT GCT G – 3'	RT-PCR, Q-PCR
Dipt (Reverse)	5' – TCC ATT CAG TCC AAT CTC GTG G – 3'	RT-PCR, Q-PCR
AttC (Forward)	5' – ATC GTC AGT CAA CAG TCA GCC – 3'	RT-PCR, Q-PCR
AttC (Reverse)	5' – GCC TTG CTT AGG TCC AAT CG – 3'	RT-PCR, Q-PCR
AttD (Forward)	5' – CAG GCT TCA GGA AAC CCA AAG – 3'	RT-PCR, Q-PCR
AttD (Reverse)	5' – GCA TTC AGA GCG GCG TTA TTG – 3'	RT-PCR, Q-PCR
CecA1 (Forward)	5' – AAC ATC TTC GTT TTC GTC GCT C – 3'	RT-PCR, Q-PCR
CecA1 (Reverse)	5' – ATT GTG GCA TCC CGA GTG TG – 3'	RT-PCR, Q-PCR
CecA2 (Forward)	5' – CGT CGC TCT CAT TCT GGC – 3'	RT-PCR, Q-PCR
CecA2 (Reverse)	5' – AAC CTC GAG CAG TGG CTG – 3'	RT-PCR, Q-PCR
CecC (Forward)	5' – CCA CAG CAG CTA AAC AGC – 3'	RT-PCR, Q-PCR
CecC (Reverse)	5' – CTC ATC CTC TGG CGG TGG – 3'	RT-PCR, Q-PCR
rp49 (Forward)	5' – TGA CCA TCC GCC CAG CAT AC – 3'	RT-PCR, Q-PCR
rp49 (Reverse)	5' – TTC TTG GAG GAG ACG CCG TG – 3'	RT-PCR, Q-PCR
DiptprKpn1 (Forward)	5' – GCA CGG TAC CCT GCA GTT GAA AAA CAT ACA AA – 3'	Cloning of the diptericin promoter into pGL3 vector
DiptprHindIII (Reverse)	5' – GCA CAA GCT TTG CTG ACT GAT ACC TTT GCT GC – 3'	Cloning of the diptericin promoter into pGL3 vector
AttDprKpnI (Forward)	5' – GCA CGG TAC CCA GGT GAC AAC AAT CAG TAC G – 3'	Cloning of the attacinD promoter into pGL3 vector
AttDprHindIII (Reverse)	5' – GCA CAA GCT TGA CTG CAT ATT TCC GAC GGT CG – 3'	Cloning of the attacinD promoter into pGL3 vector
CecA1prKpnI (Forward)	5' – GCA CGG TAC CGT ATT TTG GCC ATT TTC GGG G – 3'	Cloning of the cecropinA1 promoter into pGL3 vector
CecA1prBglII (Reverse)	5' – GCA CAG ATC TGA CTG CGA TAC AAA AGG CGA G – 3'	Cloning of the cecropinA1 promoter into pGL3 vector
PDE11 (Forward)	5' – CAA CAT ACC AGA TGC TTA CCA GGA C – 3'	RT-PCR, Q-PCR
PDE11 (Reverse)	5' – TGT GGG TGA GAA TGC GGA AG – 3'	RT-PCR, Q-PCR

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