Generation and characterization of the antibacterial activity of a novel hybrid antimicrobial peptide comprising functional domains from different insect cecropins

Richard M. Plunkett, Stuart I. Murray, and Carl A. Lowenberger

Abstract: The search for new antimicrobial compounds involves finding novel sources of chemotherapeutic compounds or manipulating and combining structures from existing molecules. Small antimicrobial peptides (AMPs) are components of innate immune defenses characterized in greatest detail in insect-derived AMPs. We have generated hybrid AMPs (hAMPs) by combining functional motifs from different insect AMPs as a proof of principle that we can generate molecules with lower minimum inhibitory concentrations, and with different activity and target specificity than either parent molecule. A two-helix, cecropin-like hAMP was created by linking the N-terminal α-helix of cecropin A from Aedes aegypti to the C-terminal α-helix of cecropin A1 from Drosophila melanogaster. This molecule exhibits antibacterial activity at sub-micromolar concentrations with a target specificity that differs from either parent molecule. Antibacterial activity of the hybrid molecule was found to be greater against Gram-negative than Gram-positive bacteria. No hemolysis was observed in sheep red blood cells exposed to concentrations up to 50 μmol/L, suggesting the peptide is not detrimental to eukaryotic cells.

Key words: hybrid antimicrobial peptides, cecropin, antimicrobial activity, fusion expression.

Introduction

The development of resistance by different groups of pathogens to commonly available and inexpensive antibiotics has forced us to search for novel sources of chemotherapeutic compounds. This search may be done by modifying existing antibiotics or examining properties of naturally existing molecules from nontraditional sources. Insects comprise one of the most successful groups of organisms on earth in part because of their ability to recognize and eliminate potential pathogens, including viruses, bacteria, fungi, and eukaryotic parasites. Insects rely solely on innate immunity of which the expression of potent wide-spectrum antimicrobial peptides (AMPs) is a major component (Bulet and Stocklin 2005; Chernysh et al. 2002; Hancock 2001; Zasloff 2002). Hundreds of insect AMPs belonging to several different families have been identified (Bulet et al. 1999), and this has propelled the consideration and evaluation of insect-derived AMPs as the basis on which to develop novel antimicrobial agents.


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Most insect AMPs are small (20–100 amino acids) cationic peptides that contain at least one region that forms an α-helical structure that binds to hydrophobic surfaces, such as microbial membranes. While the specific modes of action are not completely understood, it is generally accepted that they first bind to the bacterial cell surface and then either disrupt or penetrate the membrane. Toxicity may result from damage to external membranes or by action against intracellular structures or processes (Brogeden 2005; Tossi et al. 2000). For example, cecropins attack membranes, but also have been shown to affect cellular processes in Escherichia coli by altering gene transcription (Hong et al. 2003). Cecropins have one of the simplest AMP structures, which comprises 2 short α helices joined by a non-helical hinge. Cecropins were isolated first from the moth Hyalophora cecropia (Boman and Steiner 1981; Steiner et al. 1981), and later described in other lepidopterans (Dickinson et al. 1988; Sumida et al. 1992) and dipterans (Liang et al. 2006; Lowenberger et al. 1999; Vizioli et al. 2000; Zhou et al. 1997).

While the general two-helix structure is conserved in all cecropins, the primary sequence of amino acids can differ significantly among cecropins from different insect orders (Bulet and Stocklin 2005). These modifications may contribute to the different spectrum of activity of each of these molecules. In addition to sequence variability, certain structural modifications of cecropins may contribute to determining the target specificity and structural stability (Bulet and Stocklin 2005; Imler and Bulet 2005). Many cecropins from dipterans and lepidopterans are post-translationally amidated at the C terminus, which may help maintain the stability and cationic nature of the molecule. An N-terminal tryptophan residue is present in the first or second position of most lepidopteran and dipteran cecropins but is absent in cecropin D from the silkworm Bombyx mori and in mosquito cecropins.

Whereas other studies have combined structures from diverse AMPs in non-natural configurations (Boman et al. 1989; Hongbiao et al. 2005; Otvos et al. 2005; Shin et al. 1999), we designed hybrid AMPs (hAMPs) by combining components of homologous peptides from different insects. This approach allows us to select structures and tailor the design of hAMPs to target specific groups of microbes (e.g., methicillin-resistant Staphylococcus aureus). Insect AMPs have been selected throughout evolution for low toxicity to eukaryotic cells and low target resistance by bacteria. As the numbers of antibiotic-resistant pathogens increase, insect AMPs, with an enormous potential of combinations and permutations of structures that can be employed, may provide us with the basis of future antibiotics. A consideration when developing hybrid AMPs for evaluation is their production in large quantities. Synthetic peptides are useful because they can be obtained in high purity, but at a high cost. Bacterial expression systems are commonly used for producing large amounts of recombinant protein, also with high purity and at a lower cost. When the recombinant peptide is intended to have antimicrobial properties, it is important to protect the expression host from the toxic effects of product. We have used a bacterial system to express an antimicrobial peptide in E. coli as a fusion protein with a large nontoxic fusion partner bound to the hAMP, which effectively reduces its antibacterial activity against the expression host.

Here, we report the generation of a chimeric cecropin-like hAMP that was created by linking the N-terminal α-helical domain (residues 1–26 of the active peptide) of cecropin A from Aedes aegypti (Liverpool strain) (Lowenberger et al. 1999) by a proline hinge to the C-terminal α-helical domain (residues 27–40 of the active peptide) of cecropin A1 from Drosophila melanogaster (Canton-S) (Kylsten et al. 1990). This molecule exhibits antibacterial activity at sub-micromolar concentrations different to the activity of either parent molecule. We present the design, expression, and characterization of this hAMP in terms of its specific antibacterial activity against selected Gram-negative and Gram-positive bacteria. We also provide the results of tests evaluating its activity against eukaryotic cells using hemolytic activity assays.

Materials and methods

Synthetic peptides

For initial studies of antibacterial activity, peptides corresponding to individual N- and C-terminal α-helical domains of cecropins from lepidopteran (Hyalophora cecropia cecropin, UniprotKB/Swiss-Prot acc. No. P01507) and dipteran insects (Ae. aegypti cecropin A, UniprotKB/Swiss-Prot acc. No. P82592; D. melanogaster cecropin A1, UniprotKB/Swiss-Prot acc. No. P14954). Synthetic peptides were obtained from the Peptide Array/Peptide Synthesis Facility at the Brain Research Centre, University of British Columbia. Synthetic full-length AC(1–26)-DC(27–40) was purchased from GenScript Corp. (Piscataway, New Jersey) and used in antibacterial and hemolysis assays.

Generation of the chimeric gene

We created an artificial chimeric gene (Fig. 1) using a combination of overlap and recursive PCR methods (Prodomou and Pearl 1992; Warrens et al. 1997). Figure 2 is a schematic representation of the PCR process used in this study, and Table 1 describes the oligonucleotide primers used. We extracted RNA from the fat body of immune-activated Ae. aegypti, and generated cDNA as described previously (Cooper et al. 2007; Lowenberger et al. 1999). In the initial PCR reaction, we designed primers RP008F and RP002R to amplify the N-terminal α helix of Ae. aegypti cecropin A (GenBank acc. No. AF117886). The expression vector we used in this study (pET-32 Ek/LIC; Novagen, San Diego, California) utilizes a ligation-independent cloning (LIC) mechanism, which allows cloning without the need for restriction enzyme digestion. It was therefore necessary to generate PCR products with appropriate nucleotide sequences at each end, as described in the manufacturer’s directions. The forward primer RP008F included a sequence corresponding to a 3’ linker sequence to facilitate insertion into the cloning site of the pET-32 expression vector for subsequent expression studies. The PCR conditions were 95 °C (2 min) followed by 30 cycles of 95 °C (10 s), 55 °C (10 s), and 72 °C (30 s) and a final extension step of 72 °C (2 min). This PCR product was size-fractionated on 1.2% agarose, stained with ethidium bromide. A band of the expected size (106 bp) was excised from the gel and the DNA purified (QiAQuick Gel Extraction Kit; QIAGEN, Mississauga, Ontario). This DNA was used in a subsequent over-

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lap PCR reaction using the initial RP008F primer and a 67 bp reverse primer (RP007R) that overlapped the template DNA by 22 bp, incorporated a proline hinge, continued with the C-terminal a helix of D. melanogaster cecropin CecA1 (GenBank acc. No. NM 079849), and incorporated a 5’ linker sequence subsequent expression studies. The PCR conditions were identical to those described above for the initial PCR reaction.

The 152-bp product of the overlap PCR, our synthetic gene designated AC(1–26)-DC(27–40), was extracted from the gel and purified as described above, ligated into pGEM-T Easy (Promega, Madison, Wisconsin), transformed into JM109 competent cells (Promega), and grown overnight as described (Lowenberger et al. 1999). Colonies were screened for inserts, and positive transformants were grown overnight in Luria–Bertani broth containing 50 μg/mL ampicillin (LB–AMP). Subsequent purified plasmid (SV Minipreps; Promega) was sequenced using Big Dye version 3.1 chemistry (ABI, Foster City, California). We used the plasmid as template for a PCR reaction using the insert-specific primers RP008F and RP009R that amplified the entire synthetic gene that was cloned into the protein expression vector pET-32 Ek/LIC according to the manufacturer’s instructions. The plasmid designated pET-32 AC-DC encodes our hAMP with an N-terminal thioredoxin (Trx) fusion partner, a 6×His tag, and an enterokinase cleavage site. Purified pET-32 AC-DC plasmids were subsequently used to transform BL21(DE3) cells (Novagen, San Diego, California) for the expression of the recombinant fusion proteins.

Expression and purification of recombinant fusion proteins

Starter cultures for recombinant protein expression were made by inoculating LB–AMP with a single colony from an overnight plate of transformed BL21(DE3) cells (Novagen). Cells were incubated at 37 °C with shaking at 250 rev/min until they reached an optical density at 600 nm (OD600) of 0.6. Cells from 3.0 mL of the starter cultures were collected by centrifugation (5000 g for 5 min at 4 °C), washed with 3.0 mL phosphate-buffered saline pH 7.4 (PBS), and resuspended in 3.0 mL LB–AMP. These were used to inoculate 500 mL LB–AMP, which was incubated with shaking at 37 °C and allowed to grow to an OD600 of 0.6, whereupon expression was induced by adding IPTG to a final concentration of 0.4 mmol/L, and cultures were incubated at 21 °C for 16 h after induction. Cells were harvested by centrifugation at 5000 g for 20 min at 4 °C, and the supernatant was retained. The pellet was washed with 1 culture volume of PBS and stored at –80 °C. Harvested cells were resuspended in 10 mL PBS and mechanically disrupted by sonication in the form of 30 s bursts followed by 30 s rest (for a total of 12 min of sonication) while the sample was kept on ice. The soluble fraction was separated from cell debris by centrifugation at 7500 g for 40 min at 4 °C. After centrifugation, the soluble fraction was either immediately subjected to nickel-affinity column purification or stored at ~20 °C (with no apparent loss of protein recovery up to 2 months).

The recombinant fusion protein contains a polyhistidine tag that binds to nickel ions, allowing purification of the
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Fig. 2. Overlap PCR method used to generate pET-32 Trx-AC(1–26)-DC(27–40) construct. (A) First round of PCR used Aedes aegypti cDNA as the template to produce a fragment containing a partial Ae. aegypti cecropin gene (white) with a 3′ overlap for the Drosophila melanogaster cecropin gene (grey) and a 5′ linker sequence for the pET-32 Ek/LIC vector (black). (B) The product from (A) was the target for an overlapping PCR to produce the full-length AC(1–26)-DC(27–40) with both Ae. aegypti and D. melanogaster segments and 5′ and 3′ pET-32 linkers. (C) The product of (B) was used to generate the pET-32 Trx-AC(1–26)-DC(27–40) construct, featuring a thioredoxin fusion partner (Trx), a polyhistidine tag (6×His) for purification, and an enterokinase cleavage site (Ek) to release the recombinant AC(1–26)-DC(27–40) peptide.

(A) 3′ RP008F A. aegypti cDNA template RP002R 5′

(B) RP008F PCR product from “A” RP007R

(C) AC(1–26)-DC(27–40) (PCR product from “B”)

pET-32 Trx AC(1–26)-DC(27–40)

proteins from cell debris with a nickel affinity column. A volume of 2.5 mL of cleared cell lysates was bound to a bed volume of 500 μL of nickel–nitrotriacetic (Ni–NTA) agarose (QIAGEN) that had been equilibrated by washing twice with water and once with binding buffer (20 mmol/L Tris pH 7.9, 0.5 mol/L NaCl, and 5 mmol/L imidazole). The mixture then was added to cleared lysate containing 10 mmol/L imidazole. The combined Ni–NTA agarose and sample lysate were mixed on a rotator for 1 h at 4 °C then applied to a 10 mL gravity flow column, which had been washed with water and pre-equilibrated with binding buffer. The column was washed twice with 20 mmol/L Tris pH 7.9, 0.5 mmol/L NaCl, and 30 mmol/L imidazole. Bound protein was eluted from the column with 20 mmol/L Tris pH 7.9, 0.5 mol/L NaCl, and 100 mmol/L imidazole. Column flow-through, washes, and eluted fractions were collected for analysis by SDS–PAGE. After affinity column purification, the eluted fusion protein was concentrated by centrifugation in an Amicon Ultra filter device (Millipore Corp., Billerica, Massachusetts) with a molecular mass cutoff of 10 kDa. Samples were centrifuged at 7500 g at 4 °C to approximately one-tenth volume then washed in the filter device 3 times with 2.5 mL PBS by centrifugation, then resuspended in 250 μL PBS.

The hAMP region of the fusion protein was separated from the Trx-6×His fusion partner by cleavage with recombinant enterokinase (rEK) (Novagen). Cleavage reactions took place in 1× rEK Cleavage/Capture buffer with 1 unit rEK per 50 μg protein; the reactions were incubated for 16 h at 21 °C followed by removal of rEK with EKapture agarose (Novagen) according to the manufacturer’s instructions.

Protein concentrations were determined by the BioRad Protein Assay (BioRad, Mississauga, Ontario), based on the method of Bradford (1976).

SDS–PAGE and immunoblot analysis

For visualization of Trx-AC(1–26)-DC(27–40), 10 μL of eluate from the nickel-affinity column was electrophoresed on a 15% SDS–polyacrylamide gel using a PageRuler broad-range molecular marker as a standard (BioRad), then stained with Coomassie blue to make protein bands visible. Immunoblot detection was used to specifically identify the thioredoxin fusion protein Trx-AC(1–26)-DC(27–40). Ten microliters of the Trx-AC(1–26)-DC(27–40) eluate from the nickel-affinity column was electrophoresed on a 15% SDS–polyacrylamide gel and transferred to Hybond C+ extra nitrocellulose membrane (Amersham, Picataway, New Jersey) in transfer buffer (25 mmol/L Tris, 192 g glycine, 0.01% SDS, 20% methanol, H2O to 1 L) for 60 min using the Bio-Rad semi-dry blotting system (Bio-Rad). Membranes were pre-blocked overnight at 4 °C in 5% blocking solution (5% Carnation skim milk powder in 1× Tris-buffered saline + 0.5% Tween-20 (TBST)). To detect Trx-AC(1–26)-DC(27–40), the membranes were incubated in an 1:5000 dilution of monoclonal mouse antibodies to Ae. aegypti cecropin A for 50 min with gentle agitation, washed 3 times (for 5 min each wash) in 15 mL of 1× TBST, and then incubated with a 1:10 000 dilution of goat anti-mouse antibody-HRP conjugate (Novagen) for 50 min with gentle agitation. The membranes were washed 3 times (for 5 min) in 15 mL of 1× TBST and once in 15 mL ddH2O. Trx-AC(1–26)-DC(27–40)-HRP conjugate was detected using the Transcend Chemiluminescent substrates according to manufacturer’s instructions and exposed using Kodak Biomax light-1 film.

Antibacterial activity assays

We evaluated the antibacterial activity of our hAMP against various Gram-negative and Gram-positive bacteria obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). The Gram-negative bacteria included Alcaligenes faecalis ATCC 8750, Citrobacter freundii ATCC 8090, Enterobacter aerogenes ATCC

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bacterial growth was evaluated by measuring optical density were incubated at 25°C (10 g/L bactotryptone, 5 g/L NaCl, pH 7.4). The plates exponential-phase bacterial cultures in Poor Broth medium conducted to determine minimum inhibitory concentrations inhibition of bacterial growth. Broth dilution assays were conduced to determine minimum inhibitory concentrations (MIC) as previously described (Hetru and Bulet 1997) for these, several pairs of peptides were selected for MIC determination. The highest relative levels of antimicrobial activity were observed in bacteria exposed to the N-terminal α-helical portion of cecropin A from Ae. aegypti in combination with an equimolar amount of the C-terminal α-helix of cecropin A from D. melanogaster, showing activity against E. coli, though no activity was detected against S. epidermidis (Table 2). On the basis of these data, a chimeric gene was designed to incorporate the coding sequences for the N-terminal α-helical portion of Ae. aegypti cecropin A linked by a proline hinge to the C-terminal α-helical portion of D. melanogaster cecropin A (Fig. 1).

Results

Antibacterial assays of α helices from insect cecropins
Experiments investigating relative antibacterial activity were performed using synthetic peptides representing α-helical domains from insect cecropins. They were tested by the disk diffusion method either alone or combined in pairs for activity against Gram-positive (E. coli) and Gram-negative (S. epidermidis) bacteria; relative activity was determined by comparing the diameters of zones of inhibition. From these, several pairs of peptides were selected for MIC determination.

Expression and purification of recombinant proteins from chimeric genes
Successful expression of the hAMP was achieved by using the Trx-AC(1–26)-DC(27–40) construct, which added an N-terminal thioredoxin fusion partner. The expressed Trx-AC(1–26)-DC(27–40) fusion protein was detected by SDS–PAGE (Fig. 3A), revealing a band corresponding to the predicted 21.2 kDa protein that was not visible in unin-
duced cultures. The recombinant thioredoxin fusion protein was also detected by immunoblot analysis using antibodies to Ae. aegypti cecropin (Fig. 3B).

Antimicrobial activities of α helices Trx-AC(1–26)-DC(27–40) and AC(1–26)-DC(27–40)
The MICs of the AC and DC α helices were evaluated individually against several bacterial species. The AC frag-

Table 1. Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP002R</td>
<td>CTTGGTGGAGCGATAGGAAGAGCCTTCTC</td>
<td>5’ end of Drosophila melanogaster cecropin A1 C-terminal α helix followed by proline hinge codon with sequence matching 3’ end of Aedes aegypti cecropin A N-terminal α helix</td>
</tr>
<tr>
<td>RP007R</td>
<td>GAGGAGAAGCCCGTCAACCTCGGGCAGTTGCCGGG ACATTGGCCTTGTGAGCQATCAGGAGACG</td>
<td>3’ end of Ae. aegypti cecropin A N-terminal α helix followed by proline hinge codon with D. melanogaster cecropin A1 C-terminal α helix and 3’ pET vector linker sequence</td>
</tr>
<tr>
<td>RP008F</td>
<td>GAGCGACGCAAGATGGTTGCCCTCAAGAAGCTGG</td>
<td>5’ pET vector linker sequence followed by start codon and 5’ end of Ae. aegypti cecropin A N-terminal α helix</td>
</tr>
<tr>
<td>RP009R</td>
<td>GAGGAGAAGCCGGTCAACCTCGGGCAGTTG</td>
<td>3’ pET vector linker sequence followed by 3’ end of D. melanogaster cecropin A1 C terminal α helix</td>
</tr>
</tbody>
</table>

13048, Escherichia coli ATCC 11303, Pseudomonas fluorescens ATCC 13525, and Serratia marcescens ATCC 13880. The Gram-positive bacteria included Bacillus coagulans ATCC 7050, Bacillus subtilis ATCC 6051, Lactococcus lactis ATCC 11454, Micrococcus luteus ATCC 4698, and Staphylococcus epidermidis ATCC 14990.

Hemolytic activity assays
Hemolysis assays were done in a manner similar to one previously described (Hetru and Bulet 1997) for Trx-AC(1–26)-DC(27–40) and AC(1–26)-DC(27–40), disks contained 5 μL of the nickel-affinity column eluate or enterokinase cleavage reactions (after cleanup with EKapture agarose), respectively. Plates were incubated overnight at 37°C or for 24 h at 25°C (for M. luteus, P. fluorescens, and Serratia marcescens) and examined for inhibition of bacterial growth. Broth dilution assays were conducted to determine minimum inhibitory concentrations (MIC) as previously described (Hetru and Bulet 1997) for Trx-AC(1–26)-DC(27–40) and AC(1–26)-DC(27–40), disks contained 5 μL of the nickel-affinity column eluate or enterokinase cleavage reactions (after cleanup with EKapture agarose), respectively. Plates were incubated overnight at 37°C or for 24 h at 25°C (for M. luteus, P. fluorescens, and Serratia marcescens) and examined for inhibition of bacterial growth. Broth dilution assays were conducted to determine minimum inhibitory concentrations (MIC) as previously described (Hetru and Bulet 1997) for Trx-AC(1–26)-DC(27–40) and AC(1–26)-DC(27–40), disks contained 5 μL of the nickel-affinity column eluate or enterokinase cleavage reactions (after cleanup with EKapture agarose), respectively. Plates were incubated overnight at 37°C or for 24 h at 25°C (for M. luteus, P. fluorescens, and Serratia marcescens) and examined for inhibition of bacterial growth.

Hemolytic activity assays
Hemolysis assays were done in a manner similar to one previously described (Park et al. 2007). Sheep’s blood (Sigma-Aldrich) was diluted to 1% in PBS. Cells were pelleted by centrifugation at 1000g for 5 min at 4°C, washed twice with, and resuspended in, 1 volume of PBS. Washed cells were added to tubes containing 20 μL of compounds to be tested (to yield final concentrations of 0–50 μM/L) to a volume of 200 μL and incubated for 1 h at 37°C, after which unlysed cells were removed by centrifugation (1000g for 5 min). One hundred microliters of supernatant from each tube was transferred to a 96-well microtiter plate, and heme released by cell lysis was measured as absorbance at 405 nm (A405). Percent lysis was determined by comparing the A405 of test samples against the A405 of untreated cells (0% lysis) or cells treated with 0.2% Triton X-100 (100% lysis).

Expression and purification of recombinant proteins from chimeric genes
Successful expression of the hAMP was achieved by using the Trx-AC(1–26)-DC(27–40) construct, which added an N-terminal thioredoxin fusion partner. The expressed Trx-AC(1–26)-DC(27–40) fusion protein was detected by SDS–PAGE (Fig. 3A), revealing a band corresponding to the predicted 21.2 kDa protein that was not visible in unin-
duced cultures. The recombinant thioredoxin fusion protein was also detected by immunoblot analysis using antibodies to Ae. aegypti cecropin (Fig. 3B).
Table 2. Minimum inhibitory concentrations (μmol/L) of antimicrobial peptides (AMPs).

<table>
<thead>
<tr>
<th>Gram-negative bacteria</th>
<th>This study</th>
<th>Native cecropins</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alcaligenes faecalis</em></td>
<td>25–50</td>
<td>ND</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>0.781–1.563</td>
<td>ND</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>0–0.098</td>
<td>ND</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.195–0.391</td>
<td>7.8–15.6</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>ND</td>
<td>12.5–25</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gram-positive bacteria</th>
<th>This study</th>
<th>Native cecropins</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>3.125–6.25</td>
<td>ND</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>1.563–3.125</td>
<td>ND</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>ND</td>
<td>6.25–12.5</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>ND</td>
<td>10–25</td>
</tr>
</tbody>
</table>

Note: Minimum inhibitory concentration values for *Ae. aegypti* and *D. melanogaster* cecropins were determined by Lowenberger et al. (1999). The strain of *Escherichia coli* used by Lowenberger et al. was *E. coli* 1106 ATCC 35581. —, not tested; ND, not detected at highest concentration tested (50 μmol/L).

*Synthetic* *Ae. aegypti* cecropin A N-terminal α helix and *D. melanogaster* cecropin A1 C-terminal α helix.

*Synthetic* *Ae. aegypti* cecropin A N-terminal α helix (AC) or *D. melanogaster* cecropin A1 C-terminal α helix (DC).

Fig. 3. (A) Purification of recombinant AC(1–26)-DC(27–40) visualized by 15% SDS–PAGE and Coomassie blue staining: lane 1, PageRuler broad-range protein standard; lane 2, pooled Ni–NTA column eluate after filter concentration of Trx-AC(1–26)-DC(27–40) (~22 kDa); lane 3, Trx fusion tag (~17 kDa) after recombinant enterokinase (rEK) cleavage. (B) Purified Trx-AC(1–26)-DC(27–40) (~22 kDa); lane 3, Trx fusion tag (~17 kDa) after recombinant enterokinase (rEK) cleavage. Published by NRC Research Press

Hemolytic activity of AC(1–26)-DC(27–40)

To determine whether AC(1–26)-DC(27–40) affects eukaryotic cells, hemolysis assays were conducted. No hemolysis was observed when sheep red blood cells were exposed to AC(1–26)-DC(27–40), even at the highest concentration evaluated (50 μmol/L).

Discussion

We have generated a chimeric hAMP by combining the N-terminal α helix of cecropin from a mosquito, *Ae. aegypti*, with the C-terminal α helix of cecropin from a fly, *D. melanogaster*. These studies were conducted to test the activities of a hybrid AMP constructed of functional domains from insect AMPs with regard to MIC and activity spectrum when compared to the native molecules from which the domains were taken. Neither domain was highly lethal by itself nor...
when combined in a single assay. AC(1–26)-DC(27–40), however, exhibits sub-micromolar antibacterial activity yet has low toxicity against vertebrate red blood cells. This hAMP also has a different target specificity than the native parent cecropins. Therefore, rearranging and combining different AMP functional motifs can alter the antimicrobial activity spectra and MICs of native molecules. Our hAMP is effective against some Gram-negative bacteria (viz. E. coli) at MIC levels comparable to those of the native cecropins of Ae. aegypti and D. melanogaster (Lowenberger et al. 1999). Although the activity was generally greater against Gram-negative bacteria, as is the case with the native cecropins, the target specificity has changed. Our hybrid had limited activity against the Gram-positive bacteria tested (none had MICs <1 μmol/L), whereas M. luteus is susceptible to both Ae. aegypti and D. melanogaster cecropins, with MICs of 10–25 μmol/L and 5–10 μmol/L, respectively (Lowenberger et al. 1999), it was not inhibited by up to 50 μmol/L of our hAMP molecule. One characteristic that was unchanged in our hAMP was its absence of hemolytic activity, even in high concentrations. This supports the hypothesis that hAMPs and naturally occurring AMPs from eukaryotes (e.g., insects) should have low activity against eukaryotic cells, since AMPs are released into the hemolymph where they eliminate prokaryotes while remaining nonlethal to the host organism (Bulet et al. 2003). The bacterial strains used in this study represent diverse Gram-positive and Gram-negative bacteria to explore the spectrum of activity of our hybrid AMP. Future studies should include bacterial pathogens, especially antibiotic-resistant clinical isolates, to establish efficacy against infectious organisms.

Dipteran cecropins exhibit antimicrobial activity at MICs in the micromolar range, and synthetic AMPs with structures similar to cecropin are also effective at low molarities. Synthetic cecropin analogues have been designed (Hao et al. 2008) and a hybrid molecule consisting of cecropin A from D. melanogaster fused to a retro-version of the same cecropin molecule is active (Schmitt et al. 2008). Cecropins from Ae. aegypti, Anopheles gambiae, and D. melanogaster are active against Gram-negative bacteria at lower concentrations than those required against Gram-positive bacteria (Lowenberger et al. 1999; Vizioli et al. 2000); (Ekengren and Hultmark 1999; Samakovlis et al. 1992). It has been proposed that, in the presence of microbial membranes, the amphipathic N-terminal helix of cecropin aligns itself parallel to the membrane surface, and the hydrophobic C-terminal helix inserts itself into the membrane causing membrane disruption and bacterial death. Our hAMP was more effective at inhibiting bacterial growth than the individual α helices from which it was composed, or a mixture of the α helices that were not covalently bound to each other. This supports the concept that the activity is dependent on the involvement of both α helices in a two-step process to bind to and subsequently create pores in bacterial membranes. Understanding which domains bind best to specific organisms may allow us to develop pathogen-specific antibiotics.

The activity of AC(1–26)-DC(27–40) against E. coli was comparable to that of cecropin from either Ae. aegypti or D. melanogaster (Lowenberger et al. 1999), but was different from that of the parent molecules against other bacteria. This suggests that subtle changes or motif combinations may change target specificity without increasing the MIC, which is an important consideration when designing and developing antimicrobial chemotherapeutics for specific applications. The target of the native AMPs probably reflect those pathogens that the insects have had to combat over the millennia. We may be able to capitalize on these target specificities when incorporating functional domains from insect AMPs into hybrid AMPs.

Other hybrid peptides have been constructed using AMP motifs from insects and other organisms. These include cecropin–magainin hybrids (insect + amphibian) (Shin et al. 1999), cecropin–mellitin hybrids (insect + bee venom toxin) (Boman et al. 1989), a cecropin–thanatin hybrid (2 insect AMPs; one α helical and one β hairpin) (Hongbiao et al. 2005), and a pyrrhocoricin–drosocin hybrid (a proline-rich insect hAMP combining DnaK-binding + delivery domains) (Otvos et al. 2005). These studies demonstrate various levels of success and activity spectra that differ from the native molecules. The cecropin–mellitin hybrids also illustrate that the hemolytic activity of the mellitin can be attenuated or eliminated when it is part of a hybrid molecule.

We designed our hAMP based upon the cecropin model because of its simplicity, relative ease of expression, and significant data on native molecules with which we were able to compare our results (Lowenberger et al. 1999). Also, because cecropins are presumed to target generalized external cell structures, there may be a lower likelihood of the bacteria developing resistance than what might happen with a compound with a specific molecular target (for instance, the DnaK-binding domain from bacterial heat-shock protein (Otvos et al. 2005)). Our approach could be modified easily to incorporate any structure, such as β sheets or disulphide bridges, but this might require a different expression system. The expression of hAMPs in bacteria was complicated by their natural toxicity to the expressing cells. Initially, the AC(1–26)-DC(27–40) gene was inserted into the pET-46 Ek/LIC expression vector (Novagen), which includes a polyhistidine tag, but does not carry the Trx fusion partner. Upon induction with IPTG in the induced cultures (but not uninduced controls), a drop in the OD600 was observed, indicating that the cells had stopped growing, and cell lysis and death occurred. Successful expression was achieved by using the Trx-AC(1–26)-DC(27–40) construct, which added an N-terminal thioredoxin fusion partner. Others have shown that expression-host toxicity may be overcome by generating a larger nonlethal Trx fusion protein. This has been done to express a cecropin from Musca domestica (Beaulieu et al. 2007), pediocin (a bacteriocin from the lactic acid bacterium Pedicoccus acidilactici) (Morin et al. 2006), and indolicidin (a small 13-amino acid tryptophan-rich AMP found in bovine neutrophils) (Xu et al. 2007). Further characterization of therapeutic hAMPs will require inexpensive, simple, and reproducible production. At this stage, however, small-scale expression or synthesis allows us to evaluate the effects of combining motifs in a Lego-like approach to test the differential effects of adding, removing, or combining novel associations of specific structures on the activity of these molecules against general groups or very specific pathogens. Insect AMPs have served the insects for millennia in their ability to eliminate pathogens and parasites.
They now might serve as the backbones of novel therapeutic agents to treat pathogens that attack humans.

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