Two novel non-cationic defensin-like antimicrobial peptides from haemolymph of the female tick, *Amblyomma hebraeum*

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Two non-cationic defensin-like antimicrobial peptides, named *Amblyomma* defensin peptide 1 and *Amblyomma* defensin peptide 2, were identified from the hard tick, *Amblyomma hebraeum*, by a combination of suppression subtractive hybridization for differentially expressed genes and proteomics. cDNA clones encoding each of these two defensin-like antimicrobial peptides were isolated from the differentially expressed cDNA library of the tick synganglia (central nervous system). The preproteins deduced from the cDNA sequences each have 92 amino acid residues. *Amblyomma* defensin peptide 2 was purified from the haemolymph of fed female ticks. The purified peptide displayed antibacterial activity against Gram-negative and Gram-positive bacteria. *Amblyomma* defensin peptide 1 was further identified by protein chip capture combined with SELDI-TOF (surface-enhanced laser desorption/ionization–time-of-flight) MS. By

screening for differentially expressed proteins, it was found that the expression of *Amblyomma* defensin peptide 1 was upregulated during 4 days post-feeding. Our findings firstly provide two defensin-like antimicrobial peptides that are particularly novel in being anionic, together with corresponding cDNA sequences, in hard ticks, and prove that the combination of suppression subtractive hybridization and protein profiling is a powerful method to study differentially expressed proteins, especially for organisms without available genome sequence information.

Key words: anionic antibacterial peptide, defensin, protein biochip, suppression subtractive hybridization (SSH), surface-enhanced laser desorption/ionization–time-of-flight (SELDI-TOF), tick.

INTRODUCTION

A large number of antimicrobial peptides has been found in insects, amphibians and mammals. Antimicrobial peptides are widely distributed in Nature and represent an ancient mechanism of host defence. Most of the antimicrobial peptides are cationic although some anionic peptides have been reported [1]. Among these naturally occurring antibiotic peptides, defensins form a unique family of cysteine-rich cationic and structured polypeptides with three or four disulphide bridges [2]. Defensins have been isolated from mammals, insects and plants and they serve as effector molecules of innate immunity, providing an efficient initial defence against infectious pathogens [3,4]. Many defensin-like antimicrobial peptides have been identified from scorpions [5,6] and mussels [7,8]. All known defensins are cationic peptides.

Ticks are important vectors of a wide variety of disease-causing bacteria, viruses, protozoa and other pathogenic organisms. Despite the importance of ticks as vectors of disease, very little is known of their immune system. Reports indicate that ticks have the ability to control infections when challenged with various bacteria [9–12]. A family of naturally occurring antibiotic peptides, defensin-like peptides, serve an important role in innate immunity, and has been identified in one species of soft tick and two species of hard tick [13–15], although there are around 1000 species of tick worldwide. In this study, we report the characterization of two novel anionic defensin-like peptides from the fed female tick, *Amblyomma hebraeum*.

EXPERIMENTAL

Animals

Engorged adult female hard ticks (*A. hebraeum* Koch; Ixodidae), reared in the laboratory according to the method of Kaufman and Phillips [16], as used in [17], weighing 1 g or more, were allowed to detach spontaneously from the host, generally 8–10 days after attachment, and were then maintained at 26 °C and > 90 % humidity.

SMART™ cDNA synthesis

TRIzol (Life Technologies) was used to extract total RNA from synganglia dissected from fed or unfed female ticks. A SMART™ PCR cDNA synthesis kit (Clontech) was used to synthesize cDNA, using for the first-strand cDNA the 3' SMART CDS Primer II A, 5'-AAGCAGTGGTATCAACGCAGAGTACT₃₀ VN-3', and the SMART II A oligonucleotide, 5'-AAGCAGTGGT-ATCAACGCAGAGTACGCGGG-3'. The 5' PCR primer II A, 5'-AAGCAGTGGTATCAACGCAGAGT-3', was used for second-strand synthesis by amplification with Advantage™ polymerase (Clontech).

PCR-select™ cDNA subtractive hybridization and cDNA library construction for differentially expressed genes

Two populations of synganglia mRNA from fed and unfed female ticks were compared by subtractive hybridization using a Clontech

Abbreviations used: SELDI, surface-enhanced laser desorption/ionization; MALDI-TOF, matrix-assisted laser desorption/ionization–time-of-flight; RP-HPLC, reversed-phase HPLC; LB, Luria–Bertani.

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PCR Select™ cDNA Subtraction Kit to obtain clones of genes that are differentially expressed in these two populations. Tester cDNAs containing specific (differentially expressed) transcripts and driver cDNAs [18] were prepared by digesting double-stranded cDNAs with *RsaI*. Subsequently, only the tester cDNA was ligated to the adaptor provided. Adaptor-ligated tester cDNA was hybridized twice with driver cDNA, followed by selective amplification of differentially expressed cDNAs in a two-step PCR using Advantage cDNA polymerase mix according to the manufacturer's instructions. The PCR products were then cloned into pGEM®-T Easy vector (Promega). The two independent libraries constructed were a forward-subtracted library (using cDNA from fed tick synganglia as the tester and cDNA from unfed tick synganglia as the driver) and a reverse-subtracted library (using cDNA from unfed tick synganglia as the tester and cDNA from fed tick synganglia as the driver).

Differential screening of the subtracted cDNA library

Colony PCR was carried out on individual white colonies from forward- and reverse-subtracted libraries, picked from X-gal/isopropyl β -D-thiogalactoside LB (Luria-Bertani)/ampicillin plates, using the following parameters: 1 cycle at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 3 min, and 1 cycle at 72 °C for 15 min, using M13 forward and reverse primers (5'-GTAAACGACGGCCAG-3' and 5'-CAGGAAACAGCTATGAC-3', respectively). Four identical blots were created by spotting NaOH-denatured PCR products on to Electran® nylon membranes (Merck) which were denatured by soaking in 0.6 M NaOH, neutralized using 0.5 M Tris/HCl buffer, pH 7.5, washed with water, and cross-linked by UV. They were then hybridized sequentially with [α -³²P]dCTP-labelled cDNA probes generated from mRNA samples extracted from fed or unfed tick synganglia using a Ready-To-Go™ DNA-labelling kit (without dCTP; Amersham Biosciences). ULTRAhyb™ (Ambion) was used to carry out prehybridization and hybridization as recommended by the manufacturer and the blots were washed at low stringency [$2 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1 % SDS at room temperature]. Labelled spots were visualized by autoradiography and differentially hybridizing clones were selected for further study, which included sequencing the inserts using an Applied Biosystems 3700 capillary DNA sequencer.

Haemolymph collection

A small hole was cut at the base of the forelegs of engorged female ticks 4 days after detachment and, by applying gentle pressure on the tick's body, the clear haemolymph was drawn into a plastic micropipette and diluted 1:2 in ice-cold 0.2 M acetic acid. The sample was then centrifuged at 10 000 *g* for 10 min and the supernatant retained and stored at -80 °C until required.

Protein purification

Haemolymph sample collected from fed female ticks, treated with acetic acid, was filtered through a 10 kDa-cut-off Centriprep filter (Millipore, Bedford, MA, U.S.A.) and the filtrate was concentrated using a Centriprep filter (3 kDa-cut-off). Concentrated filtrate was applied to a 5 mm $\times$ 250 mm Vydac C₁₈ RP-HPLC (reversed-phase HPLC) column (Sigma) equilibrated with 0.1 % (v/v) trifluoroacetic acid/water. Elution (0.7 ml/min) was performed using 0.1 % (v/v) trifluoroacetic acid/water over 6 min, followed by a linear gradient of 10–60 % acetonitrile containing 0.1 % (v/v) trifluoroacetic acid in 0.1 % (v/v) trifluoroacetic

acid/water over 50 min, and final elution with 100 % acetonitrile containing 0.1 % (v/v) trifluoroacetic acid. UV-absorbing peaks were collected, concentrated to a small volume by vacuum centrifugation, and assayed for antimicrobial activity. The fraction with antimicrobial activities was collected and purified further by C₁₈ RP-HPLC under the same conditions and concentrated as before.

Protein sequencing and MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight) MS

Sequence analysis of the purified peptide by the automated Edman degradation method was carried out using an Applied Biosystems pulsed liquid-phase sequencer, model ABI 491. A MALDI-TOF instrument (Bruker Reflex) was used for mass measurements. The matrix solution consisted of α -cyano-4-hydroxycinnamic acid (Ciphergen Biosystems) suspended in 100 % acetonitrile.

Antimicrobial assays

The Gram-positive bacterium *Staphylococcus aureus* (Oxford), the Gram-negative bacterium *Escherichia coli* (OP 50) and the fungi strains *Candida albicans* SC 5314 and *Candida glabrato* ATCC 2001 were used in antimicrobial assays. Bacteria were grown to a D_{600} of 0.8 in LB broth from which a 10 μ l aliquot was added to 8 ml of fresh LB broth containing 0.7 % agar and poured over a 90 mm Petri dish containing 25 ml of 1.5 % agar in LB broth and left to set. A 20 μ l aliquot of the test sample, which had been filtered through a 0.22 μ m Millipore filter, was dropped on to the surface of the top agar, completely dried and then incubated overnight at 37 °C. Antimicrobial activity was observed as a clear zone formed on the surface of the top agar due to inhibition of bacterial growth. The minimal inhibitory concentration was determined in liquid LB medium by incubating the bacteria in 96-well plates with LB broth and variable amounts of the sample under test. For antifungal activity measurements, fungi were cultured with variable amounts of the test sample in yeast extract/peptone/dextrose broth. Three independent experiments were performed for each sample.

ProteinChip® Array: SELDI (surface-enhanced laser desorption/ionization) analysis of tick haemolymph

Lyophilized haemolymph samples were reconstituted in 5 μ l of 50 mM Tris/HCl buffer containing 9 M urea and 1 % CHAPS (pH 8) by vortexing. Samples were then diluted 1:10 in 50 mM Tris/HCl buffer (pH 8) and 10 μ l aliquots were incubated on spots of a weak anionic CM10 ProteinChip® Array previously equilibrated in 50 mM ammonium acetate buffer (pH 4.5; 5 μ l incubation for 5 min). Following a 30 min incubation at ambient temperature and high humidity, the sample droplet was removed and each spot subsequently washed twice with 50 mM Tris/HCl buffer (pH 8; 10 μ l each wash). The spots were finally washed once with 5 μ l of 5 mM Hepes buffer (pH 7.2) and allowed to air dry. Two 0.5 μ l applications of α -cyano-4-hydroxycinnamic acid (20 % saturated in 50 % acetonitrile/0.1 % trifluoroacetic acid; Sigma) were added to each spot. After drying, the ProteinChip® Array was inserted into the ProteinChip® Reader (PBSIIc; Ciphergen Biosystems) and analysed according to an automated data-collection protocol. The ProteinChip® Reader, a linear time-of-flight mass spectrometer equipped with a 337 nm nitrogen laser, was operated in positive-ion mode. The total accelerating potential was +20 kV and the extraction delay time was set to 528 ns. Some 65 transients were averaged over 66 % of the target area in a linear sweep to generate each spectrum.

(A)

ADP-1 ¹MATVRNSRPEAAGEPSGVSSTEGDWRHIEKRDVSYQGE⁴³GNTRR⁴³
 ADP-2 ¹...Q·REISWTF·PLYTWRT·K·YGT¹T·...TTNATSTSSKPS·...⁴³

(B)

<i>A. hebraeum</i> defensin peptide 1	FDNPF ¹ GCPA ² DEGKCFDHCNNKA-YDIGYCGGSYRATCV ³ CYRK
<i>A. hebraeum</i> defensin peptide 2	YENPY...TDEGK·FDR·NDSE-FEG...G·SYRAT·V...T
<i>B. microplus</i> defensin	GF...FNQGA·HRH·RSIR-RRG...A·LIKQT·T...N
<i>D. variabilis</i> defensin	GF...LNQGA·HNH·RSIR-RRG...S·LIKQT·T...N
<i>O. moubata</i> defensin	GY...FNQYQ·HSH·SGIRGYKG...K·TFKQT·K...
<i>M. galloprovincialis</i> defensin	GF...NN-YQ·HRH·KSIPGRCG...G·WHRLR·T...C

Figure 1 The amino acid sequences of *Amblyomma* defensin peptide 1 (ADP1) and 2 (ADP2)

(A) Comparison of signal peptide sequences in prepro-ADP1 and prepro-ADP2. The deduced amino acid sequences are shown. (B) Sequence comparison of mature *Amblyomma* defensin peptides with other defensin-like peptides. The sources of sequences are: predicted defensin from *Boophilus microplus* (accession no. AA048943), predicted defensin from *Dermacentor variabilis* (AA024323), defensin from *Ornithodoros moubata* [13] and defensin MGD 1 from *Mytilus galloprovincialis* (mussels; [8]). Gaps (–) have been introduced to optimize the sequence homology. Dots indicate identical amino acid residues.

RESULTS

Suppression subtractive hybridization

Using synganglia from fed and unfed female *A. hebraeum* ticks as the experimental material, we constructed two cDNA libraries for differentially expressed genes. From the libraries constructed from forward-subtracted hybridization (using cDNA from fed tick synganglia as the tester and cDNA from unfed tick synganglia as the driver) and reverse-subtracted hybridization (using cDNA from unfed tick synganglia as the tester and cDNA from fed tick synganglia as the driver), we obtained 1300 and 1200 transformant colonies, respectively. The plasmid inserts from these white colonies were amplified and arrayed on to a nylon membrane. Some 150 clones containing inserts encoding differentially expressed genes were obtained by differential screening of the subtracted cDNA libraries using [³²P]dCTP-labelled cDNA probes generated from total RNA extracted from synganglia of fed or unfed female ticks.

Molecular characterization of *Amblyomma* defensins

Sequencing of these differentially expressed cDNAs from the forward subtracted library revealed that two of the clones (GenBank accession numbers are AY437137 and AY437138, respectively) contained an insert cDNA fragment encoding a small predicted protein displaying some identities with defensin from other ticks (Figure 1). Conceptual translation of the cDNA sequence revealed that these two preproproteins consist of 84 amino acid residues with predicted processing sites (–Arg⁴²–Arg⁴³–) for signal peptides (Figure 1), which are conserved in insect defensins [19]. The predicted mature peptides, composed of 41 amino acid residues, from the preproproteins were named as *Amblyomma* defensin 1 and *Amblyomma* defensin 2 (Figure 1B); analysis using the ExPASy MW/pI tool (http://www.expasy.ch/tools/pi_tool.html) showed that they have predicted pI values of 6.71 and 4.44, respectively, because there are multiple acidic amino acid residues in their primary sequences, specifically seven acidic amino acid residues and only four basic amino acid residues for *Amblyomma* defensin 2. All the non-cationic amino acid residues are clustered near the N-terminus of these two

Amblyomma defensins. This is the first report of defensin-like peptides having predicted acidic pI values. The predicted signal peptide sequences (Figure 1A) of these two defensins are variable (23 % identity), but their mature sequences (Figure 1B) are quite conserved (70 % identity), and the DNA sequences of their 3'-untranslated regions are as variable as the sequences encoding the signal peptides. The predicted mature *Amblyomma* defensins have the same cysteine motif as other defensins from soft and hard ticks (Figure 1B). They have an extension composed of three amino acids at their N-terminus (Figure 1B).

Partial amino acid sequencing and molecular mass

Haemolymph collected from fed female ticks was filtered using a 10 kDa-cut-off filter and the filtrate was purified by RP-HPLC. A fraction containing antimicrobial activity was eluted at 40 % acetonitrile in 0.1 % trifluoroacetic acid and purified further by RP-HPLC on the same system (Figure 2). This fraction was collected and concentrated for protein sequencing. The N-terminal partial amino acid sequence obtained was Tyr-Glu-Asn-Pro-Tyr-Gly-Xaa-Pro-Ala⁹, which matches perfectly with the amino acid sequence of *Amblyomma* defensin 2 deduced from the cDNA sequence. It suggested that the protein occurs in the haemolymph from fed female ticks although its cDNA was isolated from the synganglia of fed female ticks. *Amblyomma* defensin 1 was not purified from the haemolymph in our experiments. The MALDI-TOF analysis of *Amblyomma* defensin 2 gave a mass of 4612.5 Da that matches well with the predicted molecular mass from the cDNA sequence after allowing for the occurrence of three disulphide bonds (4612.0 Da).

Antimicrobial assays

The antimicrobial assays were performed according to the method described in the Experimental section. The purified *Amblyomma* defensin 2 exerted its antimicrobial activity against *E. coli* and *S. aureus* with minimal inhibitory concentrations of 30 and 7.5 µM, respectively. When tested against *C. albicans* SC 5314 and *C. glabrato* ATCC 2001, no antifungal activity was found in our experiments.

Figure 2 RP-HPLC purification of *Amblyomma* defensin 2

Acetic acid-treated haemolymph from fed female ticks was filtered using a 10 kDa-cut-off Centrprep filter, and the filtrate was applied to a Vydac C₁₈ RP-HPLC column equilibrated with 0.1 % (v/v) trifluoroacetic acid/water. Elution (0.7 ml/min) was performed according to the procedure described in the Experimental section. UV-absorbing peaks were collected. The fraction with antimicrobial activities marked by a bar (A) was collected and purified further by C₁₈ RP-HPLC (B).

Figure 3 SELDI spectra of haemolymph samples bound to CM10 ProteinChip® Array

A peak of ≈ 4585.1 Da was present in the haemolymph sample from day 4 post-engorgement female ticks but absent in the haemolymph sample from day 0 (engorged) female ticks.

SELDI profiling of haemolymph to study differential expression of defensin

The haemolymph samples from female ticks (days 0 and 4 after detachment) were assayed with CM10 ProteinChip® Arrays. The ProteinChip® software provided by the manufacturer was used to identify peaks present in different relative amounts in these haemolymph samples. Among 35 clusters differentially present, one peak had a mass of 4585.1 ± 2 Da (Figure 3) that matched well with the molecular mass (4584.1 Da) predicted from the

cDNA sequence encoding *Amblyomma* defensin 1 and allowing for the occurrence of three disulphide bonds. The results from SELDI profiling are consistent with its upregulation during the 4 days post-engorgement, as revealed by differential screening during feeding using [³²P]dCTP-labelled cDNA probes. In our experiments, no peak corresponding to *Amblyomma* defensin 2 was found.

DISCUSSION

Two genes encoding defensin-like peptides, one of which has a predicted very acidic pI value, were identified from the hard tick, *A. hebraeum*. One of the peptides was purified from the haemolymph of female ticks. Compared with other defensin-like peptides, *Amblyomma* defensin peptide 2 has a predicted net negative charge, a feature that is unique compared with all other known defensin-like peptides, which are positively charged. In addition, they have a novel N-terminal extension making them different from all other known defensin-like peptides, which have a conserved N-terminal glycine. In humans, insects and plants, defensins contribute significantly to host defence against invasion by micro-organisms [20]. Defensins are described as cysteine-rich cationic antimicrobial polypeptides in all of the currently available literature. The antibacterial activity of defensins is generally ascribed to their effects on microbial membranes, although the actual antibacterial mechanism is not clear [20]. Positively charged defensin-like peptides interact with negatively charged components of microbial membranes that include lipopolysaccharide in Gram-negative bacteria, polysaccharides (teichoic acid) in Gram-positive bacteria and phospholipids (phosphatidylglycerol) [20,21]. According to the antibacterial mechanism of action of defensin, it is difficult to explain the interaction between *Amblyomma* defensin 2 and bacteria, because this defensin contains a net negative charge. However, a recent study by Romestand et al. [22], suggests that the nonapeptide corresponding to residues 25–33 of MGD 1 (mussel defensin; CGGWHLRC) exhibited

bacteriostatic activity once it was cyclized, and the bacteriostatic activity was proportional to the theoretical isoelectric point of the peptide, as deduced from the variation of activity in a set of peptide analogues of the 25–33 sequence with different numbers of cationic charges. The loop was suggested to have functional significance in the docking of MGD 1 to the bacterial membrane [22]. The loop formed by the 25–33 sequence of MGD 1 is characterized by the presence of both positively charged and hydrophobic amino acids residues. Sequence comparisons in the family of arthropod defensins including *Amblyomma* defensins in this report indicated that the presence of a positively charged and hydrophobic loop, like the sequence of residues 25–33 of MGD 1, is a conserved property. In the case of *Amblyomma* anionic defensins, perhaps they can dock to the bacterial membrane via the positively charged and hydrophobic loop near their C-terminus, even though they have a net negative charge. Another possibility is that *Amblyomma* defensins exert their antibacterial action by an alternative mechanism, such as stimulation of autolytic enzymes, interference with bacterial DNA and/or protein synthesis, inhibition of DNA synthesis leading to filamentation, or binding to and inhibition of cellular nucleic acids, as suggested by Wu et al. [23]. Nevertheless, the finding of a non-cationic defensin-like peptide in the hard tick, *A. hebraeum*, not only increases the complexity of the antimicrobial mechanism of action of defensins, but also increases the opportunity to understand the antimicrobial mechanism.

Upregulation of defensin gene expression in response to blood feeding has been observed in the blood-sucking insect *Stomoxys calcitrans* (by Northern-blot analysis) [24] and in the soft tick *Ornithodoros moubata* (by reverse transcriptase-PCR analysis) [13,19], although it was commonly believed that the gene expression in these species is only induced upon septic injury and infection [20]. The cDNAs encoding *Amblyomma* defensins are from a forward-subtracted cDNA library and their upregulation during feeding has been further confirmed by hybridization screening. Furthermore, our results using SELDI-TOF (time-of-flight) MS directly showed that there was a higher concentration of *Amblyomma* defensin peptide 1 in the haemolymph of day 4 detached female ticks than day 0 detached (i.e. newly engorged) female ticks (Figure 3). This demonstrates that the concentration of *Amblyomma* defensin peptide 1 apparently continues to increase after the end of the actual feeding period, although SELDI MS is not designed for accurate quantification. The reason why *Amblyomma* defensin peptide 2 could not be detected in the haemolymph samples is obscure. A regulatory pathway triggered by blood feeding appears to be involved in defensin gene expression, although the pathway is not yet clear. Further work is necessary to identify the regulatory pathway involved and to understand the mechanism of immunity in this important disease vector in order to develop a better method of tick control.

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