Pergamon

Isolation and characterization of a novel insect defensin from *Rhodnius prolixus*, a vector of Chagas disease

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Abstract

An antimicrobial peptide belonging to the defensin family of small cationic peptides associated with innate immunity in insects was isolated from the hemolymph of *Rhodnius prolixus*, a vector of Chagas disease. This peptide, designated *R. prolixus* defensin A, was purified and sequenced. The active peptide contains 43 residues and aligns well with other insect defensins. However the pre-pro region of the sequence has little shared identity with other insect defensins. We have identified 3 isoforms of *R. prolixus* defensin from cDNA clones obtained from RNA isolated from the whole bodies of immune activated insects. Northern analysis and Real-Time Quantitative PCR indicate that there is a very low baseline transcription of this peptide in naïve insects, and that transcription increases significantly in the fat body of immune activated insects. In addition there is a delayed induction of transcription of this peptide in the intestine 24 h post activation suggesting that the midgut/intestine of this species is active in the immune response against pathogens.
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1. Introduction

Insect immunity is the rapid, germ-line encoded anti-infection response insects use to protect themselves from pathogens and parasites (Boman, 1998). A major component of this immune response is the production of a potent arsenal of immune peptides, most of which are produced in the fat body or hemocytes of the insect and released into the hemolymph. This immune response is based on the recognition of the pathogen as nonself, the induction of suitable genes and biochemical pathways that result in the production of potent antimicrobial peptides (Hoffmann et al., 1999; Barillas-Mury et al., 2000). Immune peptides are expressed de novo, usually in the fat body and hemocytes (Dimarcq et al., 1994), and delivered to the appropriate site to defend the host in a manner that is neither learned nor acquired, unlike the hallmark characteristics of classical immunology (Boman, 1998).

Innate immunity is not limited to insects; immune peptides are highly conserved members of the innate response of highly diverse taxa, including single celled organisms (Leippe, 1999), various classes of invertebrates (for a review see Hetru et al., 1998), plants (Broekaert et al., 1995) and vertebrates (Lehrer and Ganz, 1996). However, the majority of studies over the last 10 years have been carried out on insects, especially *Drosophila melanogaster* in which strong similarities have been demonstrated between the signal transduction pathways of the innate immune response of insects and the acute phase response of vertebrates (Hoffmann et al., 1999). There are distinct advantages to the innate response; anti-microbial peptides can act with low specificity against a wide range of microorganisms, there are no memory requirements, and they can be synthesized at relatively low metabolic cost to their hosts, and can be stored in high concentrations (Shai, 1998). A keystone

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element of innate immunity is the speed with which these responses occur: transcripts can be found within minutes of stimulation, and proteins found within hours.

Several studies have suggested that inducible immune peptides can limit parasite development in vectors (Lowenberger et al., 1996, 1999b, 2001; Vizioli et al., 2001; Shahabuddin et al., 1998; Jaynes et al., 1989; Possani et al., 1998; Rodriguez et al., 1995). Most of these studies have looked at vectors, such as mosquitoes, in which the parasites have direct contact with hemolymph factors as they move from their site of development to the mouthparts or salivary glands for transmission to vertebrates.

In Chagas Disease, however, the parasites never leave the intestinal tract, and there is no such direct contact between parasite and hemocyte or hemolymph factors. Instead, the ingested parasites form epimastigotes in the vector midgut and metacyclic trypomastigotes that inhabit the hindgut and rectum (Brener, 1973; Garcia and Azambuja, 1991; Garcia et al., 1999; Azambuja et al., 1999). As the insect feeds and engorges, a fecal droplet containing infective parasites is deposited on the skin of the host and the parasites then are rubbed into the puncture or existing skin lesions.

We hypothesize that this inefficient, but successful, mode of transmission has evolved to avoid contact with lethal components of the vectors' immune response, and to test this hypothesis, requires that we first identify the repertoire and activity spectra of the inducible immune response in vectors of this parasite. We report here the isolation and characterization of a member of the insect defensin family from *Rhodnius prolixus*, a major vector of Chagas disease.

2. Methods and materials

2.1. Insect maintenance, immune activation and hemolymph collection

A colony of *R. prolixus* has been maintained at the Institute of Biology, Universidad de Antioquia, Medellín Colombia for over 5 years. Bacteria (*E. coli* and *M. luteus*) were grown at 37 °C as described previously (Lowenberger et al., 1995). Overnight cultures of each bacterial culture were combined, pelleted, and the supernatant discarded. A minuten pin (0.15 mm) was dipped into the moist bacterial pellet and inserted into the hemocoel of 4th and 5th instar nymphs of *R. prolixus*. At 6, 12, and 24 h post inoculation, a minuten pin was inserted through the thoracic pleura of the insects, and the exuding drop of hemolymph was collected. Approximately 10 μl of hemolymph was collected from each insect and the hemolymph from 30 animals was combined. Because there was rapid melanization of hemolymph the material was stored in an ammonium acetate buffer pH 3.5 (final

concentration 25 mM), supplemented with PMSF (final concentration 1.5 μM) as a protease inhibitor, and phenylthiourea (final concentration 20 μM) as a melanization inhibitor.

2.2. Peptide isolation and antimicrobial assay

After gentle stirring for 30 min in an ice-cold water bath the samples were centrifuged at 10 000 g for 20 min at 4 °C. The supernatant was loaded onto a C18 cartridge (Burdick and Jackson) previously equilibrated with acidified water (0.05% trifluoroacetic acid) and stepwise elution performed with 2, 40, and 80% ACN in acidified water (Bulet et al., 1993). The 40% fraction was analyzed by reverse-phase HPLC on an Aquapore 300 C8 column (250 × 7.0 mm) using a linear gradient of 2–100% ACN in acidified water for 160 min at a flow rate of 1.3 ml/min. The fractions were collected every minute during the run, lyophilized, and re-suspended in 100 μl of distilled water. Antimicrobial assays were carried out in two manners. Initially, sterile discs of whatman filter paper were impregnated with each fraction and placed on an agar plate previously overlayed with either *M. luteus* or *E. coli,* and incubated at 37 °C. The plates were assessed for halos of inhibition for each fraction 24 h later. Subsequently isolated fractions were assayed for activity in a liquid assay as described previously (Hetru and Bulet, 1997). Active fractions were further purified using a biphasic gradient of ACN in acidified water from 2–25% over 10 min and 25–32% over 40 min at a flow rate of 0.2 ml/min. For all HPLC analyses the effluent was measured at 225 nm and fractions hand collected and concentrated under vacuum.

2.3. Capillary zone electrophoresis and microsequence analysis

The purity of peptides was ascertained by capillary zone electrophoresis as described previously (Lowenberger et al., 1995) using a 3D Hewlett Packard Capillary electrophoresis system equipped with silica capillary. Separation was done from anode to cathode in 20 mM citrate buffer at pH 2.5. Detection was carried out at 30 °C, at 200 nm. Two very active fractions peaks were transferred to a PVDF membrane, and submitted to the Medical College of Wisconsin for sequence analysis via Edman degradation.

2.4. RNA isolation and cDNA determination

Total RNA was collected from whole bodies, fat bodies, or intestinal tracts of immune activated or non-inoculated (control) *R. prolixus* at 0, 6, 12, 24 h post inoculation using TRI REAGENT (Molecular Research Center) following manufacturers instructions. Total RNA was quantified using a Biophotometer (Eppendorf,

Germany) and 1 µg of total RNA was reverse transcribed as described previously (Lowenberger et al., 1999c) using the primer 5′-CGGGCAGTGAGCGCAACGT$_{14}$-3′. Degenerate PCR was carried out using the RT primer and two forward degenerate primers designed against the amino acid sequence we obtained via Edman degradation. The primer sequences were: Primer 1: 5′-gtnacnccnaaycaygcngg-3′, Primer 2: 5′-gcncaycayytnttymgnytngg-3′. PCR conditions were 95C-3 min, and 30 cycles of 95C (10 s), 50C (10 s) 72 C (30 s) followed by a 5 min extension period at 72 °C on an Idaho Technologies Indy Cycler® (Idaho technologies, Oregon USA). PCR products were size-fractionated on a 1.2% low melting point agarose gel and visualized on a BioDoc gel documentation system (UVP, California, USA). Bands of the predicted size were excised from the gel, placed at 65C to liquefy the fragment, vortexed briefly, and cloned directly into P-GEM-T vector (Promega, Madison WI, USA) following the manufacturers instructions. Blue-white screening of XL1-Blue cells (Stratagene, USA) was used to identify potential transformants. These colonies were grown overnight in 5 ml LB medium with 5 µl Ampicillin (100 µg/ul) and purified using the Wizard Plus Miniprep DNA Purification System (Promega, Madison WI). Sequencing of these clones was carried out on an ABI 310 sequencer (Applied Biosystems) using Big Dye chemistry. Sequences were compared with known sequences in the NCBI database. We designed specific primers for the defensin clone based on the sequences obtained from the degenerate primer PCR. Full length cDNA sequences were obtained using the Marathon cDNA synthesis kit (Clontech) using our specific primers and the flanking primers obtained with the kit. PCR amplification, cloning, transformation and sequencing were carried out as described above. Phylogenetic analysis and multiple alignment of *R. prolixus* defensins with defensins from other sources was carried out using DNA Star (Madison WI) using Clustal method with PAM 250 matrix.

2.5. Northern analysis

Northern analysis was performed as described previously (Lowenberger et al., 1999a) using 5 µg of total RNA from whole bodies and specific tissues of control or immune-activated insects. RNA was separated on a formaldehyde-agarose gels (Sambrook et al., 1989), transferred to a nylon membrane, and UV crosslinked. ^{32}P probes were made using 50 ng of the entire coding region (283 bp) of *R. prolixus* isoform A in a PCR reaction described previously (Severson and Kassner, 1995). Membranes were hybridized with the probe, and subsequently with a ^{32}P-labelled probe made from a 200 bp fragment of an actin gene isolated from *R. prolixus*. Preparation of the probe, removal of free dNTPs,

hybridization conditions and washes were carried out as described previously (Lowenberger et al., 1999c).

2.6. Quantitative PCR

For a more precise estimation of comparative transcription rates, Real-Time Quantitative PCR (QPCR) was used. Reverse transcription was done as described above with 1 µg of RNA from whole bodies or specific tissues. Initially, 1 µl of the RT reaction was used in a QPCR reaction using primers to amplify a partial fragment of a *R. prolixus* actin (Brackney, Lowenberger, and Wolff unpublished) as a control for similar amounts of cDNA in each sample. Samples were run on a BioRad *i*Cycler machine under the conditions: 95 °C (2 min), and 40 cycles of 95 °C (0.5 min), 62 °C (0.5 min) 72 °C (1 min). The PCR reagents were similar to the regular PCR with the addition of 1 µl of a 1/1000 dilution of Sybr-Green I (Sigma, USA) to measure the amounts of double stranded DNA produced in the reaction and 2.5 µl of a 1/1000 dilution of fluorescein to control for background fluorescence. Sample volumes were adjusted in subsequent PCR reactions to ensure similar amplification profiles for actin. Subsequently these volumes of the cDNAs were used to amplify a partial fragment of our defensin sequence. QPCR was carried out on samples collected from different batches of immune stimulated or naïve insects.

3. Results

In this study we assessed the hemolymph of immune activated *R. prolixus* for the presence of antimicrobial peptides. Based on our previous studies with mosquito defensins (Lowenberger et al., 1995) we concentrated our analysis on the fractions eluted with 40% ACN. This fraction was subjected to RP-HPLC analysis (Fig. 1a). Fractions (1 ml) were collected each minute during the run, dried, resuspended in ddH$_2$O, and assessed for antibacterial activity using the impregnated Whatman technique (Fig. 1b). Active fractions were subjected to a second reverse-phase chromatography, and fractions tested against *E. coli* and *M. luteus*. Capillary electrophoresis of one active peak indicated two bands that were separated on a gel, transferred to PVDF, and sequenced. We obtained a sequence: VTPNHAGCAHHLFRLGNRG. This was submitted to the NCBI BLAST program for comparisons with other reported peptides. The highest match (57% identity, 72% positives) was a segment of the insect defensin from *Pyrrhocoris apterus* (Fig. 2).

We performed PCR on the cDNA using the degenerate primers described in the Materials section. The initial amplification of a fragment of *R. prolixus* defensin cDNA using primer 1 produced clones which indicated
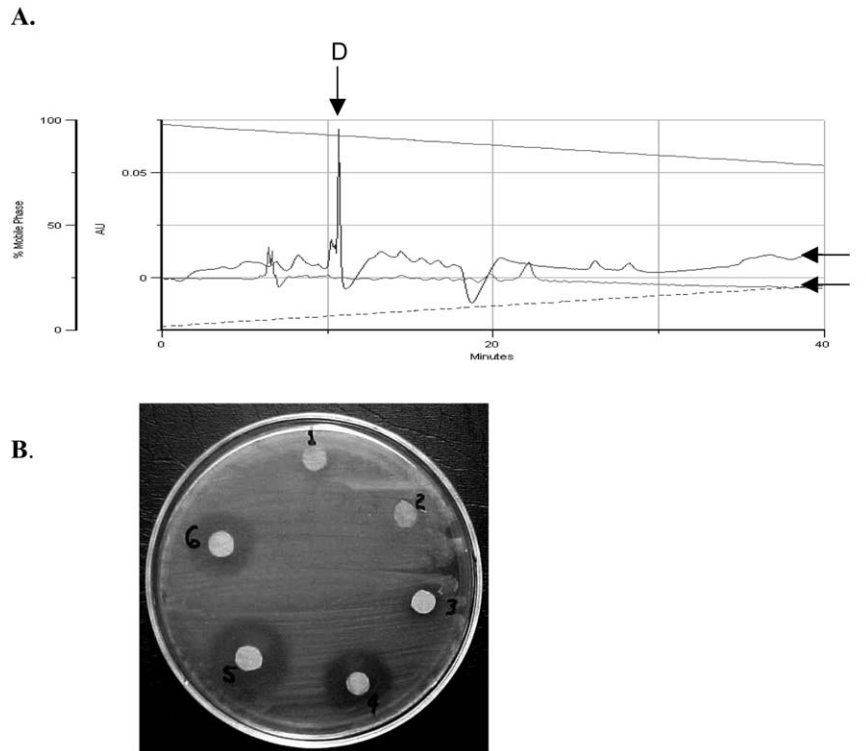
Fig. 1. A: Reverse phase chromatograph of hemolymph collected from immunized (I) or control (C) *Rhodnius prolixus*. Prepurified samples were applied to a reverse-phase C18 column and eluted with an acetonitrile gradient. Fractions (1 ml) were collected each minute, dried under vacuum and resuspended in 100 µl of water. Sterile discs were impregnated with 25 µl of this liquid and placed on an agar plate previously seeded with either *E. coli* or *M. luteus*. The presence of growth inhibition zones (Fig. 1B) indicates the presence or absence of antimicrobial activity in sequentially collected HPLC eluted fractions against *M. luteus*. Fractions demonstrating activity were reanalyzed and individual peptides obtained. The peak labeled "D" contains the *R. prolixus* defensin.

three distinct cDNA sequences (Fig. 3). We then designed 3 reverse primers that would distinguish between these three isoforms for use during the 5′ RACE protocols, using the Marathon kit (Clontech, USA) to amplify full length cDNAs. The cDNAs contain a 5′ UTR of differing lengths: 46bp (isoform A), 56bp (isoform B) and 51bp (isoform C). All isoforms have a pre-pro defensin of 153 bp beginning with the ATG start codon and terminating with AAG AGA, which translates to the K-R cleavage site present in many insect defensins. The coding region for the mature defensin is 129 bp (that translates to a peptide of 43 residues) in all isoforms, a stop codon TGA, followed by a 3′ UTR of differing lengths: 117, 113, and 129 bp, respectively, for isoforms A, B, & C. The cDNAs for isoform A and C have a polyadenylation consensus sequence (AATAAA) 16 bp before the poly-A tail, whereas isoform B has no such signal (Fig. 3).

We aligned the sequences of the mature peptide obtained with sequences available in GenBank (Fig. 2A). There is a strong conservation of the size of defensin within the insects and the position of the conserved 6 cysteines typical for members of this family of immune peptides. A phylogenetic alignment of the mature peptides (Fig. 2B) demonstrated the closest grouping of the three defensins from *R. prolixus* with other members of the Hemiptera, then with other insects, and then with other organsms.

Transcriptional Profile: Northern analysis (Fig. 4) indicates a low level of transcription in naïve insects, and 6 h after inoculation a weak response in the intestine of *R. prolixus*. However by 24 h after inoculation there is a strong transcriptional activity for defensin in the intestine. In contrast, the fat body shows a high level of transcription 6 h post inoculation and remains high for 24 h. In Real Time quantitative PCR analysis we measured transcript presence as a percentage of the controls (Fig. 5). The levels found in naive midguts and fat bodies were arbitrarily given a value of 1. Whereas transcription in immune activated midguts did not increase over controls at 6 h post stimulation, there was a 7-fold increase 24 h post stimulation. In the fat bodies there was a 25- and 29-fold increase in defensin transcripts at 6 and 24 h, respectively, after immune stimulation. These data indicate that the fat body is the major immune organ in the insect as has been reported in other insect systems. However the midgut, where pathogens and obligate symbionts reside, is also an immune competent tissue.
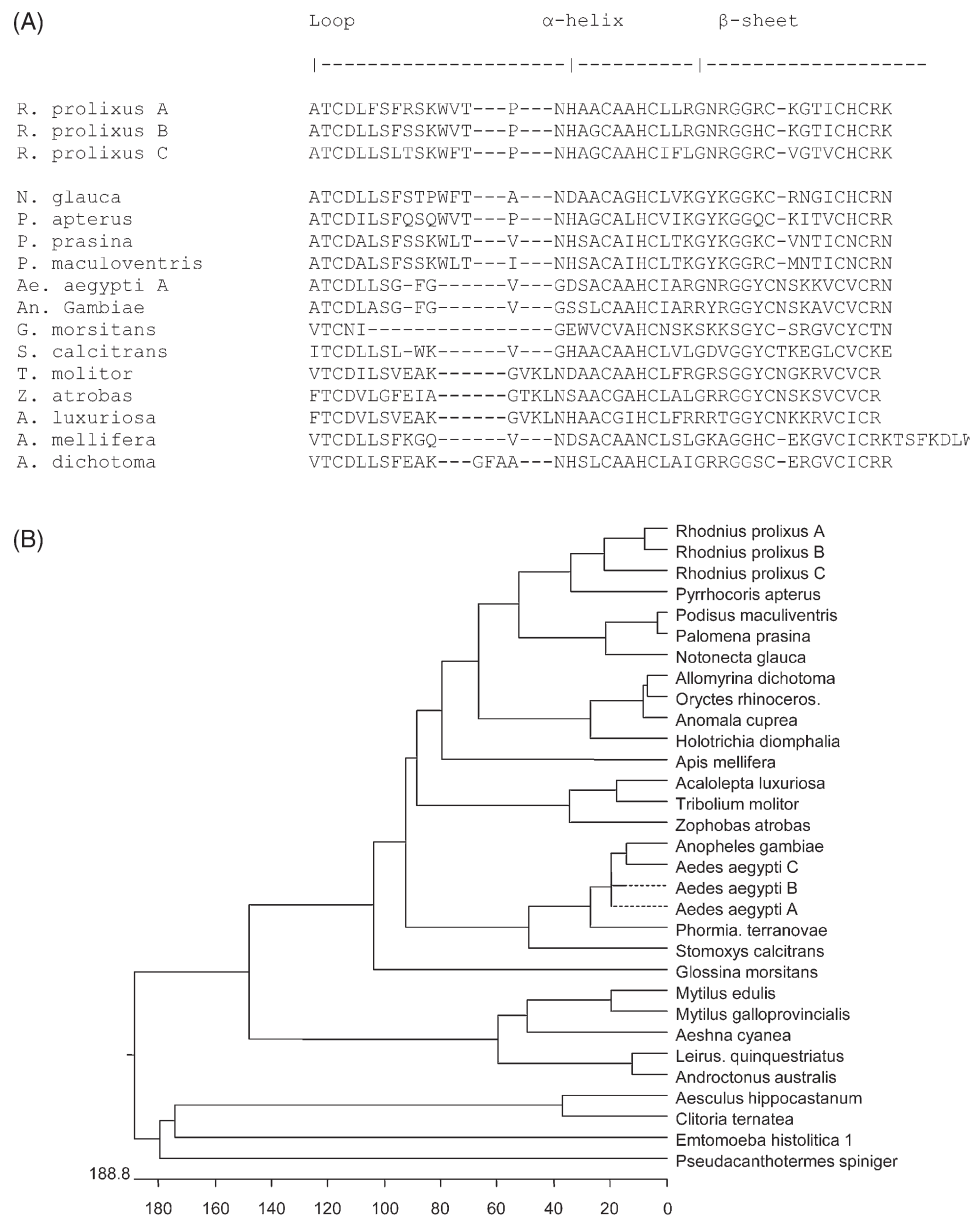
(A)

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                      Loop                 α-helix      β-sheet

                      |--------------------|----------|-------------------

R. prolixus A         ATCDLFSFRSKWVT---P---NHAACAAHCLLRGNRGGRC-KGTICHCRK
R. prolixus B         ATCDLLSFSSKWVT---P---NHAGCAAHCLLRGNRGGHC-KGTICHCRK
R. prolixus C         ATCDLLSLTSKWFT---P---NHAGCAAHCIFLGNRGGRC-VGTVCHCRK

N. glauca             ATCDLLSFSTPWFT---A---NDAACAGHCLVKGYKGGKC-RNGICHCRN
P. apterus            ATCDILSFQSQWVT---P---NHAGCALHCVIKGYKGGQC-KITVCHCRR
P. prasina            ATCDALSFSSKWLT---V---NHSACAIHCLTKGYKGGKC-VNTICNCRN
P. maculoventris      ATCDALSFSSKWLT---I---NHSACAIHCLTKGYKGGRC-MNTICNCRN
Ae. aegypti A         ATCDLLSG-FG------V---GDSACAAHCIARGNRGGYCNSKKVCVCRN
An. Gambiae           ATCDLASG-FG------V---GSSLCAAHCIARRYRGGYCNSKAVCVCRN
G. morsitans          VTCNI---------------GEWVCVAHCNSKSKKSGYC-SRGVCYCTN
S. calcitrans         ITCDLLSL-WK------V---GHAACAAHCLVLGDVGGYCTKEGLCVCKE
T. molitor            VTCDILSVEAK------GVKLNDAACAAHCLFRGRSGGYCNGKRVCVCR
Z. atrobas            FTCDVLGFEIA------GTKLNSAACGAHCLALGRRCGYCNSKSVCVCR
A. luxuriosa          FTCDVLSVEAK------GVKLNHAACGIHCLFRRRTGGYCNKKRVCICR
A. mellifera          VTCDLLSFKGQ------V---NDSACAANCLSLGKAGGHC-EKGVCICRKTSFKDLV
A. dichotoma          VTCDLLSFEAK---GFAA---NHSLCAAHCLAIGRRCGSC-ERGVCICRR
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(B)

Fig. 2.   **A**: Amino acid sequence alignments of three *Rhodnius prolixus* defensins. Alignments were compared with defensins from taxonomically related organisms. Each sequence has the classical loop, alpha helix and B sheet structure, and the highly conserved 6 cysteines that maintain the correct conformation. **B**: Phylogenetic analysis of defensins from different sources. The defensins isolated from *R. prolixus* are most closely aligned with those from other Hemipterans, and then other insect orders indicating that modifications in the defensin sequence of *R. prolixus* may have occurred after the separation of insects in evolutionary time. Sequences were compared using MegAlign (DnaStar, Madison WI) using the Clustal method with PAM 250 residue weight table.

4. Discussion

The data presented here establish that the cell free hemolymph of immune-challenged *R. prolixus* contains several compounds with activity against bacteria. One of these is a member of the defensin family, which are ubiquitous immune peptides described from several groups of invertebrates, plants and vertebrates. In common with all preprodefensin sequences there is a definite signal peptide region (residues 1–24 as predicted by Nielsen et al., 1997), a prodefensin region (residues 25–51) that terminates with a KR cleavage site. This conserved cleavage site is common among the insects (see Lowenberger et al., 1999c and Hetru et al., 1998).

A comparison with established peptides in the databanks established that our sequence is a member of the insect defensin family. At the mature protein level the *R. prolixus* peptide A shares 88 and 77% identity with defensins isloated from *P. apterus* and *Palomena prasina* respectively, both of which are members of the same insect order, the Hemiptera, and 66% shared identity with the Coleopteran, *Oryctes rhinoceros*. However we

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Defensin A:               CGCCCGGGCAGGTCTAGAATACTCCACTCAACCGCAACCAGACATC
Defensin B: CGCCCGGGCAGGTAGCACAGTACCTAGGATATTCCACTCAACCGCAACCAGACATC
Defensin C: CGCCCGGGCAGGTGTGCAGTACAGTCCTAATACCTAGCCACTCCTCACATC
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     M   K   C   I   L   S   L       T   L   F   L   V   A       L   V       S       P   A   E   W
A) ATG AAG TGC ATA CTC TCT TTG GTT ACT CTG TTC TTG GTG GCC GTC CTG GTT CAC TCT CAT CCA GCT GAA TGG
B) --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C) --- --- --- --- --- --- --- TTC --A --- --- CTG --- --T ACC --- --C TAT --C TAT --- --- --- ---

     Q       L   D  ↓ A       W   E   P   A   G   E       T   E   H           R       K   R
A) CAT CAA CAA CTA GAT GAT GCC CTA TGG GAG CCA GCC GGT GAG GTA ACC GAA GAA CAT GTC GCC CGT TTG AAG
B) CAA --- GAA TTA --C --- --T --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- CTG ---
C) CAA CAT --- TTA --- --- --- CAA --- --- --- --- --- TTA --- --- --- --C CTA TCA --- ATG ---

     A   T   C   D   L       S           S   K   W       T   P   N   H   A       C   A   A   H   C
A) GCC ACC TGT GAT CTG TTC AGC TTC CGG TCT AAA TGG GTC ACA CCT AAT CAT GCT GCC TGC GCC GCA CAT TGT
B) --- --- --- --- --- CTC --T --- TCC --- --- --- --- --- --- --- --- --A GGC --T --- --- --- ---
c) --- --- --- --- --- CTA --- CTT ACC --- --- --- TTC --- --- --C --- --- GGC --T --A --G CAT TGT

     G   N   R   G   G       C       G   T       C   H   C   R   K   *
A) CTC AGA GGA AAT AGA GGT GGC CGA TGT AAA GGA ACT ATC TGC CAT TGT AGG AAG TGA gaacattctccttagaatt
B) --- --- --- --- --- --C --- CAT --- --- --- --- --- --- --C --- --- --- atgtatttggacaatttctaacttaatt
C) TTC TTG --- --- --- --A --- --T --C GTA --- --- GTC --- --- --- --- --- atataaaaatccaaaattgaaacttttta
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A) tccttagaatttacatctttaaagcaatataccaaataaaatcatctttccggaacagtattaaataaaaatgtatctattataaataaacgaatggtttaatgatAn
B) atgtatttggacaatttctaacttaattttgttatttttgtttcttgttattgaacttcgacagattgaagatttattatatccgcagcattatt
ttgtAn
C) atataaaaatccaaaattgaaacttttatgaacgaaccaaatcaaaaaaaaatactcaatattcaagtcatttacgcatgaaaatgcattttatatt
aaatatgcgctttactatcAn
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Fig. 3. Alignment of the cDNA sequences encoding three isoforms of *Rhodnius prolixus* defensin. Dashes represent identical nucleotides in each sequence as compared with isoform A. Deduced amino acids are presented as single letter codes above each codon only when the same amino acid occurs in all three isoforms. Each sequence terminates with a stop codon (TGA) indicated by an asterisk. The putative polyadenylation consensus sequence is underlined. The position of the intron found in genomic clones is indicated by the arrow, and inserts G-intron-AT in amino acid D_{31} in the signal peptide of all three sequences.
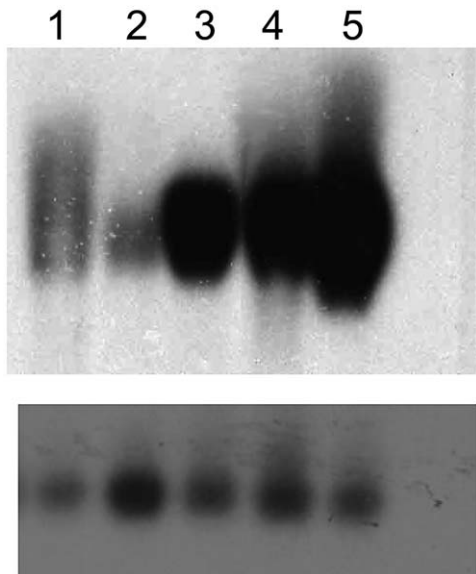

Fig. 4. Northern blot autoradiography demonstrating transcriptional activity for insect defensins in specific tissues collected from *Rhodnius prolixus* at various times after immune stimulation. Each lane contains 5 μg of total RNA run on a formaldehyde-agarose gel and probed as described in the Methods. A probe generated from a 300 bp fragment of a *R. prolixus* actin clone was used as a control. Lanes: 1) Control RNA from fat body, 2) intestine—6 h PI, 3) intestine—24 h PI, 4) fat body—6 h PI, 5) fat body—24 h PI.
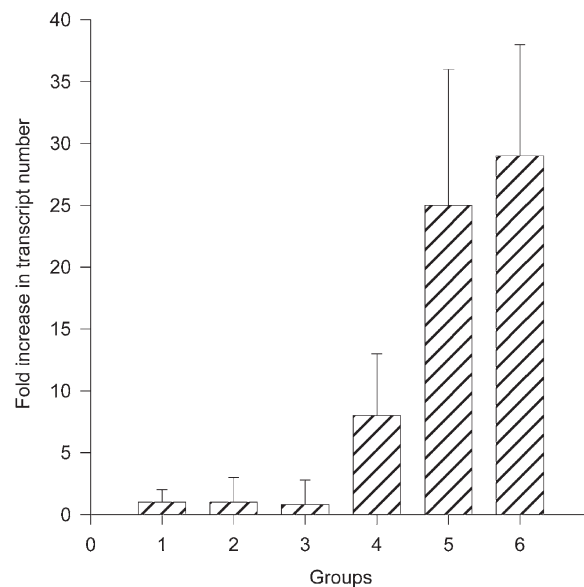

Fig. 5. Real-Time Quantitative PCR of *Rhodnius prolixus* defensin A from RNA isolated from fat bodies and intestines at various times post immune stimulation. The raw data were compared with known amounts of pure template and the values calculated for graphical purposes. Values for control lanes (fat body or intestine) were arbitrarily given values of 1 and the vertical axis represents the fold increase in the tissues as compared with the same tissue controls. Lanes: 1) Control intestine, 2) Control Fat body, 3) Intestine 6 h PI, 4) Intestine 24 h PI, 5) Fat body 6 h PI, 6) Fat body 24 h PI.

failed to obtain any positive comparisons when we subjected only the pre-pro region of our peptide to the databases, either as nucleotide or as the translated peptide sequences. Many of the sequences in the databases for closely related insects are for the mature peptide sequence only of the defensins, and this may limit the number of comparisons available.

These data suggest that whereas the mature peptide region has been highly conserved through evolutionary time, the prepro region of this peptide has been modified from a precursor molecule, and it is possible that *R. prolixus*, and possibly other members of the true bugs (Homoptera/Hemiptera), have a different pre-pro peptide than other insect orders. The exception is the KR cleavage site at the end of the pre-pro region (Fig. 3) that is highly conserved in *R. prolixus* as well as in several other very diverse species for which full-length cDNAs are available. These data and the phylogenetic analysis (Fig. 2B) suggest a very strong conservation of the mature peptide sequence among all organisms, especially the location of the 6 cysteine residues that form 3 disulphide bridges. Such conservation in all likelihood exists due to the importance of defensins as key components of the very effective immune response of invertebrates to pathogen invasion that has allowed these organisms to thrive in environments full of potential pathogens.

We tested an antibody raised against *Ae. aegypti* defensin A peptide on the hemolymph collected from naive and immune activated *R. prolixus*, and found a positive band at the expected location, albeit at a lower signal intensity that hemolymph collected from immune activated *Ae. aegypti*. The *Ae. aegypti* and *R. prolixus* peptides share approximately 55% identity, and the positive Western result indicates a high level of conservation of the peptide structure of defensins from different orders of insects.

In addition, we have isolated, by PCR, three genomic sequences containing introns of 90–97 bp. The introns are located in the same position in all three sequences; in all three sequences introns are found within the codon coding for amino acid D_{31}. This is similar to the situation with *Ae. aegypti* in which three isoforms of insect defensins were isolated (Lowenberger et al., 1995, 1999a). Similarly, there is a gene cluster encoding three cecropin genes in *Hyalophora cecropia* (Gudmundsson et al., 1991), a compact gene cluster for three cecropin genes in *D. melanogaster* (Kylsten et al., 1990) and it is conceivable that the same situation occurs in *R. prolixus* defensins as Southern analysis suggests there are three defensin genes in *R. prolixus*. We will be able to resolve these questions by obtaining full length genomic clones through screening a genomic library.

Multiple sequence alignment (Fig. 2) of mature defensin peptide amino acid sequences demonstrates a high level of conservation within closely related phylogenetic groups. Because defensin is such an ancient peptide found in a wide array of organisms, we would expect that peptides from more closely-related organisms would be most similar. However, as we encounter novel peptides, the similarities exhibited may reflect diet, environment, or previous pathogen exposure as opposed to taxonomic associations.

Transcription of defensin in *R. prolixus* was determined 0–24 h after inoculation with bacteria. Northern analyses were carried out using a probe of the entire 282 bp coding region of our clone. RpDEF-A is contained within a 463 bp message that is detectable 8 h after inoculation, and increases in strength by 24 h. Using primers designed against *R. prolixus* defensin sequences we evaluated, using Q-PCR, transcript levels in whole bodies and specific tissues of naive or immune activated nymphs at various times after immune challenge (Fig. 5). We can detect defensin transcripts at very low levels in naïve insects as was demonstrated in mosquitoes (Lowenberger et al., 1999a). However there is a significant increase due to immune activation. Immune activation not only increases transcription significantly in the fat body as we would expect, but also increases defensin transcription 4 fold in the midgut/intestine 24 h after activation, whereas there is little increase in midguts/intestine 6h after stimulation suggesting that indeed the midgut/intestine is an immune responsive tissue. There is not the speed of transcription nor the same quantity of message in the midgut/intestine that we see in the fat body. However, in these insects bacteria were injected into the hemocoel, and we would expect an extremely rapid and strong response in this important immune responsive tissue. These data suggest that there may be a systemic factor in the *R. prolixus* immune response in which tissues not stimulated directly by the inoculation (intestine) have a delayed transcription of immune peptides. This might be due to a cascade of signaling molecules released in response to the immune activation process. Alternatively, injected bacteria or other products of bacterial degradation may interact directly with the hemocoel side of the midgut/intestine inducing the expression of defensins. Because the transcriptional response in these tissues is less than that of the fat body, and assuming all transcripts are translated, it is possible that parasites in the intestine may be protected from lethal concentrations of these peptides. In addition, high levels of immune peptides in the midgut may be not be advantageous if the obligate bacterial symbionts that reside there are susceptible to the immune peptides.

We may hypothesize that the relegation of *T. cruzi* to its lifecycle in the intestine of *R. prolixus*, and its relatively inefficient mode of transmission, may be an evolutionary consequence of its susceptibility to insect immune peptides. Durvasula et al. (1997) and Beard et al. (2001) demonstrated that the presence of bacterial

symbionts engineered to express cecropin in the midgut lumen of *R. prolixus* had no stages of *T. cruzi*, whereas individuals containing non-engineered symbionts were positive for the parasite. However to test this hypothesis requires that we explore more fully the repertoire of immune molecules in this species, their ability to be produced in the intestine, or to be transported to this region, and their ability to affect the development of the parasite in vivo. The characterization of the defensin described here and the identification of novel immune peptides in vectors such as *R. prolixus* will increase our knowledge of general aspects of insect immunity. In addition, expanding these studies to vectors with different modes of parasite transmission will allow us a greater understanding of the mechanisms used by these insects to protect themselves, and the stresses placed on the parasites within the insect vectors.

Acknowledgements

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