Developmental and Comparative Immunology 60 (2016) 53–65

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Identification and functional characterization of an uncharacterized antimicrobial peptide from a ciliate *Paramecium caudatum*

Pengfei Cui [a], Yuan Dong [a], Zhijian Li [a], Yubo Zhang [a], Shicui Zhang [a, b, *]

[a] Laboratory for Evolution & Development, Institute of Evolution & Marine Biodiversity and Department of Marine Biology, Ocean University of China, Qingdao, 266003, China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, China

ARTICLE INFO

Article history:
Received 31 October 2015
Received in revised form
9 February 2016
Accepted 10 February 2016
Available online 13 February 2016

Keywords:
Multi-drug resistant microbes
Antibiotics
Antimicrobial peptides
Ciliate
Paramecium

ABSTRACT

The global ever-growing concerns about multi-drug resistant (MDR) microbes leads to urgent demands for exploration of new antibiotics including antimicrobial peptides (AMPs). Here we demonstrated that a cDNA from Ciliata *Paramecium caudatum*, designated *Pcamp1*, coded for a protein with features characteristic of AMPs, which is not homologous to any AMPs currently known. Both the C-terminal 91 amino acid residues of PcAMP1, cPcAMP1, expressed in *Escherichia coli* and the C-terminal 26 amino acid residues (predicted mature AMP), cPcAMP1/26, synthesized, underwent a coil-to-helix transition in the presence of TFE, SDS or DPC. Functional assays revealed that cPcAMP1 and cPcAMP1/26 were both able to kill *Aeromonas hydrophila* and *Staphylococcus aureus*. ELISA showed that cPcAMP1 and cPcAMP1/26 were able to bind to microbe-associated molecular pattern molecules LPS and LTA, which was further corroborated by the observations that cPcAMP1 could deposit onto the bacterial membranes. Importantly, both cPcAMP1 and cPcAMP1/26 were able to induce bacterial membrane permeabilization and depolarization, and to increase intracellular ROS levels. Additionally, cPcAMP1 and cPcAMP1/26 were not cytotoxic to mammalian cells. Taken together, our results show that PcAMP1 is a potential AMP with a membrane selectivity towards bacterial cells, which renders it a promising template for the design of novel peptide antibiotics against MDR microbes. It also shows that use of signal conserved sequence of AMPs can be an effective tool to identify potential AMPs across different animal classes.

1. Introduction

Antibiotics have been successfully used for the treatment of bacterial infections in humans, preventing bacterial epidemics for nearly 70 years. However, the emergence of multi-drug resistant (MDR) microbes caused by overuse of antibiotics has resulted in the less efficacy of major antimicrobial drugs used in clinical settings (Huh and Kwon, 2011). With the global ever-growing concerns about multi- or pan-resistant pathogens, there occur urgent demands for exploration of new antibiotics, such as phytochemicals, synthetic antibiotics, antimicrobial peptides (AMPs), and inhibitors for drug-efflux pumps (Davies and Davies, 2010; Dossey, 2010; Mangoni, 2006; Nguyen et al., 2011; Theopold et al., 2004; Torres-Larios et al., 2000; Wright, 2005). Among them, AMPs are endogenous components of innate immunity that act as a first line of defense of hosts against pathogen infection, and thus a promising class of compounds with a great potential pharmaceutic application. AMPs are usually cationic and amphipathic molecules which interact with microbial membranes, and kill microbes by direct disruption of cellular components, including the microbial membrane and DNA (Epand and Vogel, 1999; Wang et al., 2015), and thus the acquisition of resistance against AMPs is very rare, in sharp contrast to conventional antibiotics (Hancock and Sahl, 2006). Accordingly, AMPs have attracted great attention for overcoming MDR microbes.

According to the online updated Antimicrobial Peptide Database (APD) (Wang et al., 2009a,b; Wang and Wang, 2004), more than 2600 AMPs have been isolated and characterized currently. AMPs are generally short, gene-coded polypeptides that can be constitutively expressed or induced to attack invading microbes. They are usually present in natural sources, such as the skin mucosa of aquatic animals, only in small quantities, thus their isolation and characterization are often rather laborious and time-consuming

* Corresponding author. Room 205, Ke Xue Guan, 5 Yushan Road, Ocean University of China, Qingdao, 266003, China.
E-mail address: sczhang@ouc.edu.cn (S. Zhang).

http://dx.doi.org/10.1016/j.dci.2016.02.016
0145-305X/

(Pukala et al., 2006). In addition, they are often produced or induced in exotic animals or their tissues as inactive precursors, complicating sample collection and assay-based identification methods. An alternative approach is to identify the genes encoding AMPs, either by directly isolating genomic DNA from small tissue samples, or by mining the vast amount of sequence information already deposited in genomic or expressed sequence tag (EST) databases (Tessera et al., 2012).

Identification of novel AMPs in databases is basically dependent on the presence of a sufficient sequence similarity, substantial number of EST data and a query sequence of a known AMP (Fedders and Leippe, 2008). However, the sequences between orthologous AMPs are considerably divergent because they are at the interface between the host and a complex and ever changing microbial biota, and are thus under strong positive selection for variation in many animal taxa (Tennessen, 2008), leading to extremely lower similarity between orthologous AMPs of even closely related species. Fortunately, the sequences of signal peptides and proregions that AMPs have in most cases tend to be significantly more conserved than mature AMPs or full-length AMPs themselves. Tessera et al. (2012) and Juretić et al. (2011) have taken this advantage successfully to search for and identify novel AMPs from databases within the same lineages of fish and amphibians, respectively. Whether such an approach can apply to animals from different lineages of more divergent species or not remains to be tested. Therefore, this study was performed to examine the possibility via using the signal peptide of the pore-forming peptide amoebapore A, a known AMP from *Entamoeba histolytica*, a species of Amoebozoa (Leippe et al., 2005; Andrä et al., 2003; Zhai and Saier, 2000), as a query to search for AMP candidates from *Paramecium* databases. Here we report the identification of an uncharacterized AMP from a species of Ciliata *Paramecium caudatum*, named as PcAMP1 hereafter, its bactericidal activity and modes of action, proving that conserved sequences of signal peptides of AMPs can be used to identify potential AMPs across different animal classes.

2. Materials and methods

2.1. Database searching

Use of conserved signal sequences as queries has been shown to be effective in identifying putative AMPs in both teleost fish and anurans from the EST database (Tessera et al., 2012; Juretić et al., 2011). BLASTP 2.2.31 + was used to search the non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF for protein sequences using the sequence MKAIVFVLIFAVAFAVTATHQ as probe, with search set, organism *Paramecium* (taxid:5884) and algorithm parameters word size: 3, matrix: BLOSUM62, gap costs: existence 11, extension 1 (Tessera et al., 2012; Sun et al., 2007). This query is the signal sequence of pore-forming peptide ameobapore A precursor (GenBank XP_653265.1), an AMP from a parasite *E. histolytica*. Sequences in the output list were inspected via examining the conservation of signal peptides, and resulting 2 hits were used as queries to further search protein databases of *Paramecium tetraurelia,* which resulted in identification of two hypothetical proteins (XP_001431782.1 and XP_001452803.1) that were predicted to contain 202 and 220 amino acid residues, respectively, and showed the characteristics of putative AMPs.

2.2. Cloning of Pcamp1 cDNA

Based on analyses above, we tried to clone the genes encoding the homologues of XP_001431782.1 and XP_001452803.1 from *P. caudatum,* which was cultured in yeast medium as described by Yuan (2010). Total RNAs were extracted from *P. caudatum* with Trizol (Invitrogen) according to the manufacturer's instructions. After digestion with recombinant RNase-free DNase (TaKaRa) to eliminate the genomic contamination, the first-strand cDNA was synthesized with reverse transcription system (Promega) using oligo d(T) primer, and used as PCR template. The fragment of XP_001431782.1 homologue, named as *Pcamp1* hereafter, was amplified by PCR with the primer pairs P1-S and P1-AS (Table 1) that were designed using Primer Premier 5.0 program on the basis of relative sequence identified in *P. tetraurelia*. After determination of the partial cDNA sequence, rapid amplification of cDNA ends (RACE) was employed to obtain the full-length cDNA. The gene-specific primer pairs P2 and P3 (Table 1) were used in RACE reactions for the cloning of 3'-end cDNAs. The 3′-RACE-Ready cDNAs were synthesized from the total RNAs using the 3′ Full RACE Core Set (TaKaRa, Dalian, China) according to the manufacturer's instructions. The products of 3′-RACE were gel-purified, sub-cloned and sequenced, and *Pcamp1* cDNA was obtained by assembling the overlapping sequences. Unfortunately, we have failed to clone XP_001452803.1 homologue although we have tried three different pairs of primers.

2.3. Sequence analyses

Pcamp1 cDNA obtained was analyzed for coding probability with the EditSeq in DNASTAR software package (DNASTAR Inc., USA), and sequence comparison was performed using the MegAlign program by CLUSTAL W method (Chenna et al., 2003). The SMART program (http://smart.embl-heidelberg.de/) was used to predict the functional sites and domains, and the SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) used to predict the signal peptide. The molecular mass (MW) and isoelectric point (pI) of the predicted mature peptide were calculated by ProtParam (http://www.expasy.ch/tools/protparam.html), and the cut site and the position of the mature peptide predicted by PeptideCutter (http://web.expasy.org/peptide_cutter/) and CAMP (http://www.camp.bicnirrh.res.in/predict/). The Jpred program (http://www.compbio.dundee.ac.uk/www-jpred/index.html) was used to predict the secondary structure, and the QUARK ONLINE (http://zhanglab.ccmb.med.umich.edu/QUARK/) to generate three-dimensional (3D) structure model. Antimicrobial Peptide Calculator and Predictor at APD (http://aps.unmc.edu/AP/main.php) were used to calculate the total hydrophobic ratio and net charge.

Helical wheel of the predicted mature AMP was created by the internet site "helical wheel projection" (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi) developed by Armstrong and Zidovetzki (Appadu, 2012).

2.4. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed to assay the expression of *Pcamp1* in response to challenge with the microbial signature molecule LPS, as

Table 1
Sequences of the primers used in this study.

Primers	Primer sequences (5′–3′)
P1-S	TTCCCAAAGAACAAGAGG
P1-AS	ATGCCATAAAGCCAACAG
P2	TGGTCGGCATATTCATCT
P3	GCAGAATCAACTAATAGTACAAAAT
P4-S	CTCTAAATGCTTAATTGCTAGGAGGG
P4-AS	AGCCAACAGATGAATATGCCGAC
P5-S	CACTTGCTCCATCATCCA
P5-AS	TTTCGTCGTATTCGCTTC
P6	CGGAATTCCAATTGCTAGGAGGGAAAGTATCAT
P7	CCGCTCGAGTACTATCAAAGAAAACCATGAC