Cecropin P1 and novel nematode cecropins: a bacteria-inducible antimicrobial peptide family in the nematode *Ascaris suum*

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Cecropin P1 was first identified as a mammalian antimicrobial peptide isolated from the pig intestine. Much research aimed at characterizing this peptide has been reported. Recently, the workers who discovered the peptide corrected their original conclusion, and confirmed that this peptide originates in fact from the pig intestinal parasitic nematode, *Ascaris suum*. In the present study, we carried out a semi-exhaustive search for bacteria-inducible transcripts in *A. suum* by the cDNA subtraction method. The transcripts encoding cecropin P1 and novel *Ascaris* cecropins, designated cecropins P2, P3 and P4, were found to be positively induced factors. Chemically synthesized *Ascaris* cecropins were bactericidal against a wide range of microbes, i.e. Gram-positive (*Staphylococcus aureus*, *Bacillus subtilis* and *Micrococcus luteus*) and Gram-negative (*Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens* and *Escherichia coli*) bacteria, and were weakly but detectably active against yeasts (*Saccharomyces cerevisiae* and *Candida albicans*). Cecropin P1-like sequences were also detected at least in two other species (*Ascaris lumbricoides* and *Toxocara canis*) of the *Ascarididae*. All *Ascaris* cecropin precursors contain an acidic pro-region connected by a tetrabasic cleavage site at the C-terminus. Such an acidic pro-region is also reported to be present in the tunicate cecropin-type antimicrobial peptide styelin. On the basis of the evolutionary position of nematodes and tunicates, the ancestral cecropin may have contained the acidic pro-region at the C-terminus.

Key words: *Caenorhabditis elegans*, cecropin, innate immunity, molecular evolution, nematode, 5′ rapid amplification of cDNA ends (RACE).

INTRODUCTION

Not only vertebrates, but all multicellular organisms, are believed to be equipped with immune defence against microbes. In invertebrates, primitive 'innate' immunity is commonly found. Innate immunity is non-adaptive immunity with extensive recognition of microbes. Although vertebrates, including human beings, possess adaptive immunity with specific non-self recognition based on T- and B-lymphocytes, innate immunity is also present and controls the establishment of adaptive immunity [1]. Such a common distribution allows the use of invertebrate models to research innate immunity. Insects are the most widely used model. In the early stages, large insects such as silk moths and flesh flies were studied in order to explore insect responses against bacterial infection by physiological and biochemical experiments [2,3]. Later, the fruit fly, *Drosophila melanogaster*, was introduced as a genetic model to find the genes involved in innate immunity, based on the knowledge obtained using the bigger insects [4]. Many important genes, such as *toll* and *imd*, have been identified using *D. melanogaster* [4].

Nematodes are novel invertebrate models in the study of innate immunity and host–pathogen interactions [5–8]. The free-living soil nematode, *Caenorhabditis elegans*, has been used in most research. Although *C. elegans* is an excellent genetic model, this nematode is not suitable for some physiological and biochemical experiments, such as microbial injection for immunization, purification of immune-related proteins from the body fluid, etc. As the history of research on insect immunity indicates, large-nematode models that can complement *C. elegans* may be useful. We believe that the pig intestinal parasite, *Ascaris suum*, with a body length of 25–35 cm (adult female), is one such complementary model [9].

Antimicrobial peptides are produced as immune effectors against microbial infection in a wide variety of animals [10]. Previously, we isolated the antimicrobial peptides designated ASABFs (*Ascaris suum* antibacterial factors) [9]. To date, six ASABF-type antimicrobial peptides have been found in *A. suum* [11]. Six homologues have also been identified in *C. elegans* [12,13]. These peptides contain a single α-helix and a pair of β-sheets stabilized by four intramolecular disulphide bridges [14]. ASABF-α is most active against Gram-positive bacteria, especially the common pathogen *Staphylococcus aureus*, and also against Gram-negative bacteria and yeast under optimal conditions [15]. Recently, transcripts encoding ASABF-α, -β, -γ and -δ were found to be positively induced by bacterial injection, suggesting that nematodes can recognize bacterial invasion in the pseudocoelom (body cavity) and actively trigger a humoral defence reaction [11]. To date, only members of the ASABF family have been identified as bacteria-inducible factors in *A. suum*. On the other hand, many proteins (antimicrobial peptides, lysozymes, lectins, etc.) are induced in insects by bacterial injection [4]. In mammals, large numbers of acute-phase proteins (C-reactive protein, serum amyloid A, haptoglobin, etc.) are up-regulated or down-regulated during the acute-phase response [16]. Therefore bacteria-inducible factors other than ASABF-type antimicrobial peptides should also be expected in *A. suum*.

In the present study, we carried out a semi-exhaustive search for bacteria-inducible transcripts in *A. suum* by the cDNA subtraction method. As a result, another type of antimicrobial peptide, cecropin P1 and novel homologues, was identified as a factor that

Abbreviations used: ASABF, *Ascaris suum* antibacterial factor; EST, expressed sequence tag; MBC, minimum bactericidal concentration; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription–PCR.

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The nucleotide sequences reported have been submitted to the DDBJ, GenBank®, EMBL and GSBD Nucleotide Sequence Databases under accession numbers AB186032–AB186039 (eight entries).

is positively induced. Cecropin P1 was first identified as an α-helical mammalian antimicrobial peptide isolated from the pig intestine [17]. Recently, the workers who discovered the peptide corrected their original conclusion, and confirmed that this peptide originates in fact from *A. suum* [18]. Here we describe the precursor structure, gene organization, bacterial inducibility and antimicrobial activity of members of the *Ascaris* cecropin family. Cecropin-type antimicrobial peptides have also been found in insects [19,20] and tunicates [21]. We examine their phylogenetic relationship by comparison of the precursor structure revealed in this work.

EXPERIMENTAL

PCR primers

The PCR primers used in the present study were as follows: cecP1-t1-1s, 5′-GTCGCAGAGCAGGACGCGATTCA-3′; cecP1-t1-1sR, 5′-TGAATCGCGTCCTGCTCTGCGAC-3′; cecP1-t1-2s, 5′-TCTCGTGTTAGCCGAGAAGTGCC-3′; cecP1-t1-2sR, 5′-GGCACTTCTCGGCTAACACGAGA-3′; cecP1-t1-3s, 5′-TACAAGGCGGTCCGCGCCGACGT-3′; cecP1-t1-4sR, 5′-CAAAAGCAATCAAACTGAGTCGG-3′; t1flf, 5′-ATCGTTATTTCATTTGTTAAGTTC-3′; t1flr, 5′-CGTTAATATCACCAGATTGCACAA-3′; cecP1-t2-1s, 5′-TGGTGCAGCAGGACACGATTTCT-3′; cecP1-t2-1sR, 5′-AGAAATCGTGTCCTGCTGCACCA-3′; cecP1-t2-2s, 5′-CCACGCCTCGAGGTAGACGAGCG-3′; cecP1-t2-2sR, 5′-CGCTCGTCTACCTCGAGGCGTGG-3′; cecP1-t2-3s, 5′-TCTCAGAAGGCATTGCTATCGCC-3′; cecP1-t2-4sR, 5′-AGATTTGTTCTTGCACTGAGTTC-3′; t2flf, 5′-CATGCTGTGTATGATTTTCATATA-3′; t2flr, 5′-TTTGTTTTTCATTCATCCGATTGC-3′; cecP1-t3-1s, 5′-TTGGAGAGGAGGACGCGATACCT-3′; cecP1-t3-1sR, 5′-AGGTATCGCGTCCTCCTCTCCAA-3′; cecP1-t3-2s, 5′-GAGGTGAATAAATTCTTCTTGCGCA-3′; cecP1-t3-2sR, 5′-TGCGCAAGAAGAATTTATTCACCTC-3′; cecP1-t3-3s, 5′-CGCCATAAAGGGTGGCTCGCGCAG-3′; cecP1-t3-4sR, 5′-AATGTTGTTGTCAGCAACAATTAT-3′; t3flf, 5′-CGTCATGCTTGCTACTCGTCGTGC-3′; t3flr, 5′-TTTATACGTACACGTTAGCACTTG-3′; cecP1-t4-1s, 5′-GCGCACCAGGAAGAAGCATCCC-3′; cecP1-t4-1Sr, 5′-GGGATGCTTCTTCCTGGTGCGC-3′; cecP1-t4-2s, 5′-TACACGTTAAGACGGACGAGCTT-3′; cecP1-t4-2sR, 5′-AAGCTCGTCCGTCTTAACGTGTA-3′; cecP1-t4-3s, 5′-ATCGCTATACTGGGCGGTTTGCGT-3′; cecP1-t4-4sR, 5′-TTGTTCCCGTACTGTATCCGGCG-3′; cecFL, 5′-GGGGATCATTAGTTGCATTATCTCT-3′; cecFLr, 5′-GTGCGCCGTTCATTTACGAGATTGC-3′. In the following, (A, B) means a PCR reaction using primers A and B, and (A, B) × (C, D) means a nested PCR using primers A and B for the first round of PCR and primers C and D for the second round.

Culture and immune challenge of nematodes

Adult female *A. suum* were obtained from Tokyo Shibaura Zohki (Tokyo, Japan). Culture and immune challenge were carried out as described previously [11]. Briefly, the worms were asepticized by incubation for 2 days in Eagle's essential medium containing the antibiotic enrofloxacin. After incubation, heat-killed *Escherichia coli* OP50 or 0.9 % NaCl was injected into the pseudocoelom. The body walls, intestines, uteri and ovaries were collected separately from each individual 4 h after the injection.

cDNA subtraction

Each tissue isolated from bacteria- or saline-injected worms was frozen in liquid nitrogen and ground using a Coolmil (Tokken, Chiba, Japan). Total RNA was isolated using an RNeasy Protect kit (Quiagen). The RNA was reverse-transcribed and non-specifically amplified as double-stranded cDNA using a SMART cDNA synthesis kit (Clontech). cDNA subtraction was performed using a PCR-Select cDNA Subtraction kit (Clontech). In this system, transcripts whose levels were increased or decreased following bacterial injection were expected to be detected as specific PCR products in the forward or reverse subtracted samples respectively. To detect such specific PCR products, each subtracted sample was electrophoresed on a 6 % (w/v) polyacrylamide sequencing gel. The separated PCR products were visualized by silver staining using the Silver Sequence DNA Sequence System (Promega). The specific bands were dissected and re-amplified using the primer set for non-specific amplification described above. The re-amplified DNA fragments were cloned directly into the pT7Blue T-vector (Novagen). The bacterial inducibility of *Ascaris* cecropins was confirmed by virtual Northern blot, as described previously [11]. Briefly, non-specifically amplified cDNAs isolated from bacteria- and saline-injected worms were separated by agarose gel electrophoresis and transferred to a GeneScreen Plus membrane (DuPont). Portions of *Ascaris* cecropin cDNAs were amplified using PCR and used as probes labelled with [^{32}P]dCTP. The primer sets to prepare the probes were (cecP1-t1-3s, cecP1-t1-4sR) for cecropin P1, (cecP1-t2-3s, cecP1-t2-4sR) for cecropin P2, (cecP1-t3-3s, cecP1-t3-4sR) for cecropin P3, and (cecP1-t4-3s, cecP1-t4-4sR) for cecropin P4. The membrane was hybridized with the labelled DNA fragments as probes. CybS (the small subunit of cytochrome b_{558}) was used as a loading control [11].

Non-quantitative RT-PCR (reverse transcription–PCR)

To study the tissue specificity of *Ascaris* cecropin transcripts, RT-PCR was performed. Total RNA isolated from each tissue of bacteria- or saline-injected worms was reverse-transcribed using PowerScript Reverse Transcriptase (Clontech) with random hexamer primers. Cecropins were detected using nested PCR, with primers (cecP1-t1-3s, cecP1-t1-4sR) × (cecP1-t1-1s, cecP1-t1-2sR) for cecropin P1, (cecP1-t2-3s, cecP1-t2-4sR) × (cecP1-t2-1s, cecP1-t2-2sR) for cecropin P2, (cecP1-t3-3s, cecP1-t3-4sR) × (cecP1-t3-1s, cecP1-t3-2sR) for cecropin P3, and (cecP1-t4-3s, cecP1-t4-4sR) × (cecP1-t4-1s, cecP1-t4-2sR) for cecropin P4.

cDNA cloning

A partial sequence of cecropin P4 was detected by cDNA subtraction. However, we could not clone the entire sequence of cecropin P4 cDNA using standard 5′ and 3′ RACE (rapid amplification of cDNA ends). Consequently, a novel method was employed to determine the 5′ and 3′ end sequences simultaneously using a circularized cDNA library (Figure 1). Total RNA isolated from the intestine of a bacteria-injected worm was reverse transcribed with an oligo(dT)$_{30}$ primer containing a BamHI site in its anchor sequence. Oligo(dC) was added at the 3′ end using terminal deoxytransferase (Clontech). Double-stranded cDNA was synthesized using the primer, which can anneal with the oligo(dC) and carries the same anchor sequence (containing a BamHI site) as the oligo(dT)$_{30}$ primer. The double-stranded cDNA was non-specifically amplified as described above. After digestion with BamHI, the amplified cDNA was circularized by self-ligation. The 5′ and 3′ ends of cecropin P4 cDNA were cloned by inverse PCR, i.e. (cecP1-t4-1sR, cecP1-t4-2s). The primers corresponding to the 5′ and 3′ end sequences were used for cloning of the full-length cDNA of cecropin P4 by high-fidelity PCR using the single-stranded cDNA as a template, i.e. (cecFL, cecFLr). In a similar way, cDNAs for cecropins P1, P2 and P3 were cloned, i.e. (cecP1-t1-1sR, cecP1-t1-2s) and (t1flf, t1flr) for cecropin P1,

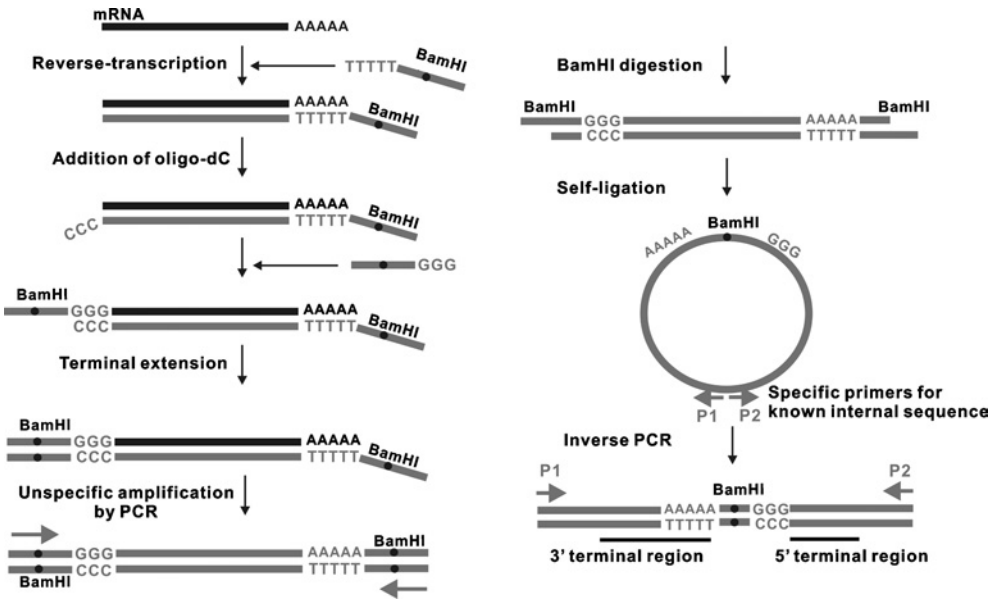

Figure 1 Cloning strategy using circularized cDNA templates

Total RNA isolated from the intestine of *A. suum* was reverse-transcribed with an oligo(dT_{30}) primer containing a BamHI site in its anchor sequence. After the addition of oligo(dC) at the 3′ end of the single-stranded cDNA, double-stranded cDNA was synthesized using the primer containing the same anchor sequence with a BamHI site. The double-stranded cDNA was non-specifically amplified using the anchor sequence as a PCR primer. The amplified cDNA was digested by BamHI and circularized by self-ligation. The 5′ and 3′ ends of *Ascaris* cecropins were cloned by inverse PCR.

(cecP1-t2-1sR, cecP1-t2-2s) and (t2flf, t2flr) for cecropin P2, and (cecP1-t3-1sR, cecP1-t3-2s) and (t3flf, t3flr) for cecropin P3.

Identification of exon–intron junctions of *Ascaris* cecropin genes

The genomic DNA of *A. suum* was prepared as described previously [11]. *Ascaris* cecropin genes were amplified by PCR, i.e. (t1flf, t1flr) for cecropin P1, (t2flf, t2flr) for cecropin P2, (t3flf, t3flr) for cecropin P3, and (cecFL, cecFLr) for cecropin P4. The genomic sequences were compared with the cDNA sequences to determine exon–intron junctions.

Computer-assisted sequence analysis

Alignment and phylogenetic analysis were carried out using ClustalW (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). The theoretical pI was estimated by ExPASy (http://www.expasy.org/tools/pi_tool.html). Blast database searches were performed to identify novel nematode cecropins (http://nema.cap.ed.ac.uk/ncbi_blast.html).

Micro-organisms

Saccharomyces cerevisiae MAFF113011 was obtained from the National Institute of Agrobiological Sciences (Ibaraki, Japan). *Escherichia coli* JM109 was purchased from Takara (Ootsu, Japan). Other strains were transferred from the National Institute of Technology and Evaluation (Kazusa, Japan): *Staphylococcus aureus* IFO12732, *Bacillus subtilis* IFO3134, *Micrococcus luteus* IFO12708, *Pseudomonas aeruginosa* IFO3899, *Salmonella typhimurium* IFO13245, *Serratia marcescens* IFO3736 and *Candida albicans* IFO1060.

Microbicidal assays

Microbicidal assays were carried out as described previously [22]. Briefly, each microbial strain in exponential phase was suspended in 10 mM Tris/HCl, pH 7.5. The microbial suspension was mixed with *Ascaris* cecropins at 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μg/ml (final concentration). Cecropin A (Bachem) derived from the cecropia moth *Hyalophora cecropia* was also subjected to the microbicidal assay as a standard. After a 2 h incubation, the suspension was diluted 100 times and inoculated on to plates of IFO702 medium (1 % polypeptone, 0.2 % yeast extract, 0.1 % $MgSO_4 \cdot 7H_2O$, 2 % agar). The number of colonies was counted, and a (peptide concentration)–(colony number) plot was created. The concentration at which no colonies were expected to be formed was estimated by extrapolation of the curve on to the x axis (colony number $= 0$) on the graph, and was defined as the MBC (minimum bactericidal concentration).

RESULTS

Cloning of *Ascaris* cecropin family members

To screen novel *Ascaris* transcripts that were induced by bacterial injection, cDNA subtraction was performed. The intestines were tested because these tissues, in which the bacterial inducibility of the antimicrobial peptide ASABF-δ was detected, were easy to collect without any contamination by other tissues [11]. cDNA fragments of transcripts that were positively or negatively induced following bacterial injection were identified as selectively amplified PCR products. These PCR products were visualized by silver staining after PAGE. Some positively and negatively induced transcripts were found. The smallest positively induced fragment (275 bp) was re-amplified, and its sequence was determined. A set of specific primers was synthesized based on the obtained sequence. The 5′ and 3′ ends of the cDNA were cloned using circularized cDNAs by inverse PCR (Figure 1). The cDNA sequence was searched for in the EST (expressed sequence tag) database of *A. suum*, and we found ESTs coding for the query cDNA sequence and also for three other homologues. One of the homologues encoded cecropin P1. The three other cecropin P1-like sequences were designated cecropins P2, P3 and P4. The

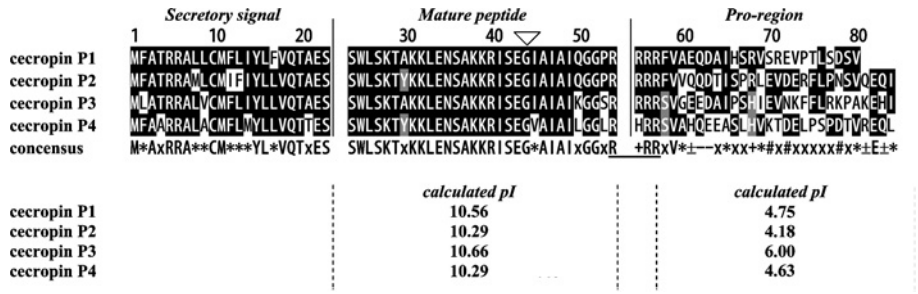

Figure 2 Alignment of *Ascaris* cecropins

Cecropin P1–P4 precursors predicted from cDNA sequences are aligned. The sequence of the mature peptide of cecropin P1 was determined experimentally by Lee et al. [17], and those of cecropins P2–P4 were deduced from comparison with cecropin P1. The common position of the intron is indicated with an inverted triangle. The tetra-basic cleavage site at the mature peptide/pro-region junction is underlined. *, Hydrophobic residue; #, hydrophilic residue; +, basic residue; −, acidic residue; ±, charged residue; x, unconserved residue.

cDNA fragment isolated by cDNA subtraction was completely identical to the 5′ region of cecropin P4 cDNA (results not shown).

The predicted amino acid sequences of the *Ascaris* cecropins were aligned (Figure 2). The mature peptide sequence of cecropin P1 has been reported [17]. Comparison of that cecropin P1 peptide sequence with the sequence predicted from its cDNA suggests that cecropin P1 is produced as a larger precursor containing a secretory signal region at the N-terminus and a C-terminal pro-region that is subsequently removed by processing. A characteristic tetra-basic site, R↓RRR (where ↓ indicates a cleavage site), which conforms to a recognition site for processing proteases, was found at the mature peptide/pro-region junction. Such tetra-basic site, i.e. R↓(R/H)RR, is conserved among cecropins P1–P4. In addition, the junctional region between the secretory signal and the mature peptide, i.e. two residues at the C-terminus of the secretory signal and six residues at the N-terminus of mature peptide (ES↓SWLSLT), is also conserved, suggesting that cecropins P2–P4 may be processed similarly to cecropin P1. The primary structure of the mature peptides, consisting of 31 residues, is highly conserved (27 out of 31 residues conserved; 87 %). Of the four non-conserved residues, amino acid 45 of the precursors is commonly a hydrophobic residue, but no conserved feature was found at the other three positions (residues 30, 50 and 53). The calculated pI of the mature peptides indicated a basic protein in all cases (pI > 10). In contrast, the C-terminal pro-region is more diversified. Only four residues are conserved in the pro-region, which consists of 26 (cecropin P1) or 30 (cecropins P2–P4) residues. However, the calculated pI of the pro-region, excluding the tetra-basic site at the mature peptide/pro-region junction, is acidic in all cecropins (pI = 4.18–6.00).

Phylogenetic relationships

To study the phylogenetic relationships between *Ascaris* cecropins, the exon–intron junctions of the genes were determined. A single intron was identified at a conserved position (phase I at the codon encoding conserved Gly[43]), suggesting that cecropins P1–P4 diversified from a common ancestor (Figure 2).

Next we explored whether *Ascaris* cecropin-like sequences are also found in other nematodes. Sequences similar to the amino acid sequence of the cecropin P1 precursor, excluding the diversified C-terminal pro-region, were searched for in parasite EST databases using the tBLASTn algorithm. We found three similar sequences in *Ascaris lumbricoides* and another three in *Toxocara canis*. *A. lumbricoides*, *T. canis* and *A. suum* are members of the same family (*Ascarididae*). A phylogenetic tree was generated from the amino acid sequences of nematode cecropins predicted from these ESTs (Figure 3). Each *Ascaris* cecropin (P1–P4) clearly exhibits higher similarity to its presumed orthologue in *A. lumbricoides* and/or *T. canis*, suggesting that the genes encoding cecropins P1–P4 duplicated prior to the divergence of these species.

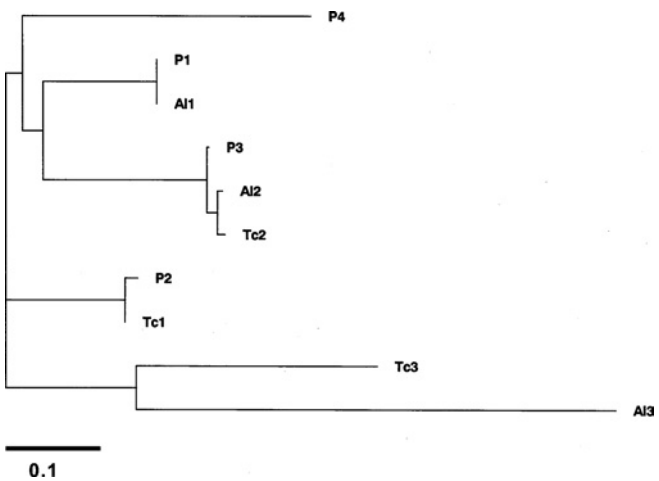

Figure 3 Neighbour-joining phylogenetic tree of nematode cecropins

The amino acid sequences of nematode cecropin precursors were analysed. The scale bar represents one substitution for every 10 amino acids. Al1–Al3 and Tc1–Tc3 are predicted from *A. lumbricoides* and *T. canis* ESTs respectively. Al1 is completely identical to cecropin P1. *A. lumbricoides* and *T. canis* cecropin-like ESTs are registered in the nematode EST database as follows: Al1, ALC00935; Al2, ALC00850; Al3, ALC00385; Tc1, TCC02159; Tc2, TCC02163; Tc3, TCC00820.

We also examined the phylogenetic relationships between *Ascaris* cecropins and other cecropin-type antimicrobial peptides isolated from evolutionarily distant organisms. Cecropins were first found in insects [19]. Zhao et al. [23] reported the presence of cecropin-type antimicrobial peptides, styelins, in tunicates. Styelins contain an acidic C-terminal extension that is not found in insect cecropins [23]. We compared the precursor organization of cecropin P1 with that of styelin D. The organization of both precursors comprises secretory signal, mature region and pro-region. In addition, the length of each portion is similar, i.e. secretory signal, 22 residues for styelin D and 23 for cecropin P1; mature peptide, 32 + Gly for styelin D and 31 for cecropin P1; anionic pro-region, 26 for both styelin D and cecropin P1. Zhao et al. [23] also pointed out that 17 residues at the N-terminus of the mature peptides of styelin C and cecropin P1 are 52.9 % identical or similar. These results suggest that *Ascaris* cecropins may have an origin identical to that of styelins.

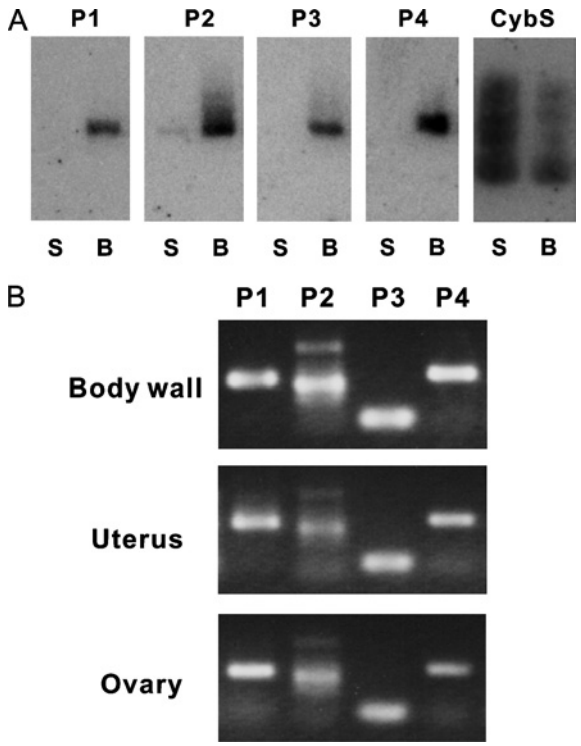

Figure 4 Expression of *Ascaris* cecropin genes

(**A**) Induction by bacterial injection in the intestine. Heat-killed *E. coli* OP50 (B) or saline (0.9 % NaCl) (S) was injected into the pseudocoelom of worms. The accumulation of *Ascaris* cecropin transcripts was assessed by virtual Northern blot. CybS (the small subunit of cytochrome b_{558}) was used as a loading control. (**B**) Tissue specificity. The *Ascaris* cecropin transcripts in the body wall, uterus and ovary were detected using non-quantitative nested RT-PCR.

Induction by bacterial injection

Cecropin P4 was identified by cDNA subtraction as a factor that is positively induced by bacteria in the intestine. We confirmed the inducibility by virtual Northern blot (Figure 4A). Inducibility in other tissues could not be evaluated because cecropin P4 transcripts were not detected by hybridization in these tissues (results not shown). However, nested RT-PCR, a more sensitive but non-quantitative method, detected cecropin P4 transcripts in the body wall, uterus and ovary (Figure 4B). Cecropins P1–P3 were also studied, and results almost identical to those for cecropin P4 were obtained (Figures 4A and 4B). In conclusion, all *Ascaris* cecropins (P1–P4) were detected in all tissues tested (intestine, body wall, uterus and ovary) and were also positively bacteria-inducible, at least in the intestine.

Antimicrobial activity

To test the microbicidal activity of *Ascaris* cecropins, cecropins P1–P4 were chemically synthesized. In addition, commercially available *H. cecropia* cecropin A was used as a standard [19]. The microbicidal activity was estimated as MBC in 10 mM Tris/HCl buffer. The antimicrobial activities of cecropin P1–P4 were similar (Table 1). All *Ascaris* cecropins and *H. cecropia* cecropin A were bactericidal against all Gram-positive and Gram-negative bacteria tested, including some human pathogens. *Ascaris* cecropins and *H. cecropia* cecropin A were also weakly active against yeasts (MBC = 200–300 μg/ml).

DISCUSSION

Cecropin P1 was first reported as a pig intestinal antimicrobial peptide. Subsequently, the origin of cecropin P1 was correctly identified as the intestinal parasite, *A. suum*. Nematode cecropins identified in the present study are definitely of *A. suum* origin. The first evidence for this is that some cecropin P4 transcripts had the 22 nt leader sequence, SL1, at their 5′ termini. Most mature mRNAs of *A. suum* are modified at their 5′ termini by the addition of SL1 by *trans*-splicing [24]. Such modification by SL1 has not been reported in vertebrates. Next, no nematode cecropin-like sequences could be detected in the human genome, the nucleotide sequence of which has been completely determined [25], although three nematode cecropins were found in the human round worm, *A. lambricoides*. In addition, we could not detect nematode cecropins in either the human or pig EST databases. These pieces of evidence suggest that the cecropins were of *A. suum* origin and not were cloning artifacts brought about by contamination with host tissues.

We identified four *Ascaris* cecropins (P1–P4) as antimicrobial peptides that were positively inducible by bacterial injection. Previously we reported that at least four ASABF-type antimicrobial peptides were also positively induced by bacteria in *A. suum* [11]. As seen in several other organisms, many antimicrobial peptides, some of which can be categorized into distinct groups, can be positively induced in nematodes to counteract rapidly evolving pathogens.

Table 1 MBCs of synthetic *Ascaris* cecropins

The indicated microbes were incubated with cecropins in 10 mM Tris/HCl, pH 7.5. After a 2 h incubation, the microbes were inoculated on to plates. Viable microbial cells were estimated by counting colonies, and MBCs were calculated. Cecropin A is an insect cecropin isolated from the cecropia moth, *H. cecropia*.

	MBC (μg/ml)				
Microbe	Cecropin P1	Cecropin P2	Cecropin P3	Cecropin P4	Cecropin A
Gram-positive bacteria					
Staphylococcus aureus IFO12732	2	8	3	3	3
Bacillus subtilis IFO3134	2	20	10	20	15
Micrococcus luteus IFO12708	8	30	8	8	8
Gram-negative bacteria					
Pseudomonas aeruginosa IFO3899	2	20	20	20	10
Salmonella typhimurium IFO13245	6	20	8	8	8
Serratia marcescens IFO3736	20	30	30	50	50
Escherichia coli JM109	6	30	9	20	20
Yeasts					
Saccharomyces cerevisiae MAFF113011	300	300	300	300	300
Candida albicans IFO1060	200	200	200	200	200

A novel procedure using a circularized template was employed to clone the 5′ and 3′ cDNA ends of *Ascaris* cecropins. This method is more useful than standard 5′ and 3′ RACE, since both the 5′ and 3′ends can be determined together. In addition, the use of a set of specific primers without universal anchor primers can facilitate successful PCR. A possible problem is that the novel method does not work if the recognition sites of restriction enzymes used for cDNA circularization also exist in the target sequences to be cloned. This problem can be resolved by the use of two or more template sets generated using distinct restriction enzymes, use of less frequent cutters such as NotI, or performing blunt-end ligation for circularization.

The precursors of *Ascaris* cecropins contain a pro-region at the C-terminus. This acidic pro-region may interact with the basic mature region. A mammalian antimicrobial peptide, α-defensin, also contains an acidic pro-region between the secretory signal and the mature region. The acidic pro-region of α-defensin inhibits the cytotoxic and bactericidal activity of this antimicrobial peptide [26–29]. Such inhibition should be protective to cells producing α-defensin. It follows that insect cecropins with a pro-region show decreased antimicrobial activity [30]. Therefore *in vivo* regulation of the biological activity of nematode cecropins by processing of the pro-region may be important, as seen with other antimicrobial peptides.

The tetra-basic site, R↓(R/H)RR, was conserved at the cleavage site between the mature peptide and and pro-region of *Ascaris* cecropins. Another nematode antimicrobial peptide, ASABF-α, also contains a short pro-region at the C-terminus [9]. A similar basic cleavage site, RG↓RRH, is also found in ASABF-α, suggesting that *Ascaris* cecropins and ASABF-α may be processed in a similar fashion. The processing mechanism may restrict the location of pro-region at the C-terminus.

The C-termini of mature insect cecropins and tunicate styelins are amidated [2,31]. This C-terminal amidation requires Gly at the C-terminus of the precursor [32]. The mature peptide of cecropin P1 has been reported to be not amidated [17]. In accordance with this, a Gly residue was not found at the C-terminus of mature cecropin P1 in the present study. The lack of a Gly residue was also observed for cecropins P2–P4, suggesting that these peptides are also not likely to be amidated.

Although cecropins P1–P4 are suggested to have undergone gene duplication before the divergence of *A. suum*, *A. lambricoides* and *T. canis* within the *Ascarididae*, we have no evidence for the distribution of nematode cecropins in other nematode families. However, it is often hard to detect homologues of antimicrobial peptides, since such peptides are rapidly evolving molecules [13]. In addition, the short length of mature cecropins also makes it difficult to identify their homologues using standard database searches by sequence comparison. Another class of nematode antimicrobial peptides, the ASABFs, have been found not only in *Ascarididae*, but also in the group V nematodes (*Strongylida* and *Rhabditida*, including the model organism *Caenorhabditis elegans*). Thus we cannot exclude the possibility that cecropins are more widely distributed in nematodes. Further isolation of antimicrobial peptides from various nematode families will address this question.

Zhao et al. [23] discussed the phylogenetic relationships of cecropin-type antimicrobial peptides isolated from evolutionarily distant organisms. In 1997, when their paper was published, cecropin P1 was still believed to be of mammalian origin, and the precursor of cecropin P1 had not been identified. Here we re-interpret the phylogeny of cecropins in the light of these findings. To date, cecropin-type antimicrobial peptides have been identified in tunicates (styelin), insects (insect cecropin) and nematodes (cecropins P1–P4). Zhao et al. [23] suggested that styelin C is

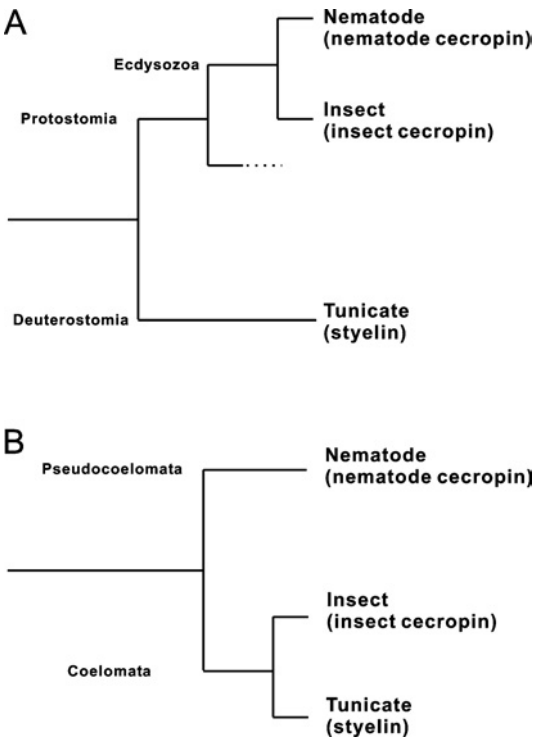

Figure 5 Phylogenetic tree of cecropin-producing organisms

(**A**) Ecdysozoa hypothesis: the moulting animals, including arthropods and nematodes, are proposed to be more closely related to each other than to tunicates. (**B**) Coelomata hypothesis. Tunicates and arthropods have a true coelom, whereas nematodes have a pseudocoelom, suggesting that nematodes are the more primitive. Thus tunicates are more closely related to arthropods according to this hypothesis.

similar to cecropin 1, isolated from the dipteran insect *Drosophila virilis*, since four of the last six residues of their mature peptide domains were identical, and 8/11 (72.7 %) were identical beginning with the last six residues of their signal sequences. However, the major difference was suggested to be the absence of the polyanionic C-terminal extension, which was conserved among styelins from precursors of insect cecropins, including cecropin 1 [23]. In the present study, such an acidic pro-region at the C-terminus was also identified in the cecropins of nematodes. The evolutionary position of nematodes has been the subject of debate. Two scenarios are dominant. One is the 'Ecdysozoa hypothesis', in which nematodes are suggested to be more proximal to insects than to vertebrates (Figure 5A) [33]. Another is the 'Coelomata hypothesis', suggesting that insects and vertebrates are more evolutionarily proximal than nematodes (Figure 5B) [33]. Since tunicates are believed to be most proximal to vertebrates, the evolutionary position of vertebrates substitutes for that of tunicates in Figure 5. In either hypothesis, the evolutionary position of nematodes is proposed to be equally or more distant from vertebrates than that of insects. Thus, if cecropin-type antimicrobial peptides of tunicates, insects and nematodes are derived from a single common ancestor that might have evolved from an early ribosomal protein L1 of an intracellular parasitic prokaryote [34], the ancestral cecropins may have contained the C-terminal acidic pro-region. Insect cecropins may have lost this pro-region during their divergence from the ancestral cecropins. Interestingly, although the C-terminal acidic region is found only in styelins and nematode cecropins, but not in insect cecropins, both mature insect cecropins and styelin D are amidated at the C-terminus, but nematode cecropins are not, as mentioned

above. Tunicate styelins seem to be partly like a mosaic of insect cecropins and nematode cecropins, whereas extensive modification of amino acid residues is unique for styelin D [31]. In our previous paper, we suggested that nematode ASABF-type antimicrobial peptides has a similar origin as the mollusc cysteine-rich antimicrobial peptides, MGD (*Mytilus galloprovincialis* defensin) and myticin, based on precursor organization, spacing of half-cystine residues, and cyteine pairings [13]. The common ancestor of ASABF and these mollusc peptides was probably generated before the divergence of the moulting animals from other protostomes, including molluscs [13]. Immunity provided by these antimicrobial peptides observed in nematodes cannot be an adaptive convergence, but could have been generated in the early stage of animal evolution with having a persisting function in some organisms.

Only limited differences were observed when considering the microbicidal activities of cecropins P1–P4, suggesting that the diversity found at amino acid positions 30, 45, 50 and 53 of the mature region does not affect activity. The positive charges of cationic antimicrobial peptides, including cecropins, facilitate access to the negatively charged microbial membrane [35]. Therefore a net positive charge is often correlated with antimicrobial activity [35]. It is curious that no significant difference was observed in bactericidal activity although Gln^{50} of cecropin P1 is replaced by Lys^{50} in cecropin P3, resulting increase of a positive charge, i.e. +5 in cecropins P1, P2 and P4, and +6 in cecropin P3. When considering analogues of magainin II amide, the α-helical antimicrobial peptide isolated from the skin of the African clawed frog *Xenopus laevis*, although an increase in charge to +5 was accompanied by a corresponding increase in antimicrobial activity, a further increase in charge to +7 did not alter the maximal activity observed [36]. Our observation with cecropins may be similar to that of magainin II analogues. Previous studies have reported that cecropin P1 exhibited little activity against *S. aureus* Cowan I in an inhibition zone assay [17]. We repeated this assay using *S. aureus* IFO12732, and confirmed that this bacterium was barely susceptible to *Ascaris* cecropins using this method (results not shown). In contrast, *S. aureus* IFO12732 was one of the most susceptible microbes in our bactericidal assay in low-ionic-strength buffer without nutrient content. In addition, both insect cecropins and cecropin P1 have been reported to be potent antifungal agents, although only a limited fungicidal effect was observed for *Ascaris* cecropins and cecropin A under our experimental conditions [37,38]. The explanation for the difference in susceptibility when measured using these two methods remains to be elucidated.

Since the first report by Boman's group in 1989, much research on cecropin P1 has accumulated [17], and cecropin P1 is one of best characterized antimicrobial peptides. In the present study, the precursor of cecropin P1 was revealed, and at least nine members of nematode cecropin family, including incompletely sequenced ESTs, were identified. We can hence promote the further investigation of cecropin P1 based on the characterization and comparison of such natural products.

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REFERENCES

1 Beutler, B. (2004) Innate immunity: an overview. Mol. Immunol. **40**, 845–859
2 Boman, H. G., Faye, I., Gudmundsson, G. H., Lee, J.-Y. and Lidholm, D.-A. (1991) Cell-free immunity in *Cecropia*. A model system for antibacterial proteins. Eur. J. Biochem. **201**, 23–31
3 Natori, S., Shiraishi, H., Hori, S. and Kobayashi, A. (1999) The roles of *Sarcophaga* defense molecules in immunity and metamorphosis. Dev. Comp. Immunol. **23**, 317–328
4 Leclerc, V. and Reichhart, J. M. (2004) The immune response of *Drosophila melanogaster*. Immunol. Rev. **198**, 59–71
5 Nicholas, H. R. and Hodgkin, J. (2004) Responses to infection and possible recognition strategies in the innate immune system of *Caenorhabditis elegans*. Mol. Immunol. **41**, 479–493
6 Kurz, C. L. and Tan, M. W. (2004) Regulation of aging and innate immunity in *C. elegans*. Aging Cell **3**, 185–193
7 Millet, A. C. M. and Ewbank, J. J. (2004) Immunity in *Caenorhabditis elegans*. Curr. Opin. Immunol. **16**, 4–9
8 Kim, D. H. and Ausubel, F. M. (2005) Evolutionary perspectives on innate immunity from the study of *Caenorhabditis elegans*. Curr. Opin. Immunol. **17**, 4–10
9 Kato, Y. and Komatsu, S. (1996) ASABF, a novel cysteine-rich antibacterial peptide isolated from the nematode *Ascaris suum*: purification, primary structure, and molecular cloning of cDNA. J. Biol. Chem. **271**, 30493–30498
10 Bulet, P., Stocklin, R. and Menin, L. (2004) Anti-microbial peptides: from invertebrates to vertebrates. Immunol. Rev. **198**, 169–184
11 Pillai, A., Ueno, S., Zhang, H. and Kato, Y. (2004) Induction of ASABF (*Ascaris suum* antibacterial factor)-type antimicrobial peptides by bacterial injection: novel members of ASABF in the nematode *Ascaris suum*. Biochem. J. **371**, 663–668
12 Kato, Y., Aizawa, T., Hoshino, H., Kawano, K., Nitta, K. and Zhang, H. (2002) *abf*-1 and *abf*-2, ASABF-type antimicrobial peptide genes in *Caenorhabditis elegans*. Biochem. J. **361**, 221–230
13 Zhang, H. and Kato, Y. (2003) Common structural properties specifically found in the CS$\alpha\beta$-type antimicrobial peptides in nematodes and mollusks: Evidence for the same evolutionary origin? Dev. Comp. Immunol. **27**, 499–503
14 Aizawa, T., Hoshino, H., Fujitani, N., Koganesawa, N., Matsuura, A., Miyazawa, M., Kato, Y., Kumaki, Y., Demura, M., Nitta, K. and Kawano, K. (2001) Structural analysis of an antibacterial peptide derived from a nematode. In Peptide Science 2000 (Shioiri, T., ed.), pp. 269–272, The Japanese Peptide Society, Nagoya, Japan
15 Zhang, H., Yoshida, S., Aizawa, T., Murakami, R., Suzuki, M., Koganezawa, N., Matsuura, A., Miyazawa, M., Kawano, K., Nitta, K. and Kato, Y. (2000) *In vitro* antimicrobial properties of recombinant ASABF, an antimicrobial peptide isolated from the nematode *Ascaris suum*. Antimicrob. Agents Chemother. **44**, 2701–2705
16 Ceciliani, F., Giordano, A. and Spagnolo, V. (2002) The systemic reaction during inflammation: the acute-phase proteins. Protein Pept. Lett. **9**, 211–223
17 Lee, J.-Y., Boman, A., Sun, C. X., Andersson, M., Jörnvall, H., Mutt, V. and Boman, H. G. (1989) Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. Proc. Natl. Acad. Sci. U.S.A. **86**, 9159–9162
18 Andersson, M., Boman, A. and Boman, H. G. (2003) *Ascaris* nematodes from pig and human make three antibacterial peptides: isolation of cecropin P1 and two ASABF peptides. Cell. Mol. Life Sci. **60**, 599–606
19 Steiner, H., Hultmark, D., Engstrom, A., Bennich, H. and Boman, H. G. (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature (London) **292**, 246–248
20 Boman, H. G. (2003) Antibacterial peptides: basic facts and emerging concepts. J. Intern. Med. **254**, 197–215
21 Lehrer, R. I., Lee, I. H., Menzel, L., Waring, A. and Zhao, C. (2001) Clavanins and styelins, alpha-helical antimicrobial peptides from the hemocytes of *Styela clava*. Adv. Exp. Med. Biol. **484**, 71–76
22 Zhang, H., Morikawa, K., Ohta, T. and Kato, Y. (2005) *In vitro* resistance to the CS$\alpha\beta$-type antimicrobial peptide ASABF-α is conferred by overexpression of sigma factor *sigB* in *Staphylococcus aureus*. J. Antimicrob. Chemother. **55**, 686–691
23 Zhao, C., Liaw, L., Lee, I. H. and Lehrer, R. I. (1997) cDNA cloning of three cecropin-like antimicrobial peptides (Styelins) from the tunicate, *Styela clava*. FEBS Lett. **412**, 144–148
24 Maroney, P. A., Denker, J. A., Darzynkiewicz, E., Laneve, R. and Nilsen, T. W. (1995) Most mRNAs in the nematode *Ascaris lumbricoides* are trans-spliced: a role for spliced leader addition in translational efficiency. RNA **1**, 714–723
25 Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A. et al. (2001) The sequence of the human genome. Science **291**, 1304–1351
26 Michaelson, D., Rayner, J., Couto, M. and Ganz, T. (1992) Cationic defensins arise from charge-neutralized propeptides: mechanism for avoiding leukocyte autocytotoxicity? J. Leukocyte Biol. **51**, 634–639
27 Valore, E. V., Martin, E., Harwig, S. S. L. and Ganz, T. (1996) Intramolecular inhibition of human defensin HNP-1 by its propiece. J. Clin. Invest. **97**, 1624–1629
28 Ayabe, T., Satchell, D. P., Pesendorfer, P., Tanabe, H., Wilson, C. L., Hagen, S. J. and Ouelette, A. J. (2002) Activation of Paneth cell α-defensins in mouse small intestine. J. Biol. Chem. **227**, 5219–5228

29 Satchell, D. P., Sheynis, T., Shirafuji, Y., Kolusheva, S., Ouellette, A. J. and Jelinek, R. (2003) Interaction of mouse Paneth cell α-defensins and α-defensin precursors with membranes: prosegment inhibition of peptide association with biomimetic membranes. J. Biol. Chem. **278**, 13838–13846
30 Boman, H. G., Boman, A., Andreu, D., Li, Z.-Q., Merrifield, R. B., Schlenstedt, G. and Zimmermann, R. (1989) Chemical synthesis and enzymic processing of precursor forms of cecropin A and B. J. Biol. Chem. **264**, 5852–5860
31 Taylor, S. W., Craig, A. G., Fischer, W. H., Park, M. and Lehrer, R. I. (2000) Sytelin D, an extensively modified antimicrobial peptide from ascidian hemocytes. J. Biol. Chem. **275**, 38417–38426
32 Bradbury, A. F. and Smyth, D. G. (1991) Peptide amidation. Trends Biochem. Sci. **16**, 112–115
33 Hedges, S. B. (2002) The origin and evolution of model organisms. Nat. Rev. Genet. **3**, 838–849
34 Pütsep, K., Normark, S. and Boman, H. G. (1999) The origin of cecropins; implications from synthetic peptides derived from ribosomal protein L1. FEBS Lett. **451**, 249–252
35 Powers, J. P. and Hancock, R. E. (2003) The relationship between peptide structure and antibacterial activity. Peptides **24**, 1681–1691
36 Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M. and Bienert, M. (2001) Optimization of the antimicrobial activity of magainin peptides by modification of charge. FEBS Lett. **501**, 146–150
37 Ekengren, S. and Hultmark, D. (1999) *Drosophila* cecropin as an antifungal agent. Insect Biochem. Mol. Biol. **29**, 965–972
38 Andra, J., Berninghausen, O. and Leippe, M. (2001) Cecropins, antibacterial peptides from insects and mammals, are potently fungicidal against *Candida albicans*. Med. Microbiol. Immunol. (Berlin) **189**, 169–173

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