Cloning and expression of three cecropin cDNAs from a mosquito cell line

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Abstract We have characterized full-length cDNAs encoding three isoforms of the antibiotic cecropin secreted by the C7–10 cell line from the mosquito, *Aedes albopictus*. The existence of two cecropin isoforms that differed from the previously described AalCecA was predicted by mass spectrometry and amino acid sequence analysis of peptides that eluted from reversed phase high performance liquid chromatography as a single peak just behind the previously described cecropin, AalCecA. Based on the amino acid sequence of the mature AalCecA peptide, we designed primers that amplified partial cDNAs encoding three different *A. albopictus* cecropins in reverse transcriptase polymerase chain reactions. Rapid amplification of cDNA ends was then used to complete the cDNA sequences of AalCecA, AalCecB and AalCecC, respectively. Each cDNA encoded a translation product containing a signal peptide, a pro region, and a mature cecropin peptide consistent with amino acid sequence data from chymotryptic digests. Although the mosquito cecropins shared 70–86% identity among each other, they shared only ∼40% identity to cecropins from *Drosophila melanogaster*. Each of the cecropins was expressed within 2 to 4 h after induction, and transcripts measuring 0.3 to 0.5 kb continued to accumulate over 24 h. The three cecropins were secreted in roughly equimolar proportions, and 30 to 90% of AalCecB was amidated at the terminal glycine residue. In contrast, amidated forms of AalCecA and AalCecC constituted a smaller proportion of these isoforms.

Key words: Innate immunity; Insect; Vector; Antibiotic; Transcription; cDNA; mRNA; α-Helical peptide

1. Introduction

Cecropins are small (∼4 kDa) α-helical peptides containing 35–39 amino acids, in which a high proportion of basic amino acids at the N-terminus confers a net positive charge, while the C-terminus is rich in hydrophobic residues. Structural analyses indicate that the N- and C-terminal α-helical structures of the well-studied cecropin A from *Hyalophora cecropia* and cecropin B2 from *Bombyx mori* are separated by a gly-pro bend [1]. The insect cecropins show broad spectrum activity against Gram-negative and Gram-positive bacteria, but differ from other antibacterial amphipathic α-helical peptides, such as magainins and dermaseptins from frog skin, in that they do not lyse erythrocytes [1,2].

Despite early evidence for mosquito cecropins based on nucleic acid hybridization [3], it was not until recently that RP-HPLC analysis and peptide sequencing allowed identification of a 35 amino acid cecropin secreted by *Aedes albopictus* C7–10 mosquito cells, which we named AalCecA, for *A. albopictus* cecropin A [4]. It is particularly interesting to note that this mosquito peptide shared only 36% amino acid identity with cecropins from *Drosophila melanogaster* and other Cyclorrhaphid flies, whose mature cecropins share, among themselves, 80% to 100% amino acid identity. Although mosquito cecropins remain to be tested directly, cecropins and their synthetic derivatives from other insects have been reported to show activity against causative agents of malaria [5–7] and Chagas disease, or South American trypanosomiasis [7]. It follows that further analysis of cecropins specifically from mosquitoes and other insect vectors will be of particular interest in the context of efforts to understand, and possibly manipulate, immunity functions in insect vectors to reduce disease transmission. In the present paper, we describe the cDNA encoding AalCecA as well as cDNAs encoding two additional cecropins secreted by the C7–10 *A. albopictus* mosquito cell line.

2. Materials and methods

2.1. Cells and induction conditions

Mosquito cells from the C7–10 *A. albopictus* cell line were maintained in E-5 medium containing 5% fetal bovine serum, as described previously [8]. Cecropin expression was induced by treating cells with heat-killed *Escherichia coli* (∼1000 bacteria/cell) in serum-free medium [9], and activity was purified from the cell culture supernatant by RP-HPLC chromatography, yielding two closely migrating peaks, A and B. As described previously [4], peak A contained a single, non-amidated peptide named AalCecA for *A. albopictus* cecropin A. Details of chromatographic isolation, proteolytic digestion, HPLC peptide mapping, amino acid analysis and protein sequencing have been described previously [4]. High resolution mass spectroscopy was performed on a Bruker Biflex III MALDI-TOF mass spectrometer (Bruker Daltronics) using external calibration and operating in reflection mode.

2.2. Polymerase chain reactions

Polyadenylated RNA was isolated from total RNA (see below) using an Oligotex mRNA Midi kit (QIAGEN, Valencia, CA, USA). Nested primers P1 (5′-GGNGGNCTGAAIAAICTGGG) and P2 (5′-CTGGGNAAIAAICTGGAIGG) were based on the amino acid sequences GGLKKLGK and LGKKKLEG, respectively. Wobble positions were assigned according to codon preferences represented by the mosquito defensin genes [10]. To obtain downstream cDNA sequences, polyadenylated RNA template was used with oligo(dT)$_{18}$ primer and Superscript II reverse transcriptase (GIBCO-BRL, Gaithersberg, MD, USA). For the first round of PCR, first-strand cDNA was heated with primer P1 and oligo(dT)$_{18}$ in a 50 μl reaction volume to 94°C for 1 min, and cycled 40 times through a reaction including

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Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; HPLC, high performance liquid chromatography; RP-HPLC, reversed phase HPLC; RACE, rapid amplification of cDNA ends

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1 min annealing at 55°C, 1 min extension at 72°C, and 1 min denaturation at 94°C. The final step involved a 10 min extension at 72°C. The resulting reaction (1 μl) was used as template for a second PCR using P2 and oligo(dT)$_{18}$ as primers, under the same reaction conditions. PCR products were cloned into p-GEM T-Easy Vector (Promega, Madison, WI, USA) and sequenced with an ABI PRISM automatic sequencing apparatus (Model 377, version 3.1). DNA upstream of the region encoding the mature cecropin peptides was obtained using primer A1 (5′AAACCGGACCATAAATC) to synthesize first-strand cDNA, which was treated with an UltraClean 15 DNA purification kit (MO BIO, Solana Beach, CA, USA) and tailed with poly(dA) using terminal deoxynucleotidyl transferase (Promega). Nested primer A2 (5′-GGCATTGCTGTTACTAACTATC) was used for the second round of PCR cycling, using the reaction conditions described above. Similarly, primers B1 (5′-GAGCATTTTCGAACGACGG) and B2 (5′ATATCCCTTCTTCCCACCC), C1 (5′-CCAAGAGCCT-TTGCTCCAGC) and C2 (5′-AAGACACGTTTGCCGGCTCCC) were used to obtain the first-strand cDNA and upstream sequences of AalCecB and AalCecC, respectively.

2.3. Northern analysis

Guanidine isothiocyanate [11] was used to obtain total RNA from C7–10 cells at times ranging from 0 to 24 h after induction with heat-killed bacteria. Total RNA (10 μg/lane) was electrophoresed on 1.5% agarose gels containing formaldehyde [12] and probed with PCR products from 5′-RACE labeled with Amersham's Multiprimer DNA Labeling System and [α-^{32}P]dATP (3000 Ci/mmol; Amersham) to a final specific activity of 2.5×10^8 cpm/μg. Blots were washed with 0.1×SSC (1×SSC is 150 mM sodium chloride, 15 mM sodium citrate) containing 0.5% SDS for 1 h at 65°C, prehybridized in 5×Denhardt's solution containing 5×SSC, 150 μg/ml denatured herring sperm DNA, 50% formamide, and 0.1% SDS at 42°C overnight, and hybridized in the same solution with labeled probe at 5×10^7 cpm/ml. Blots were washed twice with 2×SSC, 0.1% SDS at 42°C for 20 min each, and twice in 0.1×SSC, 0.1% SDS at 55°C for 20 min each.

3. Results

As described previously [4], peptides secreted into the medium by C7–10 cells after stimulation with heat-killed *E. coli* were recovered by acid precipitation, batch eluted from Sep-Pak C18 cartridges, and separated by reversed phase high performance liquid chromatography (RP-HPLC), which yielded two peaks (A and B) of antibacterial activity that eluted at 35.9 and 36.4 min, respectively. By mass spectrometry, the leading peak (A) was found to contain a single compound. Amino acid composition analysis, Edman degradation and C-terminal diphenyl phosphorylisothiocyanate (DPPITC) degradation yielded the 35 amino acid sequence GGLKKLGKKLEGVGKRVFKASEKALPVAVGIKALG

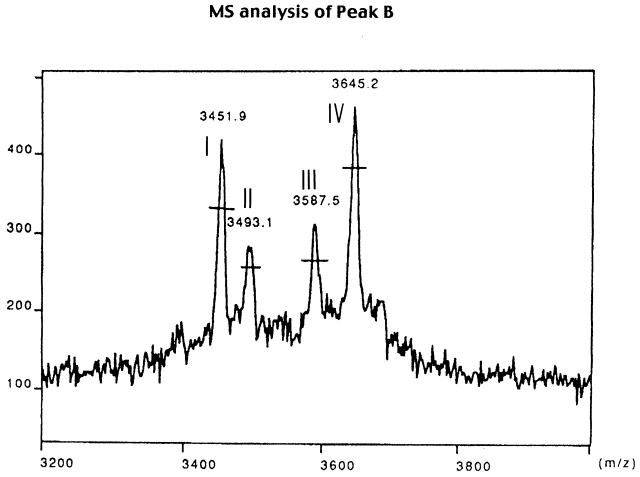

Fig. 1. Mass spectrometry of peptides from peak B. Data are from the plasma desorption time-of-flight method using an Applied Biosystems BioIon 20R operated in linear mode, with collection of between 1×10^6 to 5×10^6 counts, as described previously [4]. Calibration of this instrument on the sodium ion causes peptides in this mass range to read 2 mass units high, ±1 mass unit. The M+1 mass is given; corrected masses are shown in Table 1.

which we named AalCecA, based on its highest identity to CecA from *D. melanogaster* [4]. The C-terminus differed from other cecropins [13] in that it was not amidated, but instead retained a terminal glycine.

3.1. Mass spectrometric and protein chemical characterization of peak B

Although we noted batch-to-batch variability in the overall yield of cecropin from cultured cells, when yield was high, mass spectroscopy of peak B yielded four peaks (Fig. 1) with estimated non-protonated masses of 3448.9±1 (I), 3490.1±1 (II), 3584.5±1 (III) and 3642.2±1 (IV). The mass difference between III and IV is 57.7, quite close to the 58.1 expected for conversion of a terminal glycine to an amide (Table 1). Similarly, the estimated non-protonated mass of AalCecA measured in the same experiment differs from II by 59.7±2. The C-terminal amidation of II and III has been verified by high resolution mass spectroscopy (data not shown). Thus, Fig. 1 implies that our preparations contain as few as three structurally distinct cecropins.

Amino-terminal sequence analysis of peak B (Fig. 2)

AalCecA:	GGLKKLGKKLEGVGKRVFKASEKALPVAVGIKALG	
Peak B:	GGLKKLGKKLEG1GKRVF2A3EKALPV...	
Chymo 1b:	GGLKKL	Amino terminus
Chymo 4:	GGLKKLGKKLEGVGKRVF	Amino terminus 1
Chymo 1c:	EGVGKRVf	Amino terminus 1
Chymo 2b1:	GGLKKLGKKLEGAGKRVF	Amino terminus 2
Chymo 1a:	GKKLEGAGKRVF	Amino terminus 2
Chymo 2a:	NAAEKALPVVAXAKA...	Carboxyl terminus 1
Chymo 3,2b2:	KASEKALPVLTGY	Carboxyl terminus 2
Tryp 3:	ALPVLTGYK	Carboxyl terminus 2
Chymo 4b:	KASEKALPV...	Carboxyl terminus 3

Fig. 2. Alignment of observed sequences from peak B with the known sequence of AalCecA [4]. In the peak B sequence, the underlined values 1, 2 and 3 represent the alternatives A/V (1), K/N (2), and A/S (3), respectively, at positions 13, 19 and 21 (underlined in the alignment). Dots (...) indicate that the sequence terminated from wash-out before reaching the final residue. Peptide sequences of individual peaks resulting from digestion with chymotrypsin (Chymo) or trypsin (Tryp) are shown below the alignment of AalCecA and peak B as amino- and carboxy-terminal alignments respectively. In the amino-terminal alignment for Chymo 1c, residue f (lower case) is a presumptive F which was not directly observed. In the carboxy-terminal alignment, underlined residues represent positions 19, 21, and the amino acids immediately preceding the C-terminus. For Chymo 2a, X represents an unidentified residue.

showed a sequence nearly identical to that derived for AalCecA [4] for the 27 cycles before sample wash-out. The only exceptions were at cycle 13, which gave a 2:1 ratio of valine and alanine, at cycle 19, which gave a 2:1 ratio of lysine and asparagine and at cycle 21, which gave a mixture of alanine and serine. To resolve these alternatives, we followed the same strategy used in the structural elucidation of AalCecA, viz. chymotryptic digestion followed by reversed phase HPLC peptide mapping [4]. All major chymotryptic fragments were sequenced with the exception of the wash-through peak, which was heavily contaminated with the urea and buffer used for the digestion. An alignment of all the amino-terminal data suggested that peak B contained at least one cecropin isoform that differed from that in peak A, by the replacement of the valine in AalCecA with an alanine at position 13. The phenylalanine site for chymotrypsin cleavage at residue 18 was present in all of the peak B cecropins.

Alignment of C-terminal sequence data (Fig. 2) showed evidence for additional cecropin isoforms with the expected variability at positions 19 and 21, and additional variability in the C-terminal peptide. Carboxyl-terminus 3 was one of the largest chymotryptic peptides, migrating at a position commensurate with the intact C-terminal chymotryptic fragment of AalCecA. Based on this we presume that it is the amide derivative (Table 1) of this peptide, which was present at levels too low to allow further characterization. The overlap tryptic peptide (Tryp 3) was isolated as the major 280 nm (presumptive tyrosine-containing peptide) peak in the HPLC peptide map of a tryptic digest of peak B.

At this juncture it was clear that a combination of tryptic and chymotryptic digests and peptide maps would not provide the overlaps necessary to complete the structural elucidation of the observed carboxyl-termini, or to match the two observed amino-termini with the three observed carboxyl-termini, save in the case of the modified AalCecA. An ongoing molecular biology approach complemented this aspect of the protein chemistry, allowing unambiguous identification of three mosquito cecropins while providing additional information not available from a protein chemistry approach.

3.2. Isolation and characterization of cecropin cDNAs

To confirm the synthesis of distinct cecropins by C7–10 cells, we used a PCR-based approach to obtain corresponding cDNAs. Upstream primers P1, and then P2 (Fig. 3A) were paired with oligo-(dT_{18}) as the downstream primer in two consecutive cycles of 3′-RACE. The second cycle PCR products appeared as a broad band ranging from 200–300 bp in size. Among 20 clones sequenced (Fig. 3B), we found sequen-

Table 1
Mass spectrometry data

Peak A	Mass (MS)	Mass (calculated)
I	3549.2 ± 1	3548.4: AalCecA, intact C-terminal G, [4] GGLKKLGKKLEGVGKRVFKASEKALPVAVGIKALG
Peak B ([4], Fig. 2)		
I	3448.9 ± 1	3448.1: AalCecC, intact C-terminal G GGLKKLGKKLEGAGKRVFNAAEKALPVVAGAKALG
II	3490.1 ± 1	3490.3: AalCecA-amide
III	3584.5 ± 1	3584.4: AalCecB-amide
IV	3642.2 ± 1	3642.4: AalCecB, intact C-terminal G GGLKKLGKKLEGVGKRVFKASEKALPVLTGYKAIG

Peak A is included from a previous paper [4]. Peak B: I, II, III and IV are from Fig. 1. MS data are corrected for instrument calibration and protonation as described in the legend to Fig. 1.

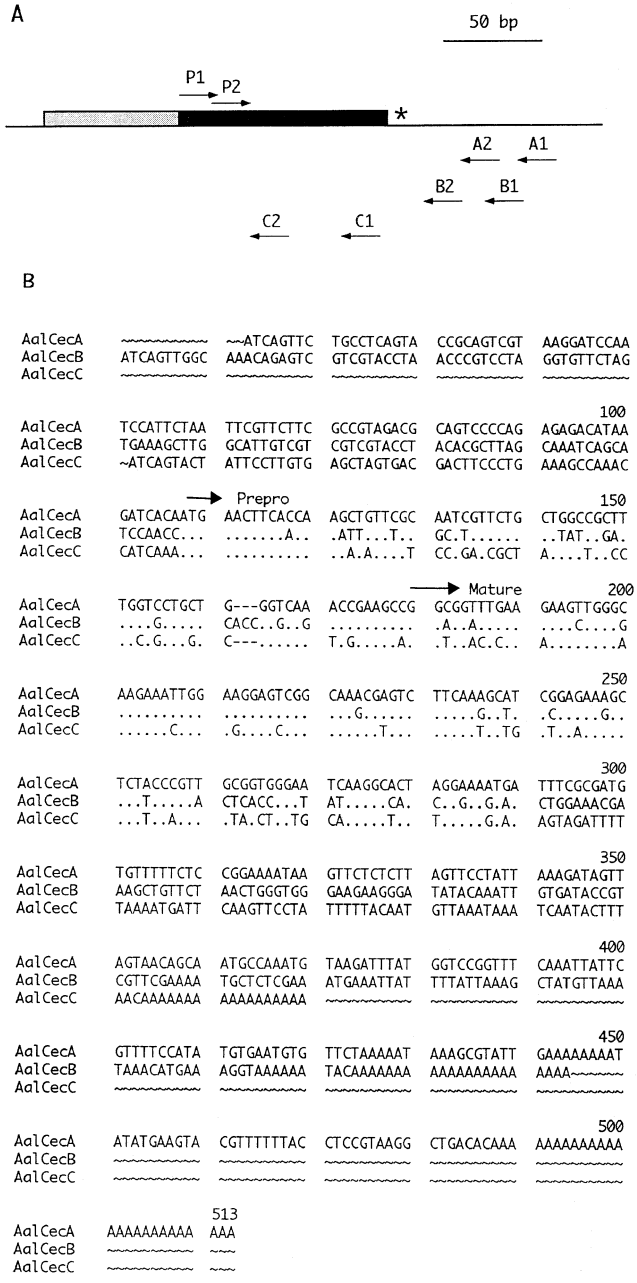

Fig. 3. Nucleotide sequences of mosquito cecropin cDNAs. Panel A shows a schematic cDNA in which the signal and pre-pro regions are shaded, and the mature region is shown in black. The asterisk indicates the stop codon. Primers P1 and P2 were used in 3′-RACE to obtain the mature domain and downstream cDNAs. For 5′-RACE, primers specific to each of the three cecropin cDNAs: A (A1 and A2), B (B1 and B2), and C (C1 and C2) were designed to obtain corresponding upstream sequence. Panel B shows nucleotide sequences aligned from the AUG codon. Arrows at top indicate pre-pro and mature regions. Within the coding region, identities are shown by dots. Gaps (∼) were introduced to maximize the alignment. Identities are not designated in the sequences upstream and downstream of the coding region. GenBank accession numbers: AalCecA: AF145802; AalCecB: AF145803; AalCecC: AF145804.

ces whose deduced translation products (Fig. 4) corresponded to AalCecA and to two additional isoforms consistent with the mass spectrometry analysis of peak B. An analogous process using 5′-RACE and the specific primer pairs A1 and A2, B1 and B2, C1 and C2 (Fig. 3A), based on known sequences at the 3′-end of the cDNAs (Fig. 3B), was used to obtain the sequence upstream of P1 and P2. Designation of the new isoforms as cecropins B and C was based on relative identity to corresponding mature peptides from *D. melanogaster* [14]. As summarized in Table 1, the masses calculated from deduced sequences were consistent with mass spectrometry values, assuming post-translational removal of the terminal lysine and, for a proportion of the mature peptides, processing of the penultimate glycine to an amide.

We note that the 5′-end of each product (Fig. 3B) was identical in the first six nucleotides: 5′-ATCAGT, suggesting that first-strand cDNA synthesis terminated at a conserved cap site. In Fig. 3B, the coding sequences are aligned from the AUG start codon, with dots indicating identical nucleotide residues. Note that in the pre-pro region, AalCecB had a three nucleotide insertion, relative to AalCecA and AalCecC, encoding an additional threonine in the signal sequence. In the mature peptide, all of the mosquito cecropins contained a five amino acid gap (Fig. 4) towards the C-terminus, relative to *D. melanogaster* CecA. Finally, we note that the mature AalCecB contained three residues that can potentially be phosphorylated: serine (position 21), threonine (position 29), and tyrosine (position 31). AalCecA also contained the serine, but none of these positions was conserved in AalCecC.

For Northern analysis, total RNA was extracted from uninduced cells, and from cells at 2, 4, 6, and 24 h after induction. Three individual sets of the five samples were electrophoresed on agarose gels containing formaldehyde and blotted as described in Section 2 (Fig. 5). PCR products from the 5′-RACE (see Fig. 3) were used as hybridization probes, and a probe from the *A. albopictus* ribosomal protein S6 (rpS6) cDNA was used as a loading control. Relative to the rpS6 control, transcript encoding AalCecB was the most abundant, and was detectable as early as 2 h after induction. In panel B, the comparable intensities of the hybridization signals allowed simultaneous exposure of the cecropin and rpS6 signals. Relative to AalCecB, AalCecA (panel A) and AalCecC (panel C) were less abundant, and the cecropin transcripts were undetectable at exposure times appropriate for rpS6 transcript. Sizes of the bands also varied slightly, from ~0.5 kb for AalCecA, 0.45 kb for AalCecB, and 0.3 for AalCecC.

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Prepro

AalCecA      MNFTKLFAIVLLAALVLL-GQTEA
AalCecB      ...N....L...IG....T.....
AalCecC      .....I.VLIAM...L.V-..S..
DmCecA       ...YNI.VF.A.ILAITI-..S..


Mature

AalCecA      GGLKKLGKKLEGVGKRVFKASEKALPVAVG-----IKALGK
AalCecB      ..........................LT.-----Y..I..
AalCecC      ............A.....N.A......VA.-----A.....
DmCecA       .W...I...I.R..QHTRD.TIQG.GI.QQAANVAAT.R.-
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Fig. 4. Deduced amino acid sequences of *A. albopictus* cecropins A, B and C, and *D. melanogaster* (Dm) CecA, aligned with AalCecA. Dots indicate identities, and gaps (–) were added to maximize alignment. The sequence extends from the AUG codon up to, but not including, the stop codon.

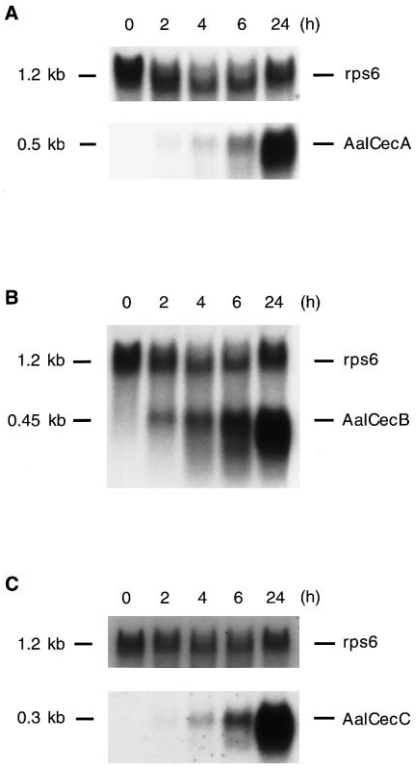

Fig. 5. Northern analysis of mosquito cecropin transcripts. Total RNA (10 μg) isolated at the indicated times after induction was run on an agarose gel containing formaldehyde, blotted, and probed with 5′-RACE PCR products. A rpS6 probe was used to control for uniformity of loading.

3.3. Relative amounts of AalCecA, B and C isoforms in peak B

Although we noted batch-to-batch variability in overall cecropin yield from different preparations of induced cells, it was of interest to estimate, at least to a first approximation, the relative proportions of the different cecropin isoforms secreted by C7–10 cells. As described previously, the material in peak A from RP-HPLC could be unambiguously defined as the non-amidated form of AalCecA [4]. In contrast, because the extent of ionization of each peptide would not necessarily correspond to its level in peak B, the proportions of different isoforms in peak B could not be inferred from MS data alone (Fig. 1). However, the fortuitous presence of unique diagnostic features in the diverse cecropins in peak B, viz. the tyrosine in AalCecB and the asparagine in AalCecC, allowed us to estimate relative proportions of AalCecB, AalCecB-amide, AalCecC, and AalCecA-amide by analysis of total amino acid composition (data not shown). In aggregate, such calculations from two independent preparations suggested that AalCecA, AalCecB and AalCecC each constituted about one-third of the total material. For AalCecA, 8 to 25% was in the amidated form, while for AalCecC, the amidated form was detected only by high resolution mass analysis of crude material. Finally, in AalCecB, the proportion of amidated material ranged from 30 to 90%.

4. Discussion

Despite the characterization of cecropins from several insect species, mosquito cecropins have only recently been described. Using PCR primers based on the amino acid sequence of AalCecA [4] we now report the cDNA encoding this peptide, as well as cDNAs encoding two additional cecropins secreted from *A. albopictus* C7–10 mosquito cells upon immune induction. Based on RP-HPLC profiles, mosquito cecropins appear to be expressed in both amidated and non-amidated forms, with the non-processed form being more abundant. Using PCR probes that spanned the divergent signal sequences, transcripts corresponding to each of the individual cecropins were detectable, ranging in approximate size from 0.3 to 0.5 kb. These transcripts appeared as early as 2 to 4 h after induction, and persisted for more than 24 h.

The cDNA sequences further confirmed that mosquito cecropins are structurally analogous to cecropins from other insects, including the signal peptide, and dipeptide, which are removed by signal peptidase and dipeptidyl aminopeptidase, respectively. We note that the signal peptides were poorly conserved, relative to the mature cecropin domains, and that the signal peptide in AalCecB contained an extra amino acid, relative to AalCecA and AalCecC.

Among the mature cecropins, identities were 100% over the first 18 amino acids, with the exception of a single conservative substitution in AalCecC. The mosquito cecropins were on average only $\sim$40% identical to *Drosophila* cecropins. Overall, most highly conserved was the N-terminus of the mature cecropin domain, which showed 71% identity over the first 14 amino acids, relative to DmCecA. Mosquito cecropins also required a five amino acid gap, relative to the *Drosophila* cecropins, to maximize amino acid alignment. The cecropin genes encoded a terminal lysine residue, which is presumably removed by carboxypeptidase, prior to processing of the penultimate glycine to yield the amidated peptides. We note that the amidated form of AalCecC has been seen only after high resolution mass analysis of unpurified material.

Although the biological factors that influence cecropin yield remain to be determined, a quantitative analysis of the available data indicates that AalCecA, AalCecB and AalCecC are secreted by C7–10 cells in roughly equimolar proportions, with only AalCecB showing substantial amidation. It remains to be learned whether the specific mosquito cecropin isoforms differ in antibiotic activity, and whether amidation affects overall activity. The extent of AalCecB processing appears to range from roughly 30% to greater than 90%. We note that in the samples from which RNA was isolated, mRNA corresponding to AalCecB appeared to be in greatest abundance, but it remains to be learned how the expression of cecropin genes and processing of the gene products are regulated, and whether regulation and processing are influenced by subtle parameters that affect overall cecropin yield. Further analyses will be required to explore these aspects of mosquito cecropin gene expression in cell culture and in intact mosquitoes. Cecropins have been of particular interest to vector biologists because they show activity against some parasitic protozoa, including species that cause malaria in humans [5–7]. In this context, it will be of particular interest to further investigate cecropins from mosquitoes representing other genera, and, because of their relatively low identity to cecropins from other insects, to test directly the specificities of mosquito cecropins against parasites.

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