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ORIGINAL ARTICLE

Amphibian cathelicidin fills the evolutionary gap of cathelicidin in vertebrate

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Abstract Cathelicidins comprise a family of antimicrobial peptides (AMPs) sharing a highly conserved cathelin domain, and play a central role in the innate defense against infection in most of vertebrates. But so far it has not yet been found in amphibians although a large number of other groups of AMPs have been identified. In the current work, the first amphibian cathelicidin (cathelicidin-AL) has been characterized from the frog skin of *Amolops loloensis*. Cathelicidin-AL (RRSRRGRGGGRRGGSGGRGGRGGGGRSGAGSSIAGVGSRGGGGGRHYA) is a cationic peptide containing 48 amino acid residues (aa) with 12 basic aa and no acidic aa. The chemical synthesized peptide efficiently killed bacteria and some fungal species including clinically isolated drug-resistance microorganisms. The cDNA encoding cathelicidin-AL precursor was cloned from the skin cDNA library of *A. loloensis*. As other cathelicidins, the precursor of cathelicidin-AL also contains highly conserved anionic cathelin domain of cysteine proteinase inhibitor followed by the AMP fragment at C-terminus. Phylogenetic analysis revealed that as connecting link, the amphibian cathelicidin predates reptilia but postdates fish cathelicidin. The peptide purification combined with gene cloning results confirms the presence of cathelicidin in amphibians and filled the evolutionary gap of cathelicidin in vertebrate, considering amphibians' special niche as the animals bridging the evolutionary land-water gap.

Keywords Cathelicidin · Amphibian · Evolution · Structure and function

X. Hao, H. Yang, and L. Wei have the same contribution to this paper.

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Introduction

Cathelicidins are a family of structurally diverse antimicrobial peptides found in virtually all species of mammals, which play a critical role in innate immune system that can provide a first line of defense against a variety of microorganisms (Ramanathan et al. 2002; Zanetti 2005). Cathelicidins are characterized by N-terminal signal peptide (30 residues), and a highly conserved cathelin domain (99–114 residues long) followed by a C-terminal mature peptide (12–100 residues) that is characterized by a remarkable

structural diversity (Zanetti et al. 2002). After the first cathelicidin, Bac5, was discovered from bovine neutrophils, a large number of cathelicidins have been identified from most of vertebrates including fish, snake, bird, and mammalian, that is, human, monkey, mouse, rat, rabbit, guinea pig, pig, cattle, sheep, goat, and horse (Turner et al. 1998; Xiao et al. 2006; Zaiou and Gallo 2002), whereas no cathelicidins have yet been found in amphibians. It seems that the cathelicidin evolution encounters a gap due to the absence of amphibian cathelicidin. The molecular evolutionary analyses of vertebrate cathelicidins will be greatly improved by obtaining cathelicidin sequences in amphibian species.

It is well known that more than 600 antimicrobial peptides belonging to more than 30 families have been characterized in amphibians, but so far no cathelicidin-derived antimicrobial peptides have ever been found in them, although some amphibian genomes have been sequenced. In the current work, we firstly report the molecular cloning and purification of the cathelicidin from amphibian species, *Amolops loloensis*.

Materials and methods

Skin secretion collection

According to previous methods (Gennaro and Zanetti 2000; Ramanathan et al. 2002; Zanetti 2005; Zanetti et al. 2002), adult specimens of *A. loloensisi* of both sexes ($n = 30$; weight range 30–40 g) were collected in Yunnan Province of China. Skin secretions were stimulated by anhydrous ether. Frogs were put into a cylinder container. A piece of absorbent cotton immersed with anhydrous ether was put on the top of the container. The container was covered with a lid and permeated with volatilized anhydrous ether. Being stimulated by anhydrous ether for 1–2 min, frog skin surface was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each frog with 0.1 M NaCl solution containing protease inhibitor cocktail (Sigma). Collected solution (500 ml) was quickly centrifuged and the supernatants were lyophilized. All the animal experiments were approved by Kunming Institute of Zoology, Chinese Academy of Sciences.

Peptide purification

Lyophilized skin secretion sample of *A. loloensisi* (1.2 g, total OD280 nm of 300) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0 (PB). The sample was applied to a Sephadex G-50 gel filtration column (Superfine, Amersham Biosciences, 2.6 cm × 100 cm) equilibrated with 0.1 M PB. Elution was performed with the same buffer, with collecting fractions of 3.0 ml. The absorbance of the eluate was monitored at 280 nm. The antimicrobial activity of fractions was determined as indicated below. The protein peak containing antimicrobial activity was pooled, lyophilized, and re-suspended in 2 ml 0.1 M PB, and purified further by C18 reverse phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C18, 30 cm × 0.46 cm) column.

Structural analysis

Amino sequence of cathelicidin-AL was determined by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. The mass spectrometry analysis was performed by positive ion and linear mode on a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF–MS) AXIMA CFR (Kratos Analytical), with specific parameters of ion acceleration voltage of 20 kV, accumulating time of single scanning of 50 and ±0.1% accuracy of mass determinations. Polypeptide mass standard (Kratos Analytical) was used as external standard.

Construction and screening of the skin cDNA library

Total RNA was extracted from the frog skin using RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The cDNA was synthesized by SMART™ techniques as per a previous report (Valenzuela et al. 2000). The synthesized cDNA was used as template for PCR to screen the cDNAs encoding the antimicrobial peptide (cathelicidin-AL). Two pairs of oligonucleotide primers, (S1: 5′-(A/C)G(A/G/C/T)(A/C)G(A/G/C/T)TC(A/G/C/T)(A/C)G(A/G/C/T)(A/C)G(A/G/C/T)GG(A/G/C/T) (A/C)G(A/G/C/T)(A/C)G(A/G/C/T)GG-3′ and primer II A: 5′-AAGCAGTGGTATCAACGCAGAGT-3′; S2: 5′-(A/G/C/T)GC(A/G)TA(A/G)TG(A/G/C/T)C(T/G)(A/G/C/T)CC(A/G/C/T)CC(A/G/C/T)CC-3′ and primer II A) were used in PCR reactions. Primers S1 and S2 are designed from the amino acid sequence of cathelicidin-AL determined by Edman degradation. The DNA polymerase used was Advantage Polymerase from Clontech. The PCR conditions were 2 min at 94°C, followed by 30 cycles of 10 s at 92°C, 30 s at 50°C, and 40 s at 72°C. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Expression profile of the tissues

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to analyze gene expression of cathelicidin-AL in *A. loloensi*. Total RNA extraction from different

tissues was extracted using TRIzol (Life Technologies, Ltd.). First-strand cDNA was synthesized by using a PrimeScript® RT-PCR Kit (Takara). The primers were, forward primer, 5′-cathelicidin: 5′-GCATGGGGCTCTCT GYAACT-3′, and reverse primer, 3′-cathelicidin: 5′-GC TCTGGCAGGTCACCACAAT-3′. PCR conditions were: 95°C (4 min), and 30 cycles of 95°C (30 s), 53°C (30 s), 72°C (45 s) followed by an 8 min extension period at 72°C. The control PCR was performed using the specific primers (forward primer, 5′-actin 5′-CCTCATGCCATC ATGCGTCT-3′, and reverse primer, 3′-actin 5′-GCTGAT CCACATCTGCTGGAA-3′) for rana actin, using the same conditions as above.

Alignment and phylogenetic analysis of amphibian cathelicidin

Multiple sequence alignments were constructed by using the ClustalW program (version 1.8) on basis of tens of known cathelicidin precursors. Multi-cathelicidin sequences were obtained from the protein database at the National Center for Biotechnology Information (NCBI). The phylogenetic trees were constructed using a neighbor-joining method (MEGA4.0), by calculating the proportion of amino acid differences (*p* distance) among all sequences. A total of 1,000 bootstrap replicates were used to test the reliability of each branch. The numbers on the branches indicate the percentage of 1,000 bootstrap samples supporting the branch.

Peptide synthesis

Cathelicidin-AL (RRSRRGRGGGRRGGSGGRGGRGGGGRSGAGSSIAG VGSRGGGGGRHYA) was synthesized by solid phase synthesis on an Applied Biosystems model 433A peptide synthesizer according to the manufacturer's standard protocols. After linker cleavage and side-chain deprotection, the crude synthetic peptide was purified on a Vydac C18-RP-HPLC column (25 cm × 1 cm) using a linear gradient of acetonitrile in water containing 0.1% Trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. Identity of the peptide was confirmed by automated Edman degradation with a protein sequencer and MALDI-TOF–MS analysis.

Antibacterial assays

A modified broth microdilution assay was used to determine the antibacterial activity of cathelicidin-AL, as previously described (Feng et al. 2011; Wang et al. 2010). Briefly, bacteria were subcultured to the midlogarithmic phase, at 37°C and suspended to 5×10^5 colony-forming units (CFU)/ml in Mueller-Hinton broth (MH). The peptide was subjected to serial dilutions in MH, and then 50 µl of the diluted sample was dispensed into a 96-well microtiter plate and mixed with 50 µl of bacteria or yeast inoculums in MH. The microtiter plate was incubated at 37°C for 18 h for bacteria; 30°C for 48 h for fungus, and the absorbance at 595 nm was measured using a microtiter plate spectrophotometer. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide that completely inhibits growth of the microbe determined by visual inspection and spectrophotometric examination.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed to study the possible mechanisms of action of cathelicidin-AL on *S. aureus* (ATCC25923) according to the methods described by Friedrich et al. with minor modification (Che et al. 2008; Friedrich et al. 2000; Li et al. 2007). Exponential phase bacteria were treated with the peptides (62.5 µg/ml) for 30 min at 37°C. This concentration was used to see an effect on a greater percentage of cells. After treatment, the bacteria were centrifuged at 300 × *g* for 10 min, and the pellets were fixed with 2.5% buffered glutaraldehyde for 1 h. The cells were then postfixed in 1% buffered osmium tetroxide for 1 h, stained en bloc with 1% uranyl acetate, dehydrated in a graded series of ethanol washes, and embedded in white resin. The buffer used was 0.1 M sodium cacodylate, pH 7.4. Thin sections were prepared on copper grids using an LKB-V microtome and stained with 1% uranyl acetate and lead citrate. The resin and grids were purchased from Marivac (Halifax, Nova Scotia, Canada). Microscopy was performed with a JEM1011 microscope under standard operating conditions.

Hemolysis

Hemolysis assays were conducted as previously described (Bignami 1993). The cathelicidin-AL (20 µg/ml) was incubated with washed human erythrocytes at 37°C for 30 min and centrifuged at 1,000*g* for 5 min. The absorbance of supernatant was measured at 540 nm; 1% (v/v) Triton X-100 was used to determine the maximal hemolysis. The experiment was repeated three times.

Cytotoxicity assay

In vitro cytotoxicity of cathelicidin-AL was examined using human umbilical vein endothelial cells (HUVEC) and raw 264.7 murine macrophage cells (RMMCs). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, 11960-044, Gibco, USA) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 U/ml of streptomycin in a humidified 5% CO_2

atmosphere at 37°C. Cells (2×10^4 per well) were seeded in 96-well plates and cultured overnight until they adhered to the plate. Various concentrations of cathelicidin-AL dissolved in the corresponding culture medium were added to the wells and the plates were incubated at 37°C for 48 h. Cytotoxicity of cathelicidin-AL was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (Mosmann 1983). IC_{50} was defined as the concentration of cathelicidin-AL at which the absorbance at 490 nm was reduced by 50%.

Statistics

Data analysis was performed using the Statistical Package for Social Science (SPSS 11.5). Statistical analysis data were presented as mean ± standard deviation (SD).

Results

Purification and structural analysis of amphibian cathelicidin-cathelicidin-AL

The supernatant of *A. loloensis* skin secretions was divided into several peaks by Sephadex G-50 and the fraction indicated by an arrow was tested to have antimicrobial activity (Fig. 1a). Thus this peak was next applied to C18 RP-HPLC column. More than 20 peaks were obtained from this separation as indicated in Fig. 1b. The eluted peak was found to exert antimicrobial activity (marked by an arrow) was collected and purified further by the same C18 RP-HPLC column (Fig. 1c). The purified peptide (marked by an arrow) was named cathelicidin-AL. The complete amino acid sequence by Edman degradation of cathelicidin-AL was determined as RRSRRGRGGGRRGGSGGRGGRGGGGRSGAGSSIAGVGSRGGGGGRHYA. The observed $[M + H]^+$ from MALDI-TOF–MS were 4453.286 (Fig. S1), which matched well with the calculated $[M + H]^+$ of 4452.77. The amino acid sequence of cathelicidin-AL was further confirmed by the cDNA cloning as described below.

Identification and characterization of the amphibian cathelicidin

The designed primers successfully amplified cathelicidin sequences from frog. As illustrated in Fig. 2, a cDNA of 651 bp was cloned from the cDNA library of *A. loloensisi* skin. This cDNA encodes a protein precursor composed of 179 amino acid residues (aa) (GenBank accession numbers JF923766). The coding sequence (CDS) for cathelicidin cDNA has only one open reading frame (ORF). As other members of cathelicidin family, precursor of cathelicidin-

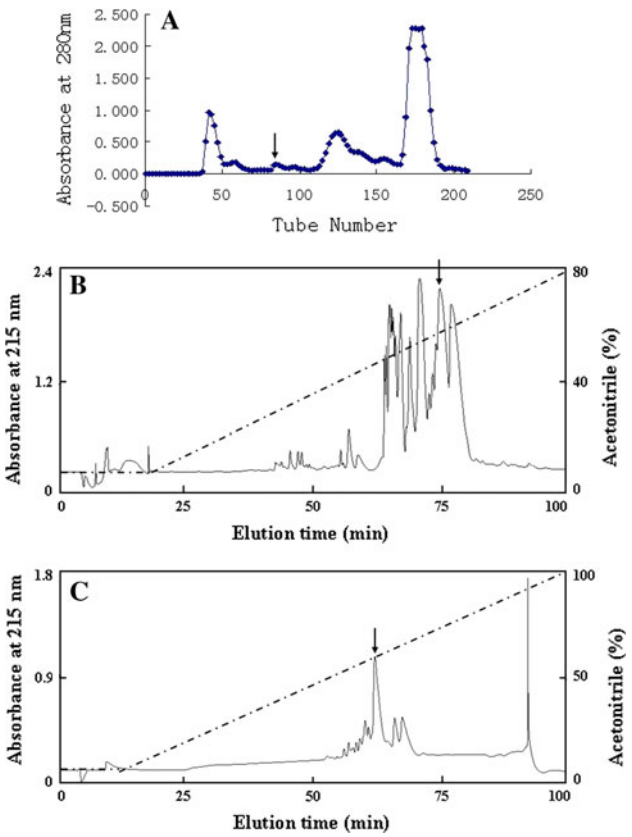

Fig. 1 Purification of the cathelicidin from skin secretion sample of *A. loloensisi*. **a** Gel filtration chromatography. Sephadex G-50 column (Superfine, Amersham Biosciences, 2.6 cm × 100 cm), equilibrated and developed with 0.1 M PBS. **b**, **c** C18 reverse phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C18, 30 cm × 0.46 cm) column, equilibrated with 0.1% (v/v) TFA/water, elution was performed with an acetonitrile liner gradient. The purified peptide with antimicrobial activity is indicated by an *arrow*

AL also includes a predicted signal peptide, a conserved cathelin domain and a cationic C-terminal mature antimicrobial peptide. Computational prediction with SignalP 3.0 software package indicates a 20 aa signal peptide located at its N-terminus (Fig. 2). Multiple cathelicidin sequences alignment of 17 typical animal species is shown in Fig. 3. Consistent with all other cathelicidins except for as CATH-1 from Atlantic salmon, cathelicidin-AL also contains four cysteine residues in the conserved region of the putative prepropeptide (Bals and Wilson 2003) (Fig. 3).

The processing of cathelicidin to generate mature antimicrobial peptides has been studied both in vitro and in vivo. Since the valine and alanine represent the most common elastase-sensitive residues (Shinnar et al. 2003), we reasoned that the valine (131) in the precursor of cathelicidin-AL is likely to be cleaved by frog elastase, which is generally considered to be responsible for such processing in fish, bird and mammals. Therefore, the mature antimicrobial peptide, cathelicidin-AL, was predicted (Fig. 2): RRSRRGRGGGRRGGSGGRGGRGGG

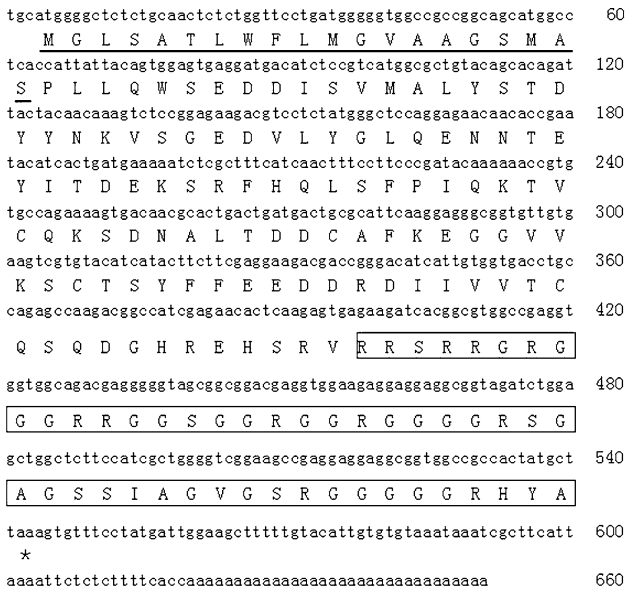

Fig. 2 The cDNA sequence encoding cathelicidin-AL and the predicted precursor amino acid sequence. The amino sequence of purified cathelicidin-AL is *boxed*. The stop codon is indicated by an *asterisk* (*). The predicted signal sequence by SignalP 3.0 software package is *underlined*

GRSGAGSSIAGVGSRGGGGGRHYA (48 aa), which supported the correction of structural characterization result of purified cathelicidin-AL. Analysis using ProtParam tool (http://au.expasy.org/tools/protparam.html) showed that theoretical pI/Mw for cathelicidin-AL is 12.70/4452.77. The putative cathelicidin-AL is a highly basic peptide, comprising as many as 12 basic Arg residues, which implies that it would be readily attracted and adhered to the negative-charged bacterial surface, and explains its high antimicrobial potency.

Expression profile of amphibian cathelicidin

Using actin as control, expression pattern of cathelicidin-AL was investigated by RT-PCR. Tissue distribution of cathelicidin-AL expression is illustrated in Fig. 4. All the selected tissues except brain, including gallbladder, liver, kidney, intestine, stomach, skin, muscle, heart, lung, spleen can express this gene. Cathelicidin-AL was found much more expressed in lung, than in kidney, intestine, heart, stomach and spleen. Comparatively, less expression was observed in gallbladder, liver and skin. Almost no cathelicidin-AL expression was detected in the brain.

Comparative and phylogenetic analysis of vertebrate cathelicidins

The phylogenetic analysis of cathelicidin-AL with almost all known cathelicidins using the neighbor-joining method was performed. Multi-sequence alignment was performed on basis of the full sequence of all cathelicidins. A condensed multifurcating tree was constructed emphasizing the reliable portion of pattern branches without considering the exact distance between each peptide. Thus, the branch lengths of the condensed tree are not proportional to the number of amino acids mutations.

The built phylogenetic tree reveals that vertebrate cathelicidins are split into two major clusters, and the sister group is represented by fish cathelicidins in a separated clade, which was potentially considered as an ancient member in the cathelicidin evolution. The second cluster is again divided into two major groups. The amphibian cathelicidin-AL marked by a triangle is coordinate by snake, avian and the most divergent mammalian cathelicidin families (Fig. 5). This phylogenetic analysis clearly indicates that as connecting link, the amphibian cathelicidin predates reptilia but postdates fish cathelicidin.

Antibacterial assays

To determine the antimicrobial activity, cathelicidin-AL was commercially synthesized and purified to >95% purity, and used for all antimicrobial assays. As listed in Table 1, cathelicidin-AL showed broad-spectrum antimicrobial activities against tested microorganisms, especially those clinical-isolated drug-resistant strains. Cathelicidin-AL showed the highest antimicrobial activity against *S. aureus* ATCC25923, *S. aureus* ATCC43300 and *P. aeruginosa* ATCC27853, all with a MIC value of 6.25 μg/ml. The MIC values against P. aeruginosa PA 01was determined to be 12.5 μg/ml. Comparatively, cathelicidin-AL showed a relatively lower activity against *E. coli* ML-35P with MIC of 25 μg/ml. However, cathelicidin-AL was observed to have weak killing activities to *B. Subtilis* and fungus *C. albicans* ATCC2002.

Transmission electron microscopy

To study the possible mechanisms of action of the cathelicidin-AL on Gram-positive, *S. aureus* (ATCC25923), transmission electron microscopy was performed on thin sections of bacteria that had been treated with the peptide for 30 min. Control cells of *S. aureus* (ATCC25923) are shown in Fig. 6a. Cathelicidin-AL produced disruption of *S. aureus* cell membrane as exemplified by Fig. 6b. Apparently, cathelicidin-AL severely destroyed the cell walls and membranes of bacteria with some mesosomes being found in the treated bacterium, and the interface between the cell walls and membranes was blurred (Fig. 6b). The mesosomes likely led to the breaking of the cell wall. Mesosomes must be regarded as being indicative

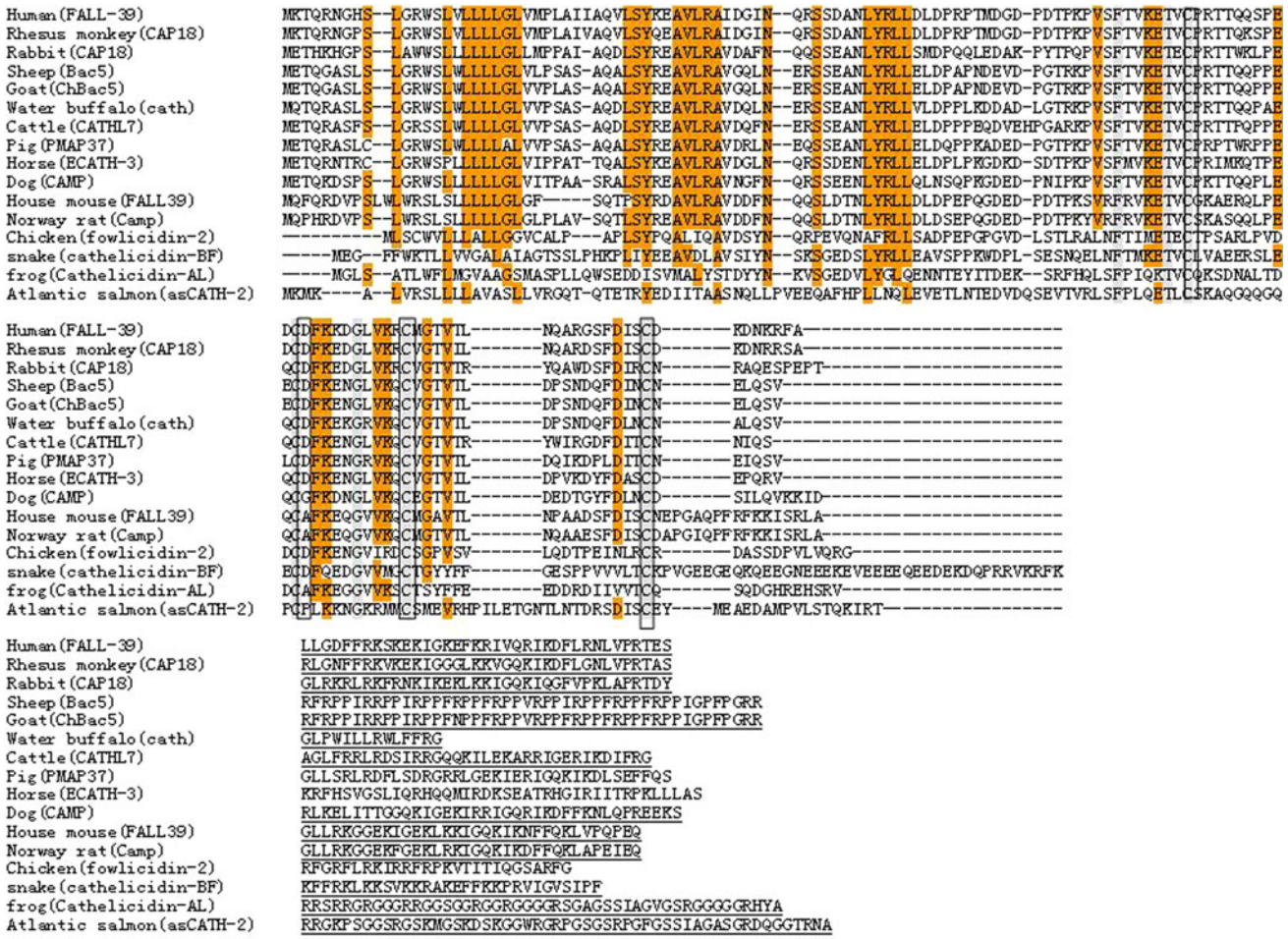

Fig. 3 Multiple sequence alignment of amphibian cathelicidin-AL with other representative cathelicidins. The conserved residues are *shaded*. The four conserved cysteine residues in cathelin domain are *framed*. Mature cathelicinds are *underlined*

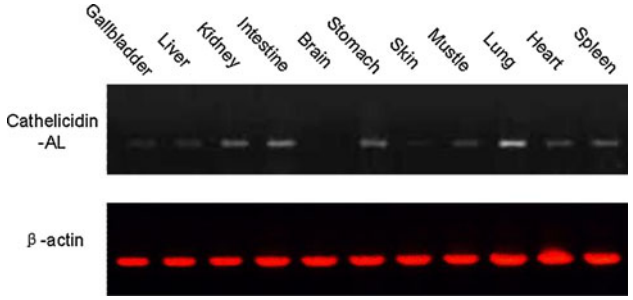

Fig. 4 RT-PCR analysis of cathelicidin gene expression pattern in various frog tissues using gene-specific primers with actin as control

of cytoplasmic membrane alteration (Friedrich et al. 2000). Blebs are clearly visible on the outer membrane, confirming the membrane damage. These results confirm that cathelicidin-AL interacts primarily at the bacterial cytoplasmic membrane level.

Hemolysis, cytotoxicity

It is generally known that the possible limitation to the clinical applications of antimicrobial peptides as antibiotics is their potential to cause injury to mammalian cell membranes. Here, the hemolytic activity of the cathelicidin-AL was examined using the freshly prepared human erythrocytes. The result showed that cathelicidin-AL displayed negligible hemolytic activity on human erythrocyte even with peptide concentrations up to 0.4 mg/ml, much higher than corresponding MICs, suggesting the considerable selectivity of cathelicidin-AL for microorganisms over mammalian cells in vitro.

The MTT method was exploited to evaluate the cytotoxicity of cathelicidin-AL toward two mammalian cell lines, HUVEC and Raw 264.7. At the same concentration (0.4 mg/ml), cathelicidin-AL was neither cytotoxic for HUVEC nor for RMMCs, suggesting the potential safety for therapeutic application.

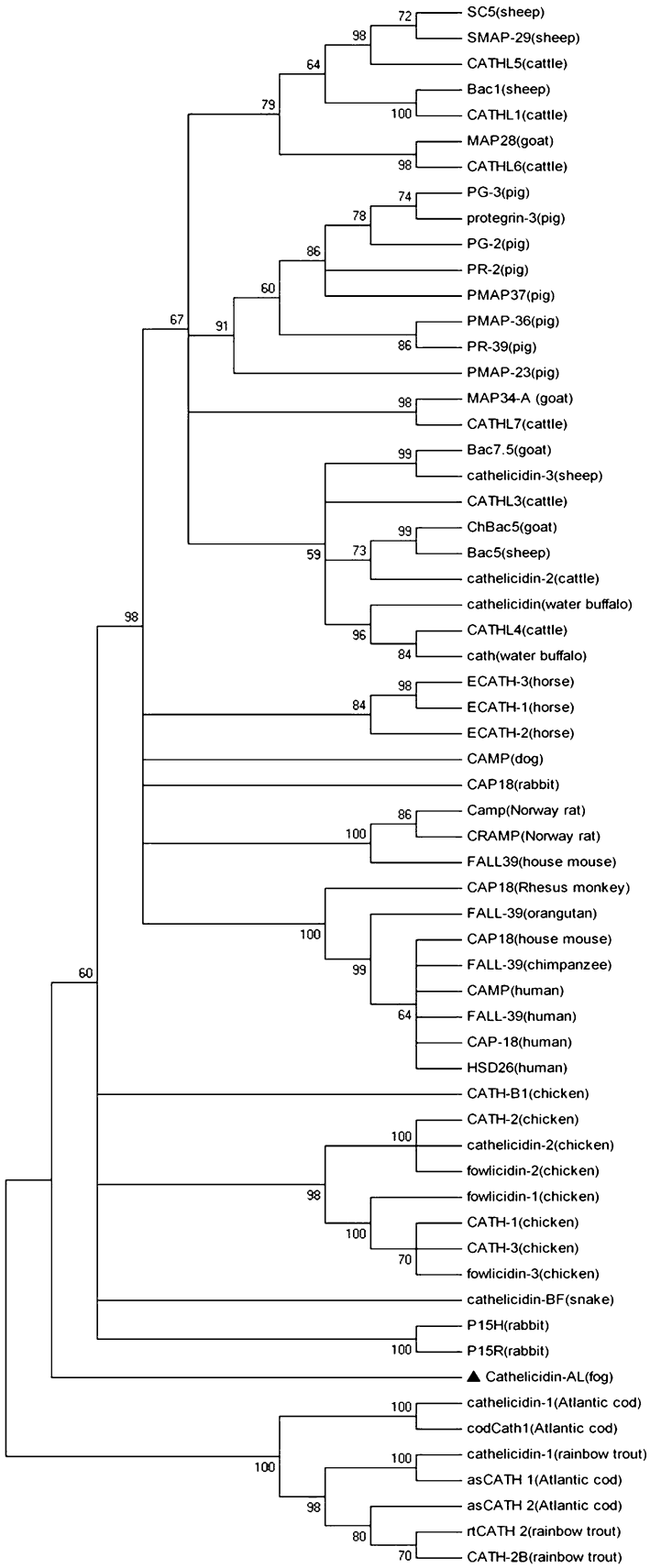

Fig. 5 Phylogenetic analysis of representative vertebrate cathelicidins. The phylogenetic dendrogram was constructed by the neighbor-joining method based on the proportion difference of aligned amino acid sites of the full sequence of prepropeptide. Only bootstrap values >50% (expressed as percentage of 1,000 bootstrap samples supporting the branch) are shown at branching points. The *bar* indicates the branch length. Amphibian cathelicidin-AL is marked with a *triangle*

Table 1 Antimicrobial activity of cathelicidin-AL

Microorganisms	MIC (μg/ml)
S. aureus ATCC25923	6.25
S. aureus ATCC43300	6.25
B. subtilis	100
C. albicans ATCC2002	50
E. coli ML-35P	25
P. aeruginosa PA01	12.5
P. aeruginosa ATCC27853	6.25

MIC minimal inhibitory concentration. These concentrations represent mean values of three independent experiments performed in duplicates

Discussion

Cathelicidins and defensins are two major AMP families in mammals (Lehrer and Ganz 2002a; Zaiou and Gallo 2002; Zanetti et al. 1995). Whereas the defensin structure is based on a common beta sheet core stabilized by three disulfide bonds (Lehrer and Ganz 2002b), cathelicidin-derived AMPs are highly heterogeneous with linear structure. Current identification of amphibian cathelicidin provided a good opportunity to elucidate the evolutionary relationship of vertebrate cathelicidins. Members of the cathelicidin family typically have an N-terminal signal peptide, a highly conserved prosequence known as the cathelin region, and a structurally variable mature peptide at the C-terminus that has broad antimicrobial activity.

Cathelicidin-AL consists of linear cationic sequences at the C termini, which are expected to be freed from the cathelin domain to become biologically active. Indeed, putatively mature cathelicidin-AL was approved by the peptide purification. Both the signal and cathelin regions of cathelicidin-AL are more distantly related to the mammalian cathelin regions than the reptile cathelicidin from snake, cathelicidin-BF (Wang et al. 2008) (Fig. 3).

Although the structure of cathelicidin-AL is significantly different with other cathelicidin family members, it still possesses potent antibacterial activities for bacteria comparable to some representative cathelicidin-derived AMPs of highly active, such as snake cathelicidin-BF (Wang et al. 2008) (Table 1), A notably atypical feature of cathelicidin-AL is that it has as many as 23 glycine residues located in mature peptide region. In addition, throughout the cathelicidin-AL mature peptide sequence, 12 arginines are only resources of positive charges, which is unlike most typical cationic AMPs that contain both lysines and arginines.

Cathelicidins are most abundantly present in circulating neutrophils and myeloid bone marrow cells (Gennaro and Zanetti 2000), and also found in mucosal epithelial cells and skin keratinocytes (Zanetti 2004). In contrast, RT-PCR

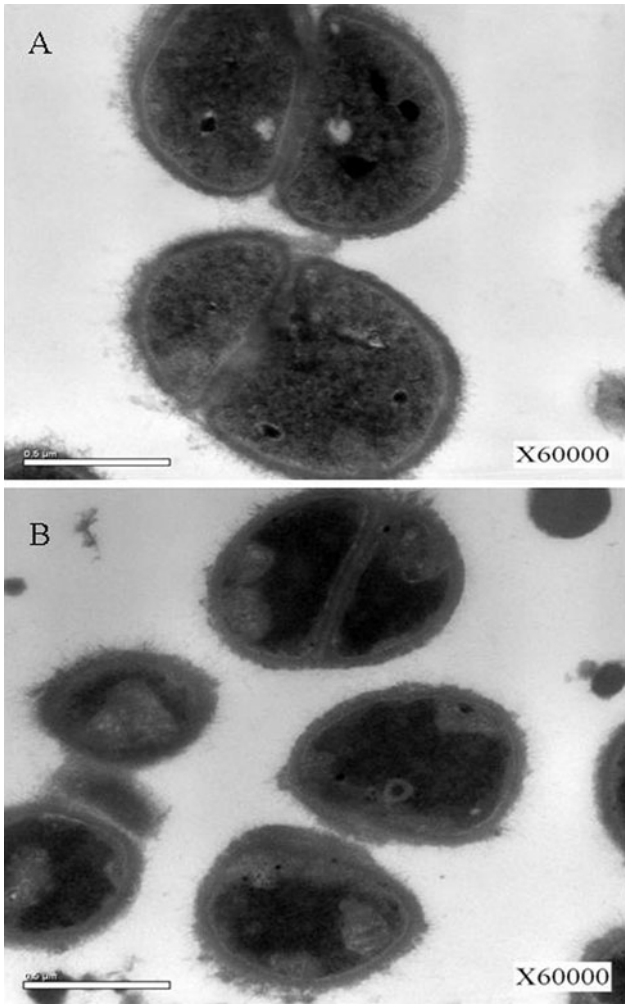

Fig. 6 Ultrastructure of *S. aureus* (ATCC25923) treated with the tested samples (62.5 μg/ml): **a** control, untreated (TEM); **b** ×10 MIC cathelicidin-AL (62.5 μg/ml) (TEM). *Bar* 500 nm

expression pattern analysis of cathelicidin-AL indicates that it is much more expressed in lung, than in kidney, intestine, heart, stomach and spleen. Comparatively, less expressions were observed in gallbladder, liver and skin, which is considered as the main reservoir for amphibian AMPs (Lu et al. 2010). This remarkable distribution pattern suggests the following hypothesis. Frog's tissues that express cathelicidin most may secrete cathelicidin-AL peptide, and then have it recruited to the skin, as a result which can be isolated by peptide purification, although cathelicidin expression level in skin is fairly low. An understanding of the mechanism for processing the antimicrobial peptide is needed to confirm or refute this hypothesis.

The phylogenetic tree constructed in this paper suggests that in the course of evolution, amphibian cathelicidins play a role of connecting bridge, predating reptilia but postdating fish cathelicidin.

In conclusion, amphibian cathelicidin was firstly identified in the present work by molecular cloning and peptide purification procedures. The nucleotide and deduced amino acid sequences of frog cathelicidin was comparatively conserved among different species. Cathelicidin-AL is an antimicrobial defense element with the functional potential and appropriate localization to guard the frog itself against invasion by viable microbes.

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Conflict of interest The authors declare that they have no conflict of interest.

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