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Cloning and characterization of Dorsal homologues in the hemipteran *Rhodnius prolixus*

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Abstract

***Rhodnius prolixus* is an ancient haematophagous hemipteran insect capable of mounting a powerful immune response. This response is transcriptionally regulated in part by transcription factors of the Rel/Nuclear Factor kappa B (Rel/NF-κB) family. We have cloned and characterized three members of this transcription factor family in this insect. Dorsal 1A is primarily expressed in early developmental stages. In contrast, dorsal 1B and 1C, both differentially spliced products of dorsal 1A, are expressed primarily in the adult fat body in response to septic injury, suggesting their exclusive role in immunity. Additionally, we identified putative κB binding sites in the 5′ upstream regions of target genes known to be involved in the innate immune response of insects.**

Keywords: insect immunity, transcription factor, gene regulation, antimicrobial peptides.

Introduction

Insects represent the largest and most varied group of animals on the planet with an estimated 4 000 000 different species, of which only 950 000 have been described and classified (Chapman, 2005). Their remarkable diversity and evolutionary success has allowed insects to exploit almost every ecological niche on our planet. In all of the diverse environments they inhabit, insects are constantly exposed to potential pathogens. Insects defend themselves from harmful organisms by behavioural avoidance, by physical defences (cuticle and epithelia) and via their potent immune response. Over the past 25 years, insect immunology has been the subject of many mechanistic studies, which have characterized the invertebrate immune response as innate (it lacks the antigen–antibody complex and the memory component characteristic of higher organisms; Boman, 1998). The activation of the immune system relies on the basic ability to recognize and discriminate self from nonself. Insects use a set of host receptors, pattern recognition receptors, that recognize highly conserved molecular patterns produced on the surface of microorganisms, but are absent from host cells. Once the invading microorganism is recognized as foreign a generalized immune response is initiated (Lemaitre & Hoffmann, 2007). Cellular immunity, mediated by haemocytes, contributes to phagocytosis, encapsulation, melanization and coagulation responses (Strand, 2008). Humoral defences are characterized by the rapid expression of antimicrobial peptides (AMPs), reactive intermediates of nitrogen or oxygen, and a complex enzymatic cascade resulting in clotting or melanization (Lemaitre & Hoffmann, 2007). Mounting an immune response is costly and the host must regulate these mechanisms to avoid wasting valuable resources. As is the case in other eukaryotic organisms, insect cells or tissues only express a small subset of their genes at any one time and specific gene activation is regulated via complex signalling pathways. This differential gene expression controls a wide variety of biological processes including the immune response. In insects, gene regulation occurs primarily at the transcriptional level. Transcription is regulated principally by two factors: a variety of *cis* regulatory DNA sequences found in the 5′ region of the gene of interest, and by *trans* regulatory proteins named transcription factors (TFs) (Harshman & James, 1998). TFs have a modular structure characterized by a DNA binding domain and an activation domain. TFs bind to their DNA target sequences as monomers, homo- or heterodimers to enhance mRNA generation. A well-characterized family of eukaryotic TFs is that of Nuclear Factor kappa B (NF-κB), named after the transcriptional activator of the immunoglobulin kappa light chain in human B-lymphocytes (Sen & Baltimore, 1986). A functional NF-κB transcription factor contains two members of the Rel family of proteins, characterized by a highly conserved 300 amino acid N-terminal region that contains the Rel homology domain (RHD) required for the formation of

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dimers, DNA binding, nuclear translocation and inhibitor binding. Rel proteins are constitutively expressed and are present in the cytoplasm as inactive zymogens bound to Inhibitor κB protein (IκB family), which masks the nuclear signal sequence. Upon release from IκB, Rel is translocated into the nucleus where it dimerizes and activates the transcription of its target genes by binding to a conserved nucleotide sequence element termed κB (Engstrom *et al.*, 1993). Members of the Rel/NF-κB family are highly conserved. In insects the first member of this family, Dorsal, was described in *Drosophila melanogaster* (Steward, 1987). Whereas Dorsal is involved in regulating the development of the dorsal–ventral axis of the *Drosophila* embryo, two other TFs, Dif and Relish, are involved in activating the transcription of AMPs (Ip *et al.*, 1993, Dushay *et al.*, 1996). Rel/NF-κB members subsequently have been identified in bees, mosquitoes, beetles and moths (Barillas-Mury *et al.*, 1996, Sagisaka *et al.*, 2004, Shin *et al.*, 2005, Tanaka *et al.*, 2005, Evans *et al.*, 2006), indicating their presence across holometabolous insect orders. In this study, we describe the molecular cloning of three different transcripts encoding new members of the Rel/NF-κB family of transcription factors in *Rhodnius prolixus*, a hemimetabolous haematophagous hemipteran insect and a major vector of the parasites that cause Chagas disease in humans. We also evaluated the role of these *R. prolixus* TFs in immune gene regulation by evaluating their expression profile in specific tissues during infection, identifying their putative target genes and confirming the functional activity of a recombinant protein.

Results

Gene cloning and sequencing

We recently isolated a 364 bp expressed sequence tag (EST) from a suppressive subtractive hybridization (SSH) cDNA library made from *R. prolixus* fat body tissue 24 h after injecting *Trypanosoma cruzi* into the haemocoel (Ursic-Bedoya & Lowenberger, 2007). One of the putative reading frames of this EST shared significant amino acid identity (39%) with *Apis mellifera* Dorsal protein splice variant B (BLASTX, *E*-value: $1e^{-12}$). We used 5′–3′ rapid amplification of cDNA ends (RACE) to obtain the full-length cDNA clones. DNA sequencing of 15 independent clones revealed three very similar molecules that differed in the 5′ untranslated region (UTR) sequence. *Dorsal 1A* mRNA is 2,323 bp long, and encodes a putative 624 amino acid protein with a predicted molecular weight of 69.4 kDa. *Dorsal 1B* is a differentially spliced version of *Dorsal 1A*, with a 75 bp deletion within intron 1, which results in the use of an alternative start methionine codon. *Dorsal 1B* mRNA encodes for a putative 611 amino acid protein with a predicted molecular weight of 67.8 kDa. *Dorsal 1C* is another differentially spliced version of Dorsal 1A and encodes a 579 amino acid protein with a predicted molecular weight of 64.6 kDa (Fig. 1). The *R. prolixus* dorsal nucleotide sequences reported in this paper have been submitted to GenBank with accession numbers EF634460, EF634461 and EF634462. The deduced amino acid sequences of all three proteins contain conserved protein domains characteristic of members of the Rel/NF-κB family of transcription factors. Analysis with Conserved Domain Database (Marchler-Bauer *et al.*, 2005) and Scan-Prosite (Hulo *et al.*, 2006) identified three main protein domains common to all three proteins. The first is the RHD (pfam 00554), characteristic of eukaryotic Rel/NF-κB transcription factors. The second conserved domain, C-terminal to RHD, is shared by the immunoglobulins, plexins and transcription factors and is termed the immunoglobulin, plexin, transcription (IPT) domain (cd01177). This is an immunoglobulin-like fold domain, responsible for DNA binding. Within this domain, as is the case with other members of this TF family, *R. prolixus* Dorsal contains several ankyrin protein binding sites, to which inhibitor proteins may bind. Inhibitors that contain the 33 amino acid ankyrin (ANK) motif bind to the ankyrin binding sites within the IPT domain, preventing the translocation of Dorsal into the nucleus (Stoven *et al.*, 2000). Upon cleavage of the inhibitor, a 19 amino acid lysine rich nuclear localization signal responsible for directing the free Dorsal molecules to the nucleus is exposed (Fig. 1).

Protein activity

To confirm the predicted protein activity of these molecules, we cloned and expressed an entire open reading frame of *R. prolixus* Dorsal and a truncated version containing the RHD and IPT domains. We produced recombinant protein in a bacterial expression system and used a nonradioactive electrophoretic mobility shift assay (EMSA) alternative assay to show binding to the mammalian consensus κB sequence 5′-GGGACTTTCC-3′. Cell lysates from bacteria expressing the entire Dorsal protein bound to the κB consensus and showed luminescence similar to that of the positive control whereas lysates from uninduced bacteria or from bacteria expressing just the RHD and immunoglobulin, plexin, transcription (IPT) domains showed very weak binding activity similar to that of the negative control (Fig. 2). These results confirm the specific ability of *R. prolixus* Dorsal to bind to a κB consensus site and suggest that the functional TF is a homodimer that requires more than the RHD and IPT domains for dimerization and/or DNA binding.

Expression profile

As Rel/NF-κB transcription factors have been shown to be involved in different insect biological processes such as embryonic development and induction of the immune system, we examined the expression of these transcriptional regulators in different stages and in different tissues of *R. prolixus*. We used real time quantitative PCR (qRT-

A

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M   N   Q   S   V   R   R   T   S   E   S   S   G   G   G   V   A   S   M   P   Y   I   K   I   I   E   Q
ATG AAC CAA TCT GTT CGG AGA ACT AGT GAA TCA AGT GGT GGA GGC GTA GCA AGT ATG CCG TAC ATT AAA ATT ATT GAA CAA
P   A   S   K   A   L   R   F   R   Y   E   C   E   G   R   S   A   G   S   I   P   G   V   N   S   T   P
CCA GCA TCT AAA GCG CTT AGG TTC AGG TAT GAA TGT GAG GGA AGA TCT GCT GGT TCT ATT CCT GGT GTA AAT TCT ACA CCA
E   N   K   T   F   P   T   I   Q   I   V   G   Y   R   G   R   A   V   V   V   V   S   C   V   T   K   D
GAA AAT AAA ACG TTT CCT ACA ATA CAG ATT GTT GGC TAC AGA GGT CGA GCA GTA GTA GTG TCA TGT GTG ACG AAG GAT
S   P   Y   R   P   H   P   H   N   L   V   G   K   E   G   C   K   K   G   V   C   T   V   E   I   N   N
AGC CCA TAC AGG CCT CAC CCA CAC AAT CTT GTT GGC AAA GAA GGT TGT AAA AAA GGT GTT TGT ACT GTC GAG ATT AAT AAT
E   T   M   T   A   A   F   A   N   L   G   I   Q   C   V   K   K   K   D   I   E   E   A   L   R   V   R
GAA ACC ATG ACA GCG GCC TTT GCA AAT CTT GGT ATT CAA TGT GTC AAA AAA AAA GAT ATT GAA GAA GCA CTT AGA GTC AGA
E   E   I   R   V   D   P   F   R   T   G   F   S   H   K   T   Q   T   S   G   I   D   L   N   S   V   R
GAA GAA ATA AGG GTA GAT CCA TTT AGG ACC GGA TTC AGT CAT AAA ACT CAA ACA AGT GGT ATA GAT TTG AAT TCA GTT CGA
L   C   F   Q   A   F   L   E   G   P   Q   R   G   K   F   T   N   P   L   S   P   I   V   S   E   P   I
TTA TGC TTT CAA GCA TTT TTA GAA GGA CCT CAA AGA GGA AAA TTT ACC AAC CCA TTA TCT CCA ATT GTA TCA GAA CCA ATT
Y   D   K   K   A   M   A   D   L   V   I   C   K   L   S   H   C   S   G   S   V   A   G   G   N   E   I
TAT GAC AAA AAA GCC ATG GCC GAT TTA GTT ATT TGT AAA CTA AGT CAT TGT TCT GGT TCA GTA GCG GGA GGA AAT GAA ATT
I   L   L   C   E   K   V   A   K   E   D   I   S   V   R   F   Y   E   E   K   D   G   Q   V   V   W   E
ATT TTA TTA TGT GAA AAA GTA GCG AAA GAG GAC ATA TCA GTG CGA TTC TAT GAA GAG AAA GAT GGC CAG GTA GTT TGG GAA
G   L   G   D   F   T   P   T   Q   V   H   K   Q   V   A   I   S   F   R   T   P   R   Y   K   T   L   E
GGA TTA GGA GAT TTC ACG CCA ACA CAA GTT CAT AAA CAA GTG GCT ATT TCA TTC CGA ACG CCT AGG TAC AAA ACA TTA GAG
I   E   Q   P   V   Q   V   L   I   Q   L   R   R   P   S   D   N   A   T   S   E   A   L   P   F   Q   I
ATA GAG CAG CCT GTG CAG GTA CTT ATA CAA CTT CGA AGG CCG TCA GAT AAC GCT ACG AGT GAA GCA TTA CCT TTT CAG ATA
T   P   L   D   S   G   R   P   F   F   W   S   L   R   R   S   I   G   Q   K   A   D   Y   R   T   F   S
ACT CCA CTT GAT TCA GGT AGG CCT TTC TTT TGG TCA TTG CGT CGA AGT ATT GGA CAA AAA GCC GAC TAC CGT ACG TTT TCA
T   I   L   Q   T   N   T   K   L   L   T   N   Q   E   R   I   D   D   D   A   N   N   N   T   N   N   S
ACA ATA CTT CAA ACG AAT ACA AAA TTA TTA ACT AAT CAA GAA AGA ATT GAC GAT GAT GCA AAC AAT AAC ACC AAC AAT AGT
N   N   N   N   E   I   K   D   V   E   V   E   D   N   V   V   S   V   A   D   I   P   Q   P   K   V   V
AAT AAT AAT AAT GAA ATT AAA GAT GTT GAA GTA GAA GAT AAT GTA GTT AGT GTA GCA GAT ATT CCA CAA CCA AAA GTT GTT
V   V   S   N   Q   E   E   E   I   K   D   E   E   E   N   C   G   Q   S   E   L   E   Q   L   L   V   K
GTA GTA TCA AAT CAA GAA GAA GAG ATA AAA GAC GAA GAA GAA AAT TGT GGT CAG TCA GAG TTA GAA CAA CTT TTA GTT AAA
D   L   E   N   I   C   N   N   N   S   D   L   G   I   Y   T   S   F   Q   M   A   L   K   N   P   C   Q
GAT TTG GAA AAT ATT TGC AAT AAT AAT AGC GAT CTT GGT ATA TAC ACA AGT TTC CAG ATG GCT TTG AAA AAT CCC TGT CAA
D   L   L   D   H   E   S   Y   E   D   I   A   P   P   R   P   T   A   A   K   P   T   L   L   Q   Q   P
GAT CTG CTT GAT CAT GAA AGT TAC GAA GAT ATT GCA CCA CCA AGA CCA ACA GCT GCT AAA CCG ACA CTT TTG CAA CAA CCA
L   S   P   V   P   H   L   D   D   E   V   L   P   P   L   P   P   K   R   A   K   R   T   G   P   D   T
TTA TCA CCA GTG CCA CAT TTA GAT GAT GAA GTT TTA CCA CCA TTA CCG CCA AAA CGA GCC AAA CGT ACT GGA CCA GAT ACG
K   T   L   P   S   P   P   Q   K   K   T   G   L   F   Q   K   L   F   N   T   N   K   R   K   K   N   K
AAG ACC CTT CCA TCA CCT CCA CAA AAG AAA ACT GGT CTT TTT CAA AAA TTA TTC AAC ACA AAT AAA AGA AAA AAA AAT AAA
E   V   Q   E   R   P   L   Q   S   R   G   S   I   K   S   L   P   A   E   M   I   I   S   S   N   E   V
GAA GTC CAA GAA CGA CCA TTG CAA TCA CGC GGA AGT ATT AAA TCA CTT CCA GCT GAA ATG ATA ATA TCA TCT AAT GAA GTA
D   V   T   E   A   E   H   Y   A   L   Y   T   S   V   A   P   H   A   T   A   S   E   F   D   E   M   S
GAT GTT ACA GAA GCT GAA CAT TAT GCT TTG TAT ACA TCT GTA GCA CCG CAT GCT ACA GCT TCT GAG TTC GAT GAA ATG TCT
F   Y   Y   S   S   V   E   Q   Q   K   V   N   stop
TTC TAT TAC AGT TCA GTC GAA CAA CAA AAA GTA AAC TAA
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B

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                  10                  20                  30                  40                  50
    ----------------+-------------------+-------------------+-------------------+-------------------+-
 1  M N N L E S F D F S L S R M D G Q N I D E S N S A I N I S D V I E V I E T D C A A G G G L M N Q S V  Do 1A
 1  - - - - - - - - - - - - - M D G Q N I D E S N S A I N I S D V I E V I E T D C A A G G G L M N Q S V  Do 1B
 1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - M N Q S V  Do 1C

                  60                  70                  80                  90                 100
    ----------------+-------------------+-------------------+-------------------+-------------------+-
51  R R T S E S S G G G V A S M P Y I K I I E Q P A S K A L R F R Y E C E G R S A G S I P G V N S T P E  Do 1A
38  R R T S E S S G G G V A S M P Y I K I I E Q P A S K A L R F R Y E C E G R S A G S I P G V N S T P E  Do 1B
 6  R R T S E S S G G G V A S M P Y I K I I E Q P A S K A L R F R Y E C E G R S A G S I P G V N S T P E  Do 1C
```

Figure 1. Deduced amino acid sequences of *Rhodnius prolixus* Dorsal isoforms. (A) Dorsal 1C is the shortest isoform, 579 amino acids long with an estimated molecular weight of 64.6 kDa. Rel homology domain in bold; IPT domain is shaded; an asparagine rich region is in italics; nuclear localization signal is underlined. (B) Alignment of the first 60 amino acids of three isoforms of *R. prolixus* Dorsal molecules. Sequence information for all three molecules has been submitted to GenBank under accession numbers EF634460, EF634461 and EF634462.

PCR) to quantify transcript levels in embryos and in selected adult tissues (salivary glands, cardia, midgut and fat body). We also analysed the expression levels in fat body tissues 24 h postbacterial inoculation. A different expression profile was discovered for each mRNA (Fig. 3). Dorsal 1A transcripts were found at low levels in all samples investigated, but significantly higher levels relative to dorsal 1B and 1C were found in embryos suggesting a role in development. No up-regulation of dorsal 1A was found after bacterial inoculation, suggesting that this isoform does not play a role in activating the immune response (Fig. 3A). Our data indicate that dorsal 1C is expressed in the greatest amount that it is mostly found in the adult fat body (Fig. 3B). As the fat body is a key tissue for insect

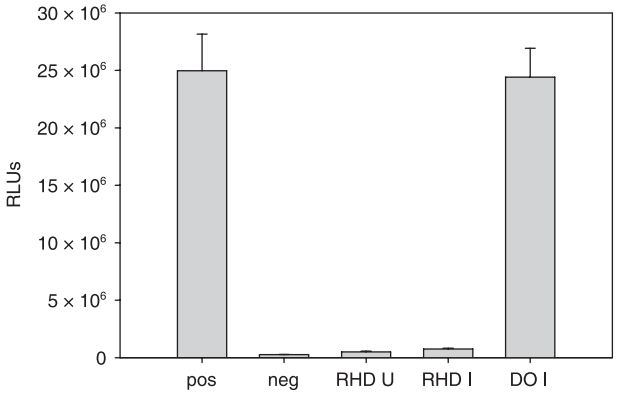

Figure 2. *Rhodnius prolixus* Dorsal 1C recombinant protein binding activity assay. Bacterial cell extracts of *Escherichia coli* engineered to express *R. prolixus* Dorsal 1C were assayed for binding to the mammalian consensus kappa B sequence 5′GGGACTTTCC3′. pos, positive reagent control; neg, negative reagent control; RHD U, Uninduced Rel homology domain cell extracts; RHD I, Rel homology and Ipt domains induced; DO I, Dorsal 1C induced. *y*-axis: relative light units (RLUs). Error bars represent the standard deviation of two independent trials in triplicate wells.

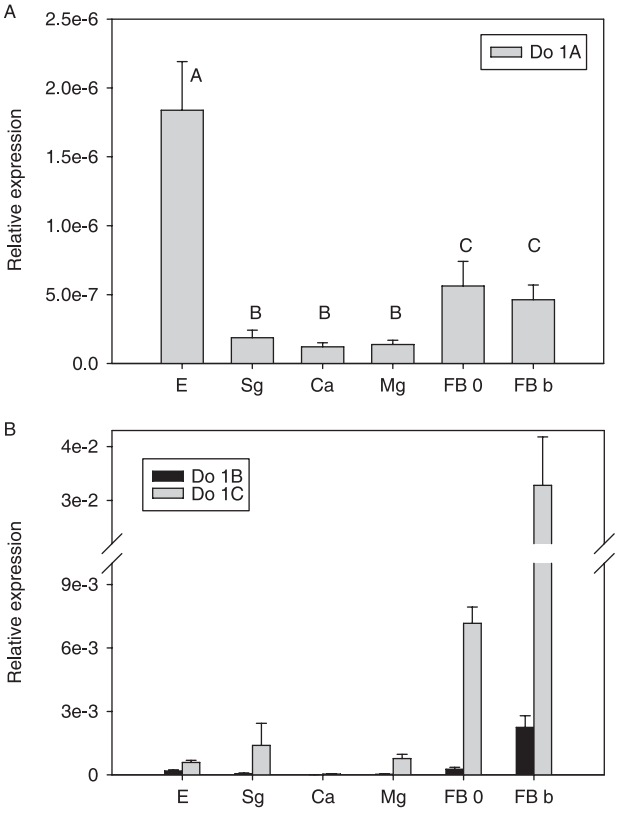

Figure 3. Quantitative analysis of dorsal transcript levels in *Rhodnius prolixus*. Each data point is presented as a normalized individual data point using the $2^{-\Delta Ct}$ method as described (Schmittgen & Livak, 2008) and represents the mean + standard deviation. (A) Differential expression of dorsal 1A and 1B transcripts in embryos and selected adult tissues; samples denoted by identical letters are not significantly different as determined by ANOVA followed by a Tukey's multiple comparison test of the Delta threshold cycle (Ct) values. (B) Expression of dorsal 1B and 1C transcripts in embryos and selected adult tissues. E, embryos; Sg, salivary glands; Ca, cardia; Mg, midgut; FB 0, fat body; FB b, fat body 24 h postbacterial inoculation.

immune responses and Dorsal is known to regulate the expression of immune genes in other insects, we then compared transcript levels of all Dorsal isoforms in this tissue in response to bacterial injection. In the fat body, both dorsal 1B and 1C are up-regulated (8.5-fold and 4.5-fold, respectively), whereas dorsal 1A is not, suggesting a role in the activation of immune genes for Dorsal 1 B and 1C.

Putative target genes

Once we confirmed the activity of the recombinant protein we set out to investigate the genes it might regulate. We used a combination of inverse PCR (iPCR) and bioinformatic analysis of the *R. prolixus* genome trace data archives to identify genomic sequences upstream of the coding region for several immune-related genes we had identified previously (Lopez *et al.*, 2003, Ursic-Bedoya & Lowenberger, 2007, Ursic-Bedoya *et al.*, 2008). When we assembled and annotated all available genomic sequences with our corresponding cDNAs into a single contig, we acquired an average of 500 bp of DNA sequence upstream of the start ATG codon. These sequences were used to search for putative TF binding sites. Bioinformatic analysis of the 5′ upstream regions using ALIBABA 2.1 identified several putative TF binding sites. Of particular interest were κB (5′-GGG(A/G)AYYYYYY-3′) and GATA (5′-(T/G)ATAA-3′) binding sites (Table 1). κB sites were identified in the 5′ upstream regions of lysozyme and defensin. GATA sites were ubiquitous with the exception of the defensin B gene. The orientation of these sites was sometimes reversed and the consensus sequence was found in the noncoding strand, suggesting a similar organizational feature to that described in *D. melanogaster* (Senger *et al.*, 2004). In addition to these immune effector genes, we also investigated the presence of putative TF binding sites in the 5′UTRs of *R. prolixus* dorsal transcript. We found a single GATA site in position –38 (5′TTTGTAGATAA3′) of Dorsal 1A.

A third type of putative binding site, CCAAT/enhancer binding protein B (C/EBP), was found abundantly in the 5′ upstream regions of all genes investigated. These sites have been shown to have immune roles in mammals, whereas in mosquitoes the closely associated κB and C/EBP sites function cooperatively to activate defensin genes (Meredith *et al.*, 2006) and possibly other genes.

To test the correlation between the presence of these putative transcription factor binding sites and the inducibility of these genes, we identified a genomic contig containing 381 bp of 5′ upstream sequence of a constitutively expressed actin gene (clone NADK-aed47c06). Our analysis did not identify any putative κB or GATA sites.

Table 1. Putative transcription factor binding sites

Gene	Clone	NF-κB	GATA
β GRP	NAAX-acc45d02		GATATAAAAA-160
			CATTAAGATTT-50
Haemolymph proteinase	NAAD-aab82b10		TGATAATGTTT-35
			TGTTTCAGATA-18
Prolixin	NAAX-ado90e10		AGCTGATAAAA-380
	NAAX-adg52b08		CTTATCTCGTG-292
Defensin A	NAAX-adj13d02	TTCTTCCTCT-479	CTATAAACAA-276
		AAGAAAATCC-372	
		GGGATTCCCCC-162	
Defensin B	NAAX-abh21d09	TGGAATCCCC-166	
		GGATATTCCAC-30	
Lys 1A	iPCR Dpn1	GGAACTTTCAA-64	TGTTTCAGATC-115
	NADD-aee07e10	ATTAGGAAATAC-49	CTTATATTTCT-42
Lys 1B	NAAX-ady62g11	TAGGAAATGAC-181	TTTGAGCAGAA-356
			TTATTATTTTT-302

Putative transcription binding sites were identified using ALIBABA 2.1 software using lazy restriction parameters.
Location of the putative binding site is indicated relative to the methionine start codon.
The clone indicators refer to the trace data files available at the NCBI trace data archives (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
GRP, glucan recognition protein; iPCR, inverse PCR; NF-κB, Nuclear Factor kappa B; %GC, G or C percentage; DefA, Defensin A; HP, haemolymph proteinase; Lys, Lysozyme.

Discussion

Differential splicing is not an uncommon occurrence in transcription factors, including the Rel/NF-κB family. *Drosophila melanogaster* has three different Rel/NF-κB TFs; Dorsal, Dif and Relish (Lemaitre & Hoffmann, 2007). Initially, the *Drosophila* transcription factor Dorsal, was described for its key role in establishing dorsoventral polarity in the early embryo. However, its alternatively spliced version, Dorsal-B is up-regulated in response to septic injury and is presumed to be involved in the immune response (Gross *et al*., 1999). Dorsal and Dorsal-B are identical in the N-terminal region. Dorsal-B however lacks the nuclear localization signal at the end of the RHD domain and the C-terminal ends are significantly different (Gross *et al*., 1999). No differentially spliced versions of Dif and Relish have been described in *D. melanogaster*; however, the relish gene encodes four transcripts that originate from alternative start sites and produce proteins of different lengths (Hedengren *et al*., 1999).

In mosquitoes, no Dif orthologue has been reported, thus splice variants of Dorsal are thought to function in a dual role in development and innate immune responses. The malaria vector *Anopheles gambiae* has only two Rel/NF-κB genes, *Rel1* and *Rel2*, which are homologues of *Drosophila*'s Dorsal and Relish respectively. *Rel2* is differentially spliced into a shorter version that lacks the ankyrin repeats and a death domain (Meister *et al*., 2005). In *Aedes aegypti*, Relish has three alternatively spliced transcripts that encode different proteins. The predominant *Ae. aegypti* Relish protein contains both the RHD domains and the IκB-like domain. Its differentially spliced version maintains the RHD domains but completely lacks the IκB-like domain. In the third transcript, a deletion replaces most of the N-terminal sequence and RHD; however, the IκB-like domain remains intact (Shin *et al*., 2002). An *Ae. aegypti* homologue of *D. melanogaster* Dorsal and *An. gambiae* REL1 has been identified. Differentially spliced versions of this homologue give rise to two isoforms that differentially activate effector genes (Shin *et al*., 2005).

In some dipteran insects, differential splicing results in the loss of functional domains of the transcription factor. This is not the case for *R. prolixus* Dorsal. A 75 and 156 bp deletion in the 5′UTR region and in the initial coding region of the protein results in the removal of 13 and 45 amino acids, respectively, but no major protein domain is affected. A similar phenomenon was recently described for *Bombyx mori* Rel proteins for which two isoforms *RelA* and *RelB* differ in the 5′ sequence. Alternative splicing removes 241 bp of this transcript resulting in the loss of 52 amino acids of RelA (Tanaka *et al*., 2005). What is noteworthy in these cases is that no functional domains seem to be removed by the differential splicing. As is the case with *Drosophila*'s Dorsal and Dif and c-Rel, RelA and RelB in vertebrates, *R. prolixus* Dorsal proteins have N-terminal RHD and IPT domains whereas their C-terminal sequences contain transcriptional activation domains.

Analysis of immune genes involved in recognition [*β1–3 glucan recognition protein* (*β-Grp*)], activation (*haemolymph proteinase*) and effectors (*defensin* and *lysozyme*) revealed the presence of putative κB binding sites only in the 5′ upstream regions of antimicrobial peptides (lysozyme and defensins), whereas GATA sites were present ubiquitously. This is consistent with a late role of Rel/NF-κB TFs in the activation and induction of AMPs in *D. melanogaster* Toll and Imd immune signalling pathways (Engstrom *et al*.,

1993). The role of GATA binding sites in insect immunity has been described as being cooperative to proximal κB sites (Kadalayil *et al.*, 1997) and important in determining tissue specificity (Petersen *et al.*, 1999, Senger *et al.*, 2006). Finding isolated GATA sites in promoter regions of immune related genes, especially those lacking apparent κB sites, is unusual and warrants further investigation.

The expression profile of *R. prolixus* dorsal 1A was similar to that of *D. melanogaster* dorsal B (Gross *et al.*, 1999) and *Ae. aegypti Rel1* transcripts (Shin *et al.*, 2005), which are found in larvae and adults challenged with bacteria. In contrast, dorsal 1B and 1C transcripts were only found in adults after bacterial insult.

The overall expression of individual Rel/NF-κB TFs may not be the only requirement as there are different reports concerning the specificity of response and activation of target genes by Rel/NF-κB TFs. The induced expression of Dorsal, Dif and Relish in transgenic *D. melanogaster*, followed by microarray analysis, suggests that some immune genes may be induced redundantly by different Rel/NF-κB TFs (Pal *et al.*, 2008). Our results indirectly agree with these studies as none of the κB sites that we identified exactly matched the core consensus site sequence used in our functional assay. However, the κB sites we identified in *R. prolixus* are an 80–100% match with sequences identified in other insects (Shin *et al.*, 2005). Our results indicate that a region of the expressed *R. prolixus* Dorsal molecule does bind to a consensus motif, suggesting that that there is a degree of plasticity in these interactions.

Our understanding of immune gene regulation in *R. prolixus* is not as detailed as for other insects, including the well-characterized *D. melanogaster*, mosquito disease vectors such as *Ae. aegypti* and *An. gambiae* or model insects such as *Manduca sexta*. However, homology-based studies have proven very valuable for the identification of related TFs and AMPs. The forthcoming release of the *R. prolixus* genome, and the genome mining that will take place, should allow us to identify more components that regulate immune genes in this species, including additional NF-κB transcription factors. As more genomes of pterygote, apterygote, holometabolous and hemimetabolous insects are annotated and released, we will have more opportunities to compare the evolution and regulation of immune-related genes and the role of transcription factors in immune gene regulation.

Experimental procedures

Molecular cloning

A 364 bp EST of RhP-dorsal was obtained originally in a study of differential gene expression in the fat body of *R. prolixus* in response to the injection of *T. cruzi* into the haemocoel of adult insects (Ursic-Bedoya & Lowenberger, 2007). We used standard techniques as described previously (Lopez *et al.*, 2003) to obtain the full-length cDNAs of our molecules using 5′–3′ RACE with the Marathon cDNA synthesis kit (Clontech, Mountain View, CA, USA). We obtained the 3′ region using PCR reactions with primers: RpDoF1 (5′gaccattgcaatcacgcgg3′) – MgdT (5′cgggcagtgagcg caacgt$_{14}$3′). Subsequently we used two primer pair combinations (dgF-rhd (5′grtttcgstacgaatgygargg3′) – RpDo-caR(5′aagttgttct aactctgact gaccac3′) and AP1 (5′ccatcctaatacgactcactatagggc3′) – RpDo-R5 (5′gagttttatgaatgaatccggtcct3′) to obtain the full-length cDNAs. Reactions were performed with either Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) or iProof DNA polymerase (Bio-Rad, Hercules, CA, USA) under the following conditions: 98 °C 60 s; 98 °C 10 s; 60–65 °C 15 s; 72 °C 45 s. Annealing temperatures were modified according to the primer pair used. Subsequent cloning into pGEM-T Easy vector (Promega, Madison, WI, USA), transformation into *Escherichia coli* JM109 cells by heat shock, plasmid DNA isolation from recombinant clones using Wizard minipreps (Promega) and DNA sequencing using BigDye v3.1 chemistry (Applied Biosystems, Foster City, CA, USA) were carried out as described previously (Ursic-Bedoya *et al.*, 2005). The overlapping sequences were aligned using the SeqMan II module of LASERGENE v5 software (DNASTAR, Madison, WI, USA) to generate the full cDNA sequence of all genes and to identify all putative open reading frames. Three different cDNAs were obtained in this manner.

Transcriptional analysis

Specific tissues were dissected from five individual one-month-old adult *R. prolixus* in cold phosphate-buffered saline (PBS). Ten embryos were collected for RNA extraction approximately 2 days after being deposited. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. For immune activated samples, adults were injected with bacteria as described previously (Ursic-Bedoya *et al.*, 2008) and fat bodies were dissected 24 h later. Total RNA was extracted and treated with DNAse (Ambion, Austin, TX, USA) to eliminate genomic DNA as described previously (Ursic-Bedoya *et al.*, 2008). Total RNA from dissected tissues (1 μg) or embryos (200 ng) was used to generate cDNAs using superscript 2 RT enzyme (Invitrogen) and a modified dT primer (MgdT 5′cgggcagtgagcgcaacgt$_{14}$3′). To perform the real-time PCR analysis, we designed forward primers against unique sequences in the 5′UTR of the three *R. prolixus* Dorsal-like molecules and specific reverse primers located within the open reading frame to prevent cross amplification:

1AF-5UTR: 5′caaataacaatgaataatttagaatcgt3′and DoMetqR: 5′acagattggttcattaagccacc3′;
1BF-5UTR: 5′acgcttttgagaatcgtttga3′ and DoMetqR;
1CF: 5′cgcttttgagttgaagttatagaa3′and 1C-r: 5′acaccagg aatagaaccagc3′.

We used β-actin (GenBank EU233794) as the internal control gene, amplified with primers: qActF: 5′ aatcaagatcattgctccaccag3′ and qActR: 5′ttagaagcatttgcggtggac3′. The real-time PCR conditions used were: 95 °C: 2 min, 40 cycles of 95 °C: 10 s, 60 °C: 15 s, 72 °C: 20 s in 25 μl reactions using PerfeCTa SYBR Green SuperMix (Quanta BioSciences, Gaithesburg, MD, USA) in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Real-time PCR results were analysed using the $2^{-\Delta Ct}$ method as described previously (Schmittgen & Livak, 2008) and are presented as normalized individual data points. Results shown represent the average and standard deviation of two independently generated cDNAs assayed at least twice where each sample was run in duplicate.

The statistical analysis was performed using JMP 7 software (SAS, Vancouver, BC, Canada). We used ANOVA to compare the Delta threshold cycle (Ct) of all samples and then a Tukey's multiple comparison test to compare Delta Ct values amongst tissues.

Putative target gene identification

In order to evaluate the role of Rp-Dorsal like molecules on the expression of immune genes, and to identify potential κB binding sites for our molecules, we examined the genomic DNA sequences upstream of selected immune genes. Although the *R. prolixus* genome is being sequenced, preliminary data are available only in trace archives. Therefore we used iPCR (Triglia, 2000) to amplify upstream genomic regions of selected immune-related genes (*β-GRP*, *lysozyme*, *defensin* and *haemolymph proteinase*; Lopez *et al*., 2003, Ursic-Bedoya & Lowenberger, 2007, Ursic-Bedoya *et al*., 2008). Restriction enzymes were selected to digest DNA within the first 500 bp of the open reading frame based on the cDNA or genomic DNA sequences of the target genes using New England Biolab's NEBcutter v2.0 (http://tools.neb.com/NEBcutter2/index.php). Inverse orientated primers were designed based on cDNA sequence of our genes or, in the case of haemolymph proteinase, the genomic sequence that we obtained by amplifying a 4.3 kb amplicon of genomic DNA with primers RpHPMet (5′atcatgatt aatcaattatcc3′) and RpHPstopR (5′gtacatcctccataagttaga3′) designed to amplify the open reading frame of the gene.

Genomic DNA was isolated from adult insects as already described (Ursic-Bedoya *et al*., 2008). One microgram of genomic DNA was digested with 10 U of a single restriction enzyme at 37 °C for 3 h. Restriction enzymes used for each gene were: lysozyme 1A: DpnI, EcoRV, RsaI; β-GRP: DpnI, EcoRI, EcoRV, RsaI; defensin A: DpnI, and for haemolymph proteinase: BamHI, DpnI. Restriction enzymes were heat inactivated where possible or DNA was isolated by a phenol : chloroform extraction. Approximately 200 ng of digested genomic DNA were self ligated with 12 U of T4 Ligase (Promega) in 100 μL reactions at 16 °C for 16 h in a thermocycler (Bio-Rad). Two microlitres of the ligation reaction were used in a PCR reaction using iProof DNA polymerase (Bio-Rad). Primer pairs used in individual reactions are listed in Table 2. Amplicons obtained for each gene were cloned into pGEM-T Easy, transformed into *E. coli* JM109 cells, and sequenced as described previously (Lopez *et al*., 2003) or directly sequenced from the original PCR amplicon with BigDye v3.1 (Applied Biosystems). In addition to the molecular approach, we used bioinformatic tools to obtain genomic data from the trace data archives of the *R. prolixus* genome sequencing project using Mega BLAST searches (www.ncbi.nlm.nih.gov/blast/mmtrace.shtml) with the first 200–300 nucleotides of the open reading frame of every gene investigated. Contigs containing the identified genomic clones and the open reading frames were constructed using the SeqMan II module of LASERGENE v. 5 software with loose assembling parameters to accommodate for large gaps corresponding to introns. Putative transcription binding sites were identified using ALIBABA 2.1 software (Grabe, 2002) with lazy restriction parameters (www.gene-regulation.com).

Table 2. Primer combinations used in inverse PCR (iPCR)

Oligo name	Sequence 5′ to 3′	Tm	%GC
iPCR Lys1A F	AACTACGACGGAAGCTATGATAATGG	65.7	42.31
iPCR Lys1A R	TAGTGAACACCCTAGCTTGTGTGG	66.1	50
iPCR GRP F	AGAATTAGAATATCTAGAAGCTGGCG	62.8	38.46
iPCR GRP R	CAGAACATGTTGCTATGAAGAGG	62.5	43.48
iPCR DefA F	AGGTAACCGAAGAACATGTCGC	66.1	50
iPCR DefA R	GGCCACCAAGAACAGAGTAACC	65.7	54.55
iPCR HP F	ATTCTAGGCATAAACCAGGAGTG	62.1	43.48
iPCR HP R	TCCAAAGCAAACTAATCCGAC	62.8	42.86

List of inverse orientated primers used for each gene investigated. Primers were designed within the first 150 bp of each gene's open reading frame.
F, forward; GRP, glucan recognition protein; R, reverse; Tm, melting temperature of the oligo.
%GC, G or C percentage; DefA, Defensin A; HP, haemolymph proteinase; Lys, Lysozyme.

Recombinant protein expression

The entire open reading frame of dorsal 1C or a region containing the putative rel homology domain (amino acids 1–336) were amplified using the primers: LICadF: 5′gacgacgacaagatgaac caatctgttcggaga3′; LICadR: 5′gaggagaagcccggtttagtttacttttg ttgttcg3′ or LIC-RHDadR: 5′gaggagaagcccggttaagttaataattttgtat tcg3′, respectively, under the conditions 95 °C: 1 min, 30 cycles of 94 °C: 20 s, 60 °C: 15 s, 72 °C: 45 s. Amplicons were cloned into pET_{32} expression vector (Novagen, Madison, WI, USA) by ligation independent cloning (LIC) as described in Novagen's pET System manual. Recombinant plasmid DNA was first transformed into non-expression host *E. coli* NovaBlue cells, grown overnight in liquid Luria-Bertani (LB) broth containing carbenicillin (50 μg/ml), and then purified using the WizardPlus Miniprep DNA Purification System (Promega). DNA sequencing of clones was carried out to confirm that the sequence was in the correct reading frame prior to transformation into the bacterial expression host. Five nanograms of plasmid DNA were transformed into *E. coli* Origami 2(DE3) by heat shock following the manufacturer's recommendations (Novagen). The recombinant bacteria were plated on LB-carbenicillin (50 μg/ml) plates and incubated overnight at 37 °C. The next morning a single colony forming unit was used to inoculate 100 ml of fresh LB-carbenicillin (50 μg/ml) liquid media and grown at 37 °C with vigorous shaking until optical density at 600 nm optical density at 600 nm (OD_{600}) ≈ 0.6. Recombinant protein expression was induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and bacterial cultures were incubated at room temperature for 6 h with shaking. Bacteria were isolated by centrifugation at 10 000 *g* for 15 min and washed once with 20 mM Tris-Cl, pH 7. The pellet was stored at –70 °C. The recombinant protein was released from the bacterial cytoplasm after lysing the cells with 5 ml per gram of pellet of BugBuster reagent (Novagen) supplemented with 1 KU of rLysozyme and 0.1 U of Benzonas e per ml (Novagen) while shaking at room temperature for 20 min. After incubation the clear cell lysate was centrifuged at 10 000 *g* at 4 °C for 20 min. The supernatant was transferred to a clean microcentrifuge or falcon tube and the pellet was resuspended in 0.5 supernatant volume of PBS; both samples were stored on ice. The protein content of each sample was estimated using Bradford reagent (Bio-Rad). Aliquots of all samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis to confirm the presence of the recombinant protein in comparison to cell extracts from non-induced recombinant bacterial cells.

In vitro *activity*

Approximately 6–10 μg of soluble cell lysate was used to assay the binding of the recombinant protein to the mammalian core NF-κB site: 5′GGGACTTTCC3′ using the NoShift II Transcription Factor

Assay System (Novagen), a nonradioactive alternative to standard EMSAs. A biotin-labelled DNA probe consisting of a double-stranded consensus transcription factor binding site and a single-stranded capture region was incubated with the cell lysate containing the recombinant transcription factor. If functionally active, the transcription factor binds specifically to the double-stranded consensus sequence. Upon addition of a double-stranded DNA specific nuclease, the DNA probe bound to the transcription factor is protected from digestion whereas the unbound probe is degraded. The reactions then were transferred to a 96-well plate coated with the complementary strand to the capture region of the probe and the probe/transcription factor complex was captured on the plate. After four washes with buffer to remove unbound biotin and digested probe, streptavidin-alkaline phosphatase was added and allowed to bind to the biotinylated probe. A second wash step was followed by the addition of a chemiluminescent alkaline phosphatase substrate. Chemiluminescence detection was performed on a Victor 3V (Perkin Elmer, Boston, MA, USA) microplate luminometer. The assay was replicated two times with every individual sample run in triplicate. Assays were validated by ensuring that the per cent digestion of the negative reagent control was above 90% as per the manufacturer's suggestion.

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