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Salt-tolerant antimicrobial peptide from *Ciona*

An exceptional salt tolerant antimicrobial peptide derived from a novel gene family of hemocytes of the marine invertebrate *Ciona intestinalis*

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A novel gene family coding for putative antimicrobial peptides was identified in the EST data base of the sea squirt *Ciona intestinalis*, and one of these genes was molecularly cloned from the Northern European *Ciona* subspecies. In situ hybridisation and immunocytochemical analysis revealed that the natural peptide is synthesized and stored in a distinct hemocyte type, the univacuolar non-refractile granulocytes. By semiquantitative RT-PCR analysis it was shown that the expression of the gene is markedly upregulated in hemocytes after immune challenge. To evaluate the antimicrobial potency of the putative defense protein, we synthesized a peptide corresponding to its cationic core region. The peptide was highly effective against Gram-negative and Gram-positive bacteria including several human and marine pathogens as well as the yeast *Candida albicans*. Notably, the antibacterial activity of the peptide was retained at salt concentrations of up to 450 mM NaCl. Using two different methods we demonstrated that the peptide kills Gram-negative and Gram-positive bacteria by permeabilizing their cytoplasmic membranes. Circular dichroism spectroscopy revealed that in the presence of liposomes composed of negatively charged phospholipids the peptide undergoes a conformational change and adopts an α-helical structure. Moreover, the peptide was virtually non-cytolytic for mammalian erythrocytes. Hence, the designed salt-tolerant antimicrobial peptide may represent a valuable template for the development of novel antibiotics.

Keywords: antimicrobial peptide, tunicates, *Ciona intestinalis*, univacuolar refractile/non-refractile granulocytes, innate immunity, peptide-membrane interaction

Abbreviations used: BSA, bovine serum albumin; CFU, colony forming units; Cy, cyanine; DAB, 3,3′-diaminobenzidine; DSM, Deutsche Sammlung von Mikroorganismen (German Collection of Microorganisms); EST, expressed sequence tag; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; MIC, minimal inhibitory concentration; MLC, minimal lethal concentration; TC, tentative consensus sequence; URG, univacuolar refractile granulocyte; UNRG, univacuolar non-refractile granulocyte; CD, circular dichroism

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INTRODUCTION

The innate immune system provides an immediate response against infections in animals and plants. Endogenous antimicrobial peptides are essential effector molecules of this first line of defense allowing the direct killing of invading microorganisms [1]. Due to the dramatic increase of multi-drug resistant bacterial strains, researchers focus on the development of alternative drugs derived from antimicrobial peptides [2]. To date various natural peptides from all kinds of organisms and synthetic derivatives thereof have been characterized concerning their potential use as novel therapeutics [3]. However, the number of candidate peptides undergoing preclinical or clinical evaluation is still very low [3]. Antimicrobial peptides are referred to several subclasses according to their amino acid composition and secondary structure. The vast majority of these peptides is cationic and has the ability to adopt an amphipathic structure. Moreover, most of these amphipathic peptides fall in one of two categories: either they exhibit an α-helical structure or they constitute a β-sheet, which is typically stabilized by disulfide bonds. The consensus view is that cationic amphipathic peptides act by binding to anionic moieties covering the surface of microorganisms and eventually killing the target cells by perturbating the integrity of their cytoplasmic membranes in different ways [4]. In recent years, several antimicrobial peptides have been isolated and characterized from different urochordate species [5-10]. Hemocytes, the circulating cells of the body fluid, were identified as the main source of these effector molecules. Particularly for the clavanins of Styela clava, it has been shown that the peptides are stored in granules of distinct hemocyte types [11]. Although the sea squirt Ciona intestinalis became recently a model organism, antimicrobial peptides have not been isolated from this species so far. Moreover, in the genome and EST databases of this tunicate genes homologous to those of known antimicrobial peptides have not been identified [12,13]. Recently, we described a method that we used successfully to identify putative antimicrobial peptide genes by screening the translated EST sequences of C. intestinalis employing characteristic structural features of these peptides as search criteria [14]. Here, we report the finding of a novel gene family potentially coding for antimicrobial peptides of the α-helical type by using the same method. The synthesis and storage of the natural entity in a distinct hemocyte type was demonstrated by in situ hybridisation and immunocytochemistry. We synthesized a 24-residue peptide representing the cationic core region of a selected member of this peptide family. The antimicrobial activity of the peptide was comprehensively analyzed with regard to its target cell spectrum and mode of action. The quality of the peptide to retain its activity under physiological salt conditions has been studied in particular as it is an important criterion for a potential use as a template for new antibiotics.

EXPERIMENTAL

Bioinformatics

The predicted amino acid sequence of the Ci-MAM-A precursor was identified in the Ciona EST database of The Computational Biology and Functional Genomics Laboratory at the Dana-Faber Cancer Institute (DFCI) (http://compbio.dfci.harvard.edu/tgi/) [15] by searching the libraries Cat# BM7 (Nori Satoh unpublished cDNA library, blood cells, 56,720 ESTs) and Cat# 9VV (Ascidian hemocytes cDNA library, 3,376 ESTs) for hemocyte library specific assemblies using the search tool provided on this website. In addition, the Ciona EST database “Ghost Database” from the Department of Zoology of the Kyoto University at http://ghost.zool.kyoto-u.ac.jp/indexr1.html [16] and the Ciona genome database from the US DOE Joint Genome Institute at http://genome.jgi-psf.org/Cioin2/Cioin2.home.html [17] were used to find other members of the peptide family in silico. Amino acid sequences were
Salt-tolerant antimicrobial peptide from *Ciona*

analyzed for putative signal peptide sequences with the help of the SignalP V3.0 program available at the homepage of the Center for Biological Sequence Analysis of the University of Denmark ([http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)) [18]. Secondary structure prediction was performed using the PSIPRED Protein Structure Prediction Server from the Bioinformatics Unit of the University College London ([http://bioinf.cs.ucl.ac.uk/psipred/psiform.html](http://bioinf.cs.ucl.ac.uk/psipred/psiform.html)) [19]. Multiple sequence alignments and helical wheel projections were made with the appropriate program of the European Bioinformatics Institute at [http://www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/) [20] and the tool created by Don Armstrong and Raphael Zidovetzki [http://rzlab.ucr.edu/scripts/wheel/wheel.cgi](http://rzlab.ucr.edu/scripts/wheel/wheel.cgi) [21], respectively. The molecular model of Ci-MAM-A24 was generated by the software package WHATIF [22] using standard helix backbone dihedral angles. The structural representations were generated with the RIBBON program [23]. Investigation of putative coiled-coil structures were performed using the coiled-coil prediction algorithm published by Lupas et al. [24].

**Peptides and antibodies**

The peptide Ci-MAM-A24 was synthesized with an amidated C terminus (WRSLGRTLLRLSHALKPLARRSGW-NH$_2$) and obtained from Biosynthan (Berlin, Germany) in a purity grade of >95%. Homogeneity and molecular identity of the synthetic peptide were verified by mass spectrometry. A second variant of Ci-MAM-A-derived peptide contained an additional cysteine at the N terminus. The latter was coupled to maleimide activated keyhole limpet hemocyanin (Pierce, Rockford, IL, U.S.A.) according to the manufacturer’s instructions. The conjugate was then used as an antigen for the immunisation of rabbits to obtain polyclonal antibodies (Eurogentec, Belgium). Ci-PAP-A22 was obtained from Biosynthan also [14]. Ci-MAM-A24 and Ci-PAP-A22 were dissolved in 10 mM HCl to a final peptide concentration of 1 mM. Cecropin P1, magainin 1 and melittin (all in HPLC grade) were purchased from Sigma-Aldrich (Deisenhofen, Germany) and dissolved in 0.01% TFA to obtain stock solutions of 100 µM.

**Microorganisms**

The bacterial strains used were: *Bacillus megaterium* (ATCC 14581), *B. subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 12600), *S. epidermidis* (ATCC 14990), *Planococcus citreus* (ATCC 14404), *Pseudomonas aeruginosa* (ATCC 27853), *Vibrio alginolyticus* (ATCC 17749), *Listonella anguillarum* (DSM No. 11323), *Yersinia enterocolitica* (96-C without plasmid, serotype 0:9), *Serratia marcescens* (both from the Research Center for Infectious Diseases, University of Würzburg), *Klebsiella pneumoniae* (from the Department of Cell Biology, University of Kassel), *Escherichia coli* K-12 strain D31, an ampicillin- and streptomycin-resistant strain, the lipopolysaccharide core of which lost part of the glucose, galactose and rhamnose [25], and *E. coli* strain ML-35p [26]. Bacteria were usually grown overnight in Luria-Bertani (LB) medium with constant shaking at 37 °C and subsequently inoculated in the same medium to reach the mid-logarithmic phase. *B. subtilis* was grown in LB medium containing 1% (w/v) glucose. The marine bacteria *P. citreus*, *V. alginolyticus* and *L. anguillarum* were grown at 28 °C in LB medium supplemented with 1.5% (w/v) NaCl instead of 1% (w/v) NaCl used in the standard recipe. Two different strains of *Candida albicans* (ATCC 10261 and SC 5314) were obtained from Bernhard Hube, Hans Knöll Institute, Jena, and cultivated on Sabouraud dextrose agar plates at 28 °C for 48 h before use.

**Assays for antibacterial and antifungal activity**

The minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) against bacterial and fungal growth were determined by a microdilution susceptibility assay in microtiter plates using 10 mM sodium phosphate, pH 5.2 or pH 7.4 as previously described [14]. In some experiments, the buffer was supplemented with 50, 100, 150, 300 or 450 mM
Salt-tolerant antimicrobial peptide from *Ciona*

NaCl, for assays with marine bacteria the buffer contained 1.5% (w/v) (257 mM) NaCl. The plates were incubated at 37 °C (or 28 °C for marine bacteria) for 24 - 48 h until the sediments of grown bacteria or fungi were clearly visible. The values were expressed as the median of at least two experiments, each performed in duplicate, with a divergence of not more than one dilution step.

Lesions of bacterial membranes were detected by monitoring the fluorescence of the membrane-impermeable, DNA-binding dye SYTOX Green (Invitrogen, Molecular Probes) as previously described [14]. The values were expressed as the mean of three independent experiments.

The kinetics of the permeabilization of the inner and outer bacterial membranes was determined by monitoring the activity of the intracellular β-galactosidase and the periplasmic β-lactamase in permease-deficient *E. coli* ML-35p in parallel [26]. Substrates used were coumarin cephalosporin fluorescein (CCF2)-FA (free-acid form, Invitrogen) for the β-lactamase and 6,8 difluoro-4-methylumbelliferyl β-D-galactopyranoside (DiFMUG) (Molecular Probes) for the β-galactosidase, respectively [27]. Stock solutions of both fluorogenic substrates were prepared in 10 mM HEPES, 25 mM NaCl, pH 7.4. *E. coli* ML-35p were grown in LB medium supplemented with 100 µg/ml ampicillin overnight, washed twice with 10 mM HEPES, 25 mM NaCl, pH 7.4, and resuspended in the same buffer. To start the reaction 25 µl of the bacterial suspension with 1 x 10^7 cells were added to 75 µl of buffer (10 mM HEPES, 25 mM NaCl, pH 7.4) containing the substrate (CCF2-FA or DiFMUG) and the respective peptide. Both reactions were performed in parallel using a black flat-bottomed 96-well plate (Costar, Corning) which was coated with 0.1% (w/v) BSA for 15 min prior to use. The final concentrations were 5 µM for each peptide and 10 µM for each substrate in a final volume of 100 µl. An equivalent volume of the peptide’s solvent was routinely tested in parallel (negative control). The time-dependent increase in fluorescence was measured every two minutes in a microtiter plate fluorescence spectrophotometer (Spectrafluor, Tecan) with shaking for a period of 10 s every minute. For both reactions an excitation wavelength of 390 nm and an emission wavelength of 465 nm were used. The values were expressed as the mean of three independent experiments, each performed in duplicate.

**Assay for hemolytic activity**

Hemolytic activity was determined against human and sheep red blood cells as previously described [14]. The values were expressed as the mean of two independent experiments, each performed in duplicate.

**Circular dichroism spectroscopy and fluorescence spectroscopy**

Circular dichroism (CD) measurements were carried out with a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan), calibrated according to [28]. CD spectra represent the average of at least three scans obtained by collecting data at 1 nm intervals with a bandwidth of 2 nm. The measurements were performed in 50 mM sodium phosphate, pH 5.2 at 25°C in a 1.0 cm tandem quartz cuvette. The two chambers of the tandem cuvette were filled with the peptide solution and the liposome suspension, respectively. After recording the first CD spectrum, the cuvette was shaken for 30 min in order to mix the peptide solution and liposome suspension, respectively. After recording the first CD spectrum, the cuvette was shaken for 30 min in order to mix the peptide solution and liposome suspension, and a second CD spectrum was recorded. The ratio of peptide, i.e. Cis-MAM-A24 or Cis-PAP-A22, and liposomes was 0.85:1 (w/w).

Fluorescence measurements were performed in 50 mM sodium phosphate buffer, pH 5.2 at 25°C using an F-2500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). In some experiments, the buffer was supplemented with 450 mM NaCl. The tryptophan residues of Cis-MAM-A24 were excited at 280 nm and the emission recorded from 300 to 400 nm in the
absence or presence of vesicles of the indicated composition after 30 min of incubation. The fluorescence data were corrected for the vesicle blank (scatter). The ratio of Ci-MAM-A24 and liposomes was 0.85:1 (w/w).

**Liposome preparation**

Liposomes were prepared essentially as described by Pick [29]. Liposomes composed of defined phospholipids were resuspended in 10 mM HEPES, 1 mM EDTA, 150 mM NaCl, pH 7.4 by passing them over a NAP-5 column (Amersham Biosciences). Phospholipids L-α-phosphatidylethanolamine (PE), 1,2-dioctanoyl-sn-glycero-3[phospho-rac-1-glycerol] (sPG), L-α-phosphatidyl-DL-glycerol (PG), L-α-phosphatidylinositol (PI), L-α-phosphatidylserine (PS), L-α-phosphatidylcholine (PC), 1,2-dilkanoyl-sn-glycero-3-phosphatidylcholine (sPC), and sphingomyeline (SM) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.).

**Assay for pore-forming activity**

Pore-forming activity of samples was determined by measuring fluorimetrically the dissipation of a valinomycin-induced membrane potential in liposomes prepared from crude soy bean phospholipids as previously described [30]. Fluorescence was measured by a fluorescence spectrophotometer (model LS 50B; PerkinElmer) using excitation and emission wavelengths of 620 nm and 670 nm, respectively. Pore-forming activity was measured as the initial change in fluorescence intensity over time after addition of the sample. As a control, the pore-forming peptide alamethicin (in HPLC-grade; Sigma–Aldrich) was used in parallel.

**Animals**

Live *Ciona intestinalis* were purchased from the Biologische Anstalt Helgoland (Alfred Wegener Institute for Polar and Marine Research, Germany) and kept in aquaria with circulating sea water (950-1000 mOsm/kg; 15 °C).

c**DNA cloning of the Northern European (NE) variant of Ci-MAM-A**

Total RNA from hemocytes of Northern European *C. intestinalis* was extracted with the RNEasy Mini Kit (Qiagen). Single-strand cDNA was synthesized from total RNA using SuperScript III RNase H Reverse Transcriptase (Invitrogen) and an anchor-dT primers 5'-GACTCGAGTCGACATCGAT-3') according to the manufacturer’s instructions. To amplify the *Ci-mam-A (NE)* transcript *Ci-mam-A (P, Pacific)* (NCBI GeneBank accession no. EU497244) -specific sense (5'-GGCACGAGGATCTCAACG-3') and antisense (5'-CACTTTGTGTTGTTCAATGACAAG-3') primers were applied in a standard polymerase chain reaction (PCR) using recombinant taq DNA polymerase (Fermentas) with the diluted cDNA as template. The PCR product (*Ci-mam-A (NE)*, NCBI GeneBank accession no. EU497243) was gel-purified with Spin Columns (Ambion), subsequently cloned into a pDrive Cloning Vector (Qiagen) and sequenced (Seqlab, Göttingen, Germany).

**Immune stimulation and semiquantitative reverse transcription (RT)-PCR**

Prior to stimulation, adult *C. intestinalis* were kept in sterile-filtered sea water for 42 h with changing water every 6 to 12 h to keep the expression of potentially inducible immune-related genes at low level. The animals were subsequently transferred to sea water contaminated with the content of a biofilter of a sea water aquarium. Additionally, 150 µl of the polluted water were injected between the tunic and the muscular epidermis of each individual. Hemocytes of three individuals each were collected and pooled from unchallenged control animals and from animals 6, 12 and 24 h after immune stimulation, respectively, for the isolation of total RNA and subsequent cDNA synthesis.
The different cDNA samples were adjusted to equal concentrations by running a polymerase chain reaction (PCR) with sense (5’-GCATCACACTTTCTACAACG-3’) and antisense (5’-TCAAGATCTTCTCAAGTAATCG-3’) primers specific for β-actin for calibration. PCR reactions were in general conducted with an initial denaturation step at 95 °C for 3 min, followed by a variable number of cycles of 95 °C (30 s), specific annealing temperature (30 s), 72 °C (30 s), and terminated by a final extension at 72 °C for 3 min. For the semiquantitative RT-PCR, one master mix including cDNA was prepared for each sample and then split and added to β-actin primers in one reaction (22 cycles, 50 °C annealing temperature) and Ci-mam-A-specific sense (5’-GCTCTTCTTCTCTTGGTGTC-3’) and antisense (5’-CAGTGGTTTTCTCGTAAAAAG-3’) primers in another reaction (32 cycles, 52 °C annealing temperature).

In situ hybridization
A DNA fragment corresponding to the nucleotides 42-323 of Ci-mam-A (NE) was amplified by PCR using the same primers as for the semiquantitative RT-PCR. The gel-purified PCR product was cloned into a pDrive Cloning Vector and sequenced, as previously described. Synthesis of digoxigenin-labelled sense and antisense RNA probes from PCR-amplified DNA fragments and following in situ hybridisation were carried out on stomach sections of C. intestinalis as described previously in detail [14].

Immunocytochemistry
Immunocytochemical analyses were performed with C. intestinalis hemocytes fixed on chamber slides exactly as previously described, using either a second antibody coupled to Cy3 or a second antibody coupled to horseradish peroxidase (HRP) and 3,3’-diaminobenzidine (DAB) as a substrate to visualize the antibody-antigen complex [14].

RESULTS

In silico identification of a putative antimicrobial peptide gene family
In a previous study, we reported on a data-mining approach that may reveal hidden families of antimicrobial peptides of C. intestinalis using EST data bases as a template [14]. The prevalent expression of the transcripts in hemocytes and certain features concerning the primary and predicted secondary structure of the putative gene products such as cationicity and amphipathicity had been taken as search criteria. As a consequence, several novel sequences were accumulated which show a hemocyte-specific expression and potentially represent precursors for small cationic peptides. Four of these putative peptide precursors displayed significant sequence similarities to each other and apparently belonged to the same peptide family (TC67543, TC67549, TC59885, TC71332). Using these translated ESTs as query sequences in BLAST search analyses, a total of seven gene family members was identified in the EST databases of C. intestinalis (Figure 1). By contrast, no genes homologous to those of any of the novel peptides have been found in other organisms by screening the NCBI data base. Hypothesizing a putative function as antimicrobial defense molecules, we termed the peptides Ciona - molecule against microbes (Ci-MAM) -A to -G. According to the DFCI Ciona intestinalis Gene Index (CinGI), five members (A, B, C, E and F) of the gene family are prevalently expressed in hemocytes while the transcripts of Ci-mam-D and -G are highly abundant in both the endostyle and in the hemocyte-derived database. A multiple alignment of all seven amino acid sequences demonstrates that the peptide family splits into two subgroups represented by the members Ci-MAM-A (TC59885), -B (TC67543), -C (TC67549) and by Ci-MAM-D to -G (TC71308, TC71332, TC71311, TC71336/TC71309). All peptides share a typical primary structure with a putative signal
Salt-tolerant antimicrobial peptide from *Ciona*

peptide followed by a cationic region and a C-terminal anionic extension (Figure 1A and 1B). Moreover, secondary structure prediction analysis revealed that the cationic part of all members, with the exception of Ci-MAM-G, is expected to form an α-helix (data not shown), which will in all cases result in a more or less defined amphipathic structure of this region (e.g. Figure 1C).

cDNA cloning of the Northern European variant of *Ci-mam-A* and design of a corresponding synthetic core peptide

Due to the potential of its cationic region to adopt a markedly amphipathic structure (Figure 1C), Ci-MAM-A was chosen as a representative for a detailed analysis. The Pacific *C. intestinalis* specimens used for the genome and the EST projects are by now considered to belong to another subspecies than those from Northern Europe we used in our studies [31-33], and to our experience differences at the nucleotide sequence and amino acid sequence level may be substantial. Therefore, at first the Northern European variant of *Ci-mam-A* was PCR-amplified from cDNA originating from animals studied here by using *Ci-mam-A* (P)-specific primers and sequenced. Of the 243 nucleotides representing the open reading frame, 18 were different between the two subspecies resulting in 8 changes at the amino acid level corresponding to a sequence identity of 90% (Figure 1B). A synthetic peptide was designed taken pattern from the well-studied processing of antimicrobial peptide precursors of the tunicate *Styela clava*. In particular, the styelins are synthesized as prepromolecules with a signal peptide and an anionic C-terminal extension which both are removed by proteolytic cleavage to obtain the mature peptide [7,34,35]. Likewise, we assumed here that the primary translation product of *Ci-mam-A* constitutes a prepropeptide. Consequently, we synthesized a peptide representing the cationic amphipathic region of Ci-MAM-A (NE) which was consistent with the first 24 amino acid residues following the putative signal peptide (Figures 1A and 1B). The synthetic peptide was constructed as a C-terminally amidated version (WRSLGRTRLLSHALKPLARRSGW-NH$_2$) and provisionally named Ci-MAM-A24. Helical wheel projection revealed potential amphipathic structure with one face of the helix being remarkably leucine rich (Figure 1 C). However, the bioinformatic tools used here did not detect a potential to form a coiled-coil structure that would be relevant for the putative biological activity.

Semiquantitative RT-PCR

To get a first impression whether transcription of the *Ci-mam-A* gene is inducible, hemocytes originating from untreated *C. intestinalis* and from specimens 6, 12, and 24 h after challenge were harvested and used for the isolation of total RNA. The expression level of the Ci-MAM-A (NE) transcript in the different samples was compared by semiquantitative RT-PCR with β-actin used for calibration of the amount of cDNA employed. This analysis revealed that the expression of *Ci-mam-A* (NE) is markedly upregulated 6 h after immune challenge. While the number of *Ci-mam-A* (NE) transcripts was still at a higher level 12 h after treatment it declined to the level of non-stimulated animals after 24 h (Figure 1D).

Immunolocalization of natural Ci-MAM-A (NE)

Using the synthetic Ci-MAM-A24 as an antigen, we generated specific polyclonal antibodies in rabbits. The specificity of the antiserum obtained was verified by Western blotting and ELISA (data not shown). Moreover, the antiserum was routinely incubated with KLH prior to its use to exclude reactivity due to antibodies against *C. intestinalis* hemocyanin-like proteins [36]. Trials to identify the parent molecule in acid extracts derived from hemocytes and from cell free hemolymph failed when Western blotting and Ci-MAM-A (NE)-specific affinity chromatography techniques employing the specific antibodies were used. However, by performing immunocytochemistry on fixed cells, we successfully localized the natural peptide
to a subpopulation of Ciona hemocytes, which were recently termed univacuolar non-refractile granulocytes (UNRGs) [14]. This cell type is characterized by possessing only one large compartment which, in contrast to the very similar univacuolar refractile granulocytes (URGs), is free of visible granules (Figures 2A-2D). Moreover, the immunocytochemical analyses indicated that only a subpopulation of small UNRGs possesses the peptide. By comparing the results of the alternative developments using Cy3 (Figures 2A and 2B) or DAB and HRP (Figures 2C and 2D), respectively, undesirable effects of autofluorescence or endogenous peroxidase activity could easily be recognized and disregarded. Furthermore, specificity of the signal was demonstrated by using antiserum preincubated with Ci-MAM-A24, which led to the complete disappearance of the staining (data not shown).

**In situ hybridization**

To confirm the results obtained by the immunocytochemical analyses we used in situ hybridization as another, substantially different technique. In situ hybridization was performed on stomach sections as this tissue is known to accumulate higher amounts of hemocytes than others [24]. In accordance with the results obtained by immunochemistry, we localized the transcripts coding for Ci-MAM-A (NE) to the cytoplasm of small univacuolar non-refractile granulocytes (UNRGs) (Figures 2E and 2F).

**Antimicrobial activity and target cell spectrum of Ci-MAM-A24**

The minimal inhibitory concentration (MIC) and the minimal lethal concentration (MLC) of the synthetic peptide Ci-MAM-A24 were determined for a panel of Gram-negative and Gram-positive bacteria, including several pathogens of humans and marine invertebrates and fish, and two different strains of the facultative human-pathogenic yeast *Candida albicans*. Moreover, the antimicrobial activity was tested at two pH values, of which pH 5.2 represents approximately the pH present inside the vacuoles of tunicate blood cells and pH 7.4 corresponds to the pH of the hemolymph of *C. intestinalis* [14] as well as to human physiological conditions. Ci-MAM-A24 exhibited potent antimicrobial activity at both pH values and against all tested strains with minimal inhibitory concentrations in the low micromolar to nanomolar range (Tables 1, 2). Furthermore, by determining the MLC it became evident that the microorganisms are efficiently killed by the peptide excluding an activity that is entirely based on growth inhibition. No consistent pH preference of Ci-MAM-A24 was observed, but, remarkably, some bacteria were more susceptible to the peptide at pH 5.2 and others at pH 7.4. As the marine bacteria *V. alginolyticus, L. anguillarum* and *P. citreus* do not grow properly in an acidic milieu, they were tested at pH 7.4 only. In general the MIC values were comparable to that of well-known antimicrobial peptides such as cecropin P1 and magainin 1 (Table 2; [14]).

With regard to the potential use of peptides as alternative therapeutics against microbial infections, it appeared important to investigate the potent antimicrobial activity of Ci-MAM-A24 also under human physiological conditions, in particular in the presence of 150 mM NaCl. As *C. intestinalis* lives osmoconform in sea water, it was, from a biological point of view, interesting to determine the peptides activity at even higher salt concentrations. In our MIC assay, the activity of Ci-MAM-A24 turned out to be exceptionally salt resistant. Human physiological salt concentrations did not impair the peptides activity against *B. megaterium* and *E. coli*. Interestingly, *E. coli* was even more efficiently killed by Ci-MAM-A24 when 50, 100 or 150 mM NaCl were added to the buffer. At a salt concentration of 450 mM NaCl, the peptide still showed 50% of the activity when compared to the standard conditions without additional salt. In contrast to Ci-MAM-A24, the efficacy of the control peptides magainin 1 and cecropin P1 decreased substantially with increasing salt concentrations (Figure 3).
Characterization of the peptide’s mode of action

The mode of action of Ci-MAM-A24 against bacteria was investigated using two different methods, both of which can evidence whether microbial membranes are compromised by the peptide studied. In the first assay, we used the fluorescent dye SYTOX Green and B. megaterium as a target bacterium. After membrane damage the dye can enter the bacterial cell and can intercalate into the DNA whereby its fluorescence will be enhanced. Using this representative, we confirmed, that Ci-MAM-A24 is potently active against Gram-positive bacteria. Notably, the experiment demonstrated that the peptide rapidly permeabilizes bacterial membranes (Figure 4A). Furthermore, the efficacy of the Ciona peptide to induce membrane damage in viable bacteria was found to be comparable to that of cecropin P1 and magainin 1 (Figure 4B). In strong contrast to the control peptides, the membranolytic activity of Ci-MAM-A24 did not decrease when the assay was performed with buffer containing 150 mM NaCl instead of 25 mM (Figure 4B). These data impressingly confirmed the results obtained by the MIC assays in the presence of additional salt (see Figure 3).

In the second assay, we analyzed the mechanism of lysis of Gram-negative bacteria in more detail. We used the permease–deficient strain E. coli ML-35p which possesses a cytosolic β-galactosidase and a plasmid-encoded periplasmic β-lactamase. By monitoring the activity of these enzymes after adding a membranolytic peptide, permeabilization of the outer and the inner bacterial membrane can be dissected. The kinetics of membrane permeabilization monitored here by enzymatic amplification of the signal clearly show that all peptides studied were active against E. coli and compromised the barrier function of both membranes of the cells. For the outer membrane, the kinetics of Ci-MAM-A24 was intermediate between those of cecropin P1 and magainin 1. By contrast, the inner membrane was most efficiently permeabilized by Ci-MAM-A24 (Figure 5).

The lytic activity of Ci-MAM-A24 against sheep erythrocytes and human erythrocytes was inferior. At a relative high concentration of 12.5 µM of Ci-MAM-A24, only a very small percentage of human red blood cells were affected comparable to the effect of the other antimicrobial peptides used. By contrast, melittin, the major cytolytic peptide from bee venom, employed here in parallel as a positive control, lysed virtually all red blood cells at such concentrations (Figure 6).

To further characterize the peptide’s mode of action we also investigated the peptide-membrane interaction by using liposomes as a minimalistic membrane system. In order to determine the secondary structure of Ci-MAM-A24 in solution in the absence and presence of liposomes, we used CD spectroscopy. For these measurements liposomes composed of different phospholipids were used. In the absence of liposomes the peptide shows a CD spectrum typically for a random-coil structure. In the presence of liposomes composed of negatively charged phospholipids (PG and PS), the peptide undergoes a distinct conformational change and exhibits CD spectra indicating an α-helical structure (Figure 7A). The same holds true for Ci-PAP-A22 [14] which we studied in parallel for comparison (Figure 7B). In the presence of PI liposomes, for both peptides large changes in the CD spectra were observed, but the spectra differ remarkably in shape and intensity. Ci-PAP-A22 undergoes almost the same conformational change in the presence of PI as observed with PG and PS liposomes. In contrast, Ci-MAM-A24 shows a CD spectrum in the presence of PI and sPG indicative for an intermediate state of peptide/membrane interaction [38]. All other phospholipids used did not induce any structural change in both peptides.

Ci-MAM-A24 contains two tryptophan residues, one located at the N terminus and the other at the C terminus (Figure 7F). We used the tryptophan fluorescence as internal indicators of an environmental change of the peptide upon interaction with liposomes. In agreement with the results obtained by CD spectroscopy, we observed the most pronounced shift of the fluorescence in case of PS and PG liposomes (15 nm), indicating that both tryptophans are in a hydrophobic environment (Figure 7C). Interestingly, in case of PI and sPG this shift is less...
pronounced (10 nm) suggesting that only one of the two tryptophan residues is inserted in the membrane (Figure 7C). Analogous experiments were performed with liposomes composed of defined phospholipids but in the presence of 450 mM NaCl. The results clearly indicate that the ability of Ci-MAM-A24 to interact with membranes of liposomes is not affected by high salt concentrations (Figure 7D).

For measuring the potential pore-forming activity of Ci-MAM-A24 and Ci-PAP-A22, we used lipid vesicles composed of azolectin, a crude phospholipid mixture from soy bean, and monitored the liposome depolarization induced by the peptides. At both pH values tested, Ci-MAM-A24 displayed some but weak activity in this assay when compared with alamethicin, a prototype of a pore-forming peptide that was used as a positive control. However, the activity of Ci-MAM-A24 was slightly higher than that of the antimicrobial peptides Ci-PAP-A22, magainin 1 and cecropin P1 (Figure 7E).

As Ci-MAM-A24 apparently adopts an α-helical structure upon interaction with negatively charged membranes, we modeled the 24-residue peptide accordingly (Figure 7F).

DISCUSSION

In our initial in silico approach to find putative antimicrobial peptide genes in *C. intestinalis* by screening the translated EST sequences we found several genes which may well code for precursors of small cationic peptides [14]. Based on a comparable screen for hidden antimicrobial peptides, we report here the identification of a novel peptide family, all members of which resemble typical precursors of antimicrobial peptides. These often constitute a prepropeptide comprising a signal peptide, a cationic region and an anionic extension or propiece, which is removed by proteolytic cleavage to obtain the mature and active peptide. The α-defensins belong to the first antimicrobial peptides, for which this kind of peptide processing was described and investigated in detail [39,40]. Here the anionic part is proposed to neutralize the cytotoxicity of the mature cationic peptide during its biosynthesis and transport through acidic intracellular compartments [41,42]. Comparable examples from tunicates are the precursors of the styelins and clavanins which have been described from the solitary sea squirt *Styela clava* [6,7,34,35,43]. Although there is no significant sequence identity between the styelins and the Ci-MAM peptide family, there are apparent similarities concerning overall length, domain structure of the putative precursors including an anionic extension, and the amphipathic potential of the cationic region.

Accordingly, the observation that the transcription of the gene in hemocytes is markedly upregulated after challenge, gave a first hint that Ci-MAM-A may be a defense molecule. Importantly, we localized the transcript of the gene as well as the natural peptide to a distinct hemocyte type, the small univacuolar non-refractile granulocytes (UNRGs). According to our observations these cells represent a cell type closely resembling univacuolar refractile granulocytes (URGs) [44], which were initially termed compartment cells [45,46]. These cells are described as being a particularly immune competent but small subpopulation of hemocytes that are involved in synthesis of opsonic and pathogen-killing factors as well as in phagocytosis [14,44,46-49].

The synthetic peptide Ci-MAM-A24 designed according to the core region of Ci-MAM-A (NE) efficiently killed a variety of different microbes including important pathogens of humans as well as of marine fish and invertebrates. Moreover, we demonstrated that its mode of action involves membrane permeabilization. Although many of the membrane-active peptides lyse both, Gram-negative and Gram-positive bacteria, they often show preferences for either one or the other group. In most cases, Gram-positive bacteria are more efficiently affected as the outer membrane of Gram-negatives constitutes a critical barrier for many peptides. This is not the case with Ci-MAM-A24 as evidenced by the assay in which
Salt-tolerant antimicrobial peptide from *Ciona*

transgenic *E. coli* ML-35p were used to monitor separately the kinetics of permeabilization of the two membranes of Gram-negatives. Compared to cecropin P1, which is known to be exceptionally active against Gram-negative bacteria [50], Ci-MAM-A24 shows a faster perforation of the inner membrane and a less rapid but still efficient damage of the outer membrane. Ultimately, both membranes are permeabilized by the peptide, which may explain the rather uniform activity against Gram-positives and Gram-negatives as reflected by the overall low MIC values.

Notably, we observed a marked but inconsistent effect of the pH value on the efficacy of Ci-MAM-A24 towards the various bacterial species. A pH-dependent activity of antimicrobial peptides has often been ascribed to the presence of histidines, which become protonated below pH 6. The positive net charge of a cationic peptide may be substantially enhanced, and histidine-rich peptides may even switch from inactive to active molecules as it allows the binding to the negatively charged bacterial membranes [51,52]. In addition to six cationic residues the synthetic peptide Ci-MAM-A24 possesses one histidine which may be the reason for the markedly increased activity against four bacterial strains (*B. megaterium*, *B. subtilis*, *E. coli* and *P. aeruginosa*) at pH 5.2 always corresponding to three dilution steps in the MIC assay. By contrast, when activity was found to be higher at pH 7.4 as observed with four other strains the differences in the MIC values are rather small when compared to the situation found at pH 5.2. In general, there is a strong correlation between cationicity of α-helical peptides and their antimicrobial activity, but this relationship is known to be not linear and increasing positive charge does not confer increased activity beyond a certain limit [53]. Additionally, our results may indicate that the optimal positive charge of an antimicrobial peptide is dependent on the species-specific composition of the bacterial target membrane.

In a minimalistic system using the depolarization of liposomes composed of a crude phospholipid mixture as a measure of pore-forming activity, it became evident that Ci-MAM-A24 exerts detectable but relatively weak activity. The mechanism of membrane permeabilization appears to be different from that of alamethicin, the prototype of a pore-forming peptide which acts according to the barrel-stave model [54-56], and one may assume that the peptide rather acts according to the carpet or toroidal-type mechanism which may result in transient pores [4].

When we studied the interaction of Ci-MAM-A24 with liposomes of defined compositions by CD spectroscopy it became evident that the peptide undergoes a distinct conformational change in the presence of anionic phospholipids (PS, PG, sPG, and PI). As described by Chen *et al.* [38] the observed spectra in the presence of these liposomes are indicative for different orientations of helical peptides with respect to the membrane surface. In case of PG and PS, Ci-MAM-A24 is in a parallel orientation to the membrane surface, whereas in case of sPG the peptide adopts a perpendicular orientation. The same behaviour was observed with Ci-PAP-A22, the *Ciona* peptide which we measured in parallel. However, in liposomes composed of PI, the peptides behave differently: Ci-MAM-A24 is in a perpendicular and Ci-PAP-A22 in a parallel orientation. This interpretation is strengthened by the tryptophan fluorescence spectra observed for Ci-MAM-A24. The largest shift (15 nm) was observed in the presence of anionic PS and PG liposomes corresponding to both tryptophan residues embedded in a hydrophobic environment. The smaller shift (10 nm) corresponding to the embedding of only one tryptophan residue is observed for PI and sPG.

The selectivity of membranolytic cationic peptides is based on their electrostatic interaction with the negatively charged surface of the microorganisms. Hence, free ions in the surrounding media often decrease binding efficacy of the peptides and finally impair their killing efficiency. For its biological function and for potential pharmacological applications it is necessary that an antimicrobial peptide is still active at the corresponding physiological salt concentrations. As we performed our antibacterial assays under conditions of various salinities, the activity of Ci-MAM-A24 turned out to be exceptionally salt tolerant.
Salt-tolerant antimicrobial peptide from *Ciona*

The aforementioned results were further confirmed by investigating the interaction of Ci-MAM-A24 with liposomes in the presence of high salt concentration. The ability of the peptide to attach to the membrane surface of PS and PG liposomes in a parallel orientation was not impaired when the buffer contained 450 mM NaCl. With PI liposomes some quenching of fluorescence became apparent at high salt conditions.

Recently, it has been reported that an exchange from arginines to lysines in human α-defensin-1 led to a drastic decrease of its antimicrobial activity and a higher sensitivity to increasing salt concentrations [57]. Interestingly, the positive net charge of Ci-MAM-A is mainly based on arginine rather than lysine residues. One may assume that the evolutionary selection of arginines may be an adaptation to the high salt concentrations in sea water.

Another crucial point regarding the development of membrane-active antimicrobial therapeutics is that they must not destroy the membrane of mammalian cells. Compared to its antimicrobial activity, Ci-MAM-A24 showed virtually no cytotoxic activity against mammalian erythrocytes. Conclusively, the synthetic derivative of Ci-MAM-A (NE) constitutes a selective and potent broad-spectrum antimicrobial peptide, the activity of which is retained at various pH values and salt concentrations. The peptide may therefore represent a promising template for the design of novel anti-infectives.

**ACKNOWLEDGEMENTS**

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Salt-tolerant antimicrobial peptide from *Ciona*

Figure legends

**Figure 1**  Predicted secondary structure of Ci-MAM-A, multiple sequence alignment of the members of the Ci-MAM peptide family, helical wheel representation of the synthetic peptide Ci-MAM-A24 and induced transcription of the Ci-mam-A (NE) gene after immune challenge
(A) Amino acid sequence and predicted secondary structure of Ci-MAM-A (P) (NCBI GeneBank accession no. EU497244). Predicted α-helices are indicated by cylinders and the putative signal peptide is shown in dark grey (B) Alignment of amino acid sequences of Ci-MAM-A variants NE (NCBI GeneBank accession no. EU497243) and P with Ci-MAM-B to -G (TC67543, TC67549, TC71308, TC71332, TC71311 and TC71336/TC71309 from the DFCI *Ciona intestinalis* Gene Index (CinGI)). Predicted signal peptides are shaded grey and the amino acid sequence of the synthetic peptide Ci-MAM-A24 is highlighted by an underline. (C) The helical wheel projection of Ci-MAM-A24 demonstrates the amphipathic potential of the peptide’s structure. Residues are numbered starting from the N terminus of Ci-MAM-A24 and at their position on the wheel their features are indicated by dark pentagons (cationic), light rhombs (hydrophobic) and light circles (others). (D) Transcription profiling of Ci-mam-A (NE) in hemocytes of *C. intestinalis* 0, 6, 12, and 24 h post immune stimulation. The amount of template cDNA was adjusted to equal amounts using β-actin for calibration.

**Figure 2** Localization of Ci-MAM-A to small univacuolar non-refractile granulocytes (UNRGs)
For immunolocalization (A-D), an antisera raised against Ci-MAM-A24 was employed. (A, B) Ci-MAM-A (NE) was detected by using the specific antibody and a second antibody coupled to Cy3 (red). The peptide is exclusively accumulated inside the compartment of the cells. Hoechst 33258 was used to visualize the nuclei of the hemocytes (blue). (C, D) Alternatively, the anti-Ci-MAM-A antibody was detected by using a second antibody coupled to HRP. Immunoreactivity was evidenced using DAB as a substrate, which resulted in a brown precipitate. In D, besides one Ci-MAM-A-positive UNRG (black arrow), a Ci-MAM-A-negative univacuolar refractile granulocyte is shown (white arrow). Refractile inclusions of the unstained cells look greenish. (E, F) Detection of the transcript of Ci-mam-A in UNRGs by in situ hybridization on stomach sections. Hybridized DIG UTP-labelled RNA probes were detected with an anti-DIG antibody coupled to alkaline phosphatase. NBT/BCIP was used as a substrate, and a positive reaction became evident as a blue to purple precipitate. Here, the staining is restricted to the cytoplasm of the cells. All scale bars represent 5 µm. Note that the hemocytes in stomach sections slightly shrunk due to the long fixation time.

**Figure 3** Effect of salt on the antimicrobial activity of Ci-MAM-A24
Activity of Ci-MAM-A24 (circles) was compared to that of cecropin P1 (rhombs) and magainin 1 (triangles). Data were obtained from MIC assays using *B. megaterium* (black symbols) and *E. coli* (white symbols) as target cells and performed at pH 7.4 with 0, 50, 100, 150, 300 and 450 mM NaCl added. Here, the values were expressed as the median of relative activity of at least two experiments, each performed in duplicate. Activity measured using buffer without NaCl was set as 1.

**Figure 4** Membrane permeabilization of *B. megaterium* induced by Ci-MAM-A24
Membrane damage of the bacteria was measured fluorometrically using the dye SYTOX Green. The binding of the dye to the DNA in membrane-compromised target cells resulted in an increase of fluorescence. Antibacterial activity of the peptides is expressed as a percentage of permeabilized bacteria. (A) Time kinetics of membrane permeabilization induced by Ci-MAM-A24 measured for different doses at various incubation periods. (B) Effect of salt on
the membrane-permeabilizing activity of Ci-MAM-A24, cecropin P1 and magainin 1. Efficiencies of membrane permeabilization at standard conditions (25 mM NaCl) (black symbols) and in the presence of 150 mM NaCl (white symbols) are compared after 10 min of incubation of B. megaterium with each peptide at various concentrations.

Figure 5  Kinetics of permeabilization of the two membranes of gram-negative bacteria induced by Ci-MAM-A24
The efficacy of permeabilization of the outer membrane and the inner membrane of E. coli ML-35p by Ci-MAM-A24 in comparison to that of cecropin P1 and magainin 1 was measured. The final concentration of each peptide was 5 µM. Permeability of the different membranes was monitored with the fluorogenic substrates for β-lactamase and β-galactosidase at pH 7.4. As a control, bacteria were incubated under identical conditions but in the absence of the peptides.

Figure 6  Hemolytic activity of Ci-MAM-A24
Activity of Ci-MAM-A24 was determined against human (HRBC) and sheep red blood cells (SRBC) in comparison to that of other antimicrobial peptides. All peptides were tested at various concentrations up to 12.5 µM. Melittin was used as a positive control.

Figure 7  CD spectra and fluorescence spectra of Ci-MAM-A24 in the absence and presence of liposomes composed of various phospholipids, pore-forming activity and molecular model of Ci-MAM-A24
CD spectra of peptides alone are depicted in dotted lines, spectra after mixing with the different liposomes are presented as displayed in the legend. (A) CD spectra of Ci-MAM-A24. (B) For comparison, CD spectra of Ci-PAP-A22 were also recorded. (C) Tryptophan fluorescence spectra of Ci-MAM-A24 in the presence of liposomes. (D) Tryptophan fluorescence spectra of Ci-MAM-A24 in the presence of liposomes and 450 mM NaCl. (E) Liposome depolarization induced by Ci-MAM-A24 and control peptides employing azolectin vesicles. The dissipation of a valinomycin-induced diffusion potential was monitored over one minute after adding the peptides. Experiments were performed at pH 5.2 (open bars) and pH 7.4 (shaded bars). Bars and brackets represent the mean and standard deviation, respectively, of at least three independent experiments. Note the logarithmic scale of the y-axis. (F) Molecular model of Ci-MAM-A24 representing the α-helical structure adopted upon membrane interaction.
Salt-tolerant antimicrobial peptide from *Ciona*

**Figure 1**

**A**

![Diagram of the peptide](image)

**B**

**Predicted signal peptide**

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<th>Peptide</th>
<th>Sequence</th>
<th>Score</th>
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<td>Ci-MAM-A (P)</td>
<td>MDRKIVFALLLLVSLNVSTYVG</td>
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<tr>
<td>Ci-MAM-A (NE)</td>
<td>MDRKIVFALLLLVSLNVSTYVG</td>
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<td>Ci-MAM-B</td>
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<td>Ci-MAM-C</td>
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<tr>
<td>Ci-MAM-D</td>
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<td>Ci-MAM-E</td>
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<td>Ci-MAM-F</td>
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<td>Ci-MAM-G</td>
<td>MDRKIVFALLLLVSLNVSTYVG</td>
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**C**

**Putative peptide precursor**

<table>
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<th>Sequence</th>
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<tr>
<td>Ci-MAM-A (P)</td>
<td>WSSLGRSLRRLTHALKPLARSGWDQRAASLNPAPIDSELEASMDRIMQQMDEKKD</td>
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<tr>
<td>Ci-MAM-A (NE)</td>
<td>WSSLGRSLRRLTHALKPLARSGWDQRAASLNPAPIDSELEASMDRIMQQMDEKKD</td>
<td>81</td>
</tr>
<tr>
<td>Ci-MAM-B</td>
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<td>Ci-MAM-C</td>
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<tr>
<td>Ci-MAM-D</td>
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**D**

![Beta-actin graph](image)
Salt-tolerant antimicrobial peptide from *Ciona*
Figure 3

Salt-tolerant antimicrobial peptide from *Ciona*
Salt-tolerant antimicrobial peptide from *Ciona*

Figure 4

**A**

![Graph A](image)

**B**

![Graph B](image)
Salt-tolerant antimicrobial peptide from *Ciona*

**Figure 5**

**Outer membrane**

- Ci-MAM-A24
- Cecropin P1
- Magainin 1
- Control

**Inner membrane**

- Ci-MAM-A24
- Cecropin P1
- Magainin 1
- Control
Salt-tolerant antimicrobial peptide from *Ciona*

Figure 6

![Graph showing lysis percentage (%) against peptide concentration (µM) for different peptides and types of cells: Ci-MAM-A24 SRBC, Ci-MAM-A24 HRBC, Cecropin P1 SRBC, Cecropin P1 HRBC, Magainin 1 SRBC, Magainin 1 HRBC, Melittin SRBC, and Melittin HRBC.](graph.png)
Figure 7
Salt-tolerant antimicrobial peptide from *Ciona*

**TABLE 1. Antimicrobial activity of the *Ciona* peptide**

<table>
<thead>
<tr>
<th>Organism</th>
<th>pH 5.2 MIC</th>
<th>pH 5.2 MLC</th>
<th>pH 7.4 MIC</th>
<th>pH 7.4 MLC</th>
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<td><strong>Gram-positive bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus megaterium</em></td>
<td>0.02</td>
<td>0.02</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>0.02</td>
<td>0.02</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1.6</td>
<td>3.1</td>
<td>0.4</td>
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<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Gram-negative bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>0.05</td>
<td>0.05</td>
<td>0.4</td>
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<td><em>Yersinia enterocolitica</em></td>
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<td>0.8</td>
<td>0.4</td>
<td>0.4</td>
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<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>1.6</td>
<td>1.6</td>
<td>0.8</td>
<td>0.8</td>
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<td><em>Pseudomonas aeruginosa</em></td>
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<td>0.1</td>
<td>0.8</td>
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<td><em>Serratia marcescens</em></td>
<td>3.1</td>
<td>3.1</td>
<td>1.6</td>
<td>1.6</td>
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<td><strong>Fungi</strong></td>
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<tr>
<td><em>Candida albicans</em> (ATCC 10261)</td>
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<tr>
<td><em>Candida albicans</em> (SC 5314)</td>
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</table>

Growth inhibiting and microbicidal activity of Ci-MAM-A24 was determined using a microdilution susceptibility assay. MIC, minimal inhibitory concentration; MLC, minimal lethal concentration.
Salt-tolerant antimicrobial peptide from *Ciona*

TABLE 2. Activity of the *Ciona* peptide against marine bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (MLC), µM</th>
<th>Ci-MAM-A24</th>
<th>Magainin 1</th>
<th>Cecropin P1</th>
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</thead>
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<tr>
<td>Gram-positive bacteria</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Planococcus citreus</em></td>
<td>0.1 (0.1)</td>
<td>1.6</td>
<td>1.6</td>
<td></td>
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<tr>
<td>Gram-negative bacteria</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>1.6 (1.6)</td>
<td>12.5</td>
<td>0.2</td>
<td></td>
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<tr>
<td><em>Listonella anguillarum</em></td>
<td>3.1 (3.1)</td>
<td>&gt;25</td>
<td>25</td>
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</table>

Growth inhibiting and microbicidal activity was determined using a microdilution susceptibility assay. MIC, minimal inhibitory concentration; MLC, minimal lethal concentration; >, no inhibition found at the concentration indicated.