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Short sequence report

Characterization of a NK-lysin antimicrobial peptide gene from channel catfish

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Natural killer (NK) cells and cytotoxic T lymphocytes (CTL) are important and powerful components of mammalian host defenses against a broad range of microbial assaults. These cell types, upon stimulation, are known to release cytolytic granules toward target cells (reviewed in [1,2]). These granules contain proteins, including perforin and the granzyme family, capable of causing membrane perturbation and eventual cell apoptosis. Given the potency and widespread efficacy of these molecules, immunologists have long been interested in capturing and investigating other protein components contained within cytolytic granules. Examination of genes expressed late (3–5 days) after T-cell activation [3] led to the identification of an mRNA initially designated 519. Further characterization of 519 localized its protein to cytolytic granules of CTLs and NK cells and found that it possessed lytic activity against tumor cells [4,5]. The renamed protein, granulysin (reviewed in [6]), was subsequently discovered to have broad lytic abilities against bacteria, fungi, protozoa, and parasites [7–9]. Most notable was the effective killing of intracellular *Mycobacterium tuberculosis* by granulysin in combination with perforin [7].

NK-lysin, the probable porcine homolog of human granulysin, was isolated from pig intestine and possesses similar structure and antimicrobial properties [10–13]. Both granulysin and NK-lysin are

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members of the saposin-like protein (SAPLIP) family. Saposins (reviewed in [14]) are involved in sphingolipid catabolism and contain conserved cysteine and hydrophobic residues. Amoebapores, lytic peptides contained within the cytolytic granules of the protozoan parasite, *Entamoeba histolytica*, also fall within the saposin family, demonstrating the conservation of the saposin-like NK-lysin proteins.

A single copy gene for granulysin in humans encodes two alternatively spliced mRNAs, 519 and NKG5 [15]. Because of the shorter peptide sequences of 519, it has been used in much of the follow-up work. The 519 mRNA encodes a 15 kDa protein non-lytic precursor molecule predominantly present at the low pH condition of the cytolytic granules. This precursor is post-translationally processed to a 9 kDa lytic molecule (74 amino acids) which only attains its full activity upon release into the higher pH of the extracellular environment. This mature lytic molecule of 74 amino acids corresponds closely to the conserved SAPLIP domain [4,16]. This processing, therefore, represents a self-protective mechanism for the cell. Similar processing steps result in the generation of the lytic 9 kDa (78 amino acids) NK-lysin molecule from the 129 amino acid precursor in porcine. The active peptide in NK-lysin also corresponds with the SAPLIP domain and shares 35% amino acid identity to granulysin.

Although the mechanism of granulysin/NK-lysin induced cell death is not completely understood, some steps are clear. Granulysin disrupts the cell membrane, perhaps with the aid of perforin [7], increases intracellular Ca^{2+}, and directly damages the mitochondria, leading to eventual cell apoptosis [17–19].

With the emergence of antibiotic-resistant bacteria, and the scarcity of new classes of useful antibiotics, there is an increasing need to identify novel antimicrobial peptides like NK-lysin from various species for the development of alternative therapeutics for both human and animal medicine. However, sequences have been reported from only a minimal number of fish species [20–26], the largest vertebrate group containing over 23,000 species [27].

Although a bovine homolog has recently been identified [13], the status of the NK-lysin/granulysin antimicrobial class in lower vertebrates is unknown. As part of a larger effort to characterize the catfish innate immune system, we report here identification, molecular cloning and characterization, and expression of a NK-lysin gene from channel catfish (*Ictalurus punctatus*).

Three clones of channel catfish NK-lysin cDNA were initially identified from analysis of expressed sequence tags (ESTs) [28]. Sequence alignment revealed that all three cDNAs were transcripts from a single gene. All three clones harbor complete cDNA and they were completely sequenced from both strands by using universal and reverse sequence primers to obtain the full cDNA sequences. High-density filters of a channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA). Each set of filters contained 10-genome coverage of the channel catfish BAC clones from BAC library CHORI 212 (http://bacpac.chori.org/library.php?id=103). As part of ongoing efforts to physically map important genes, an overgo probe was designed based on the shared cDNA sequence and hybridized to the catfish BAC library. Sequences of the overgo primers are shown in Table 1.

Table 1
Primers and their sequences used in this study

Primer name	Sequences (5′ to 3′)
Overgo primers	CCTGTGCAATGCACATGGAATACC
	GCAGAGTCAACTCTCAGGTATTCC
RT-PCR primers	CCTGTGCAATGCACATGGAATACC
	TCTTGCAGAGACCTCGAAGG
PCR Primer for TA-cloning	TGGAACCTCCTCGTTGCTTC
	ATCTGCTTGTGGTGTTGTGG
Beta-actin primers	AGAGAGAAATTGTCCGTGACATC
	CTCCGATCCAGACAGAGTATTTG
Additional sequencing primers	AACAGGCACCAGGGAGTAGC
	CCTGTGCAATGCACATGGAATACC

Overgo hybridization was conducted as described [22,24]. Positive clones were identified according to the clone distribution instructions from CHORI, and one clone was picked out for sequencing analysis.

The single positive BAC clone, BAC_021_N08, was used for sequencing analysis. Initially, the BAC clone was sequenced directly using primers designed from the cDNA sequences. Because the BAC direct sequencing produced ambiguous sequences in some regions, the segment containing the NK-lysin gene was then amplified by PCR and cloned into a pGEM-T plasmid vector by TA-cloning. The insert of the plasmid was completely sequenced using T7 and Sp6 primers plus additional sequencing primers (Table 1). Fourteen picomoles of each primer were used for sequencing reactions. BAC sequencing was performed in a 10 μl reaction using the Bigdye Terminator v3.0 Ready Reaction kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

Bioinformatic analysis of sequences was conducted by using BLAST and the DNASTAR software package as we previously described [29]. BLAST searches [30] were conducted to determine gene identities, and to determine if the cDNA contained a full open reading frame. The DNASTAR software package was used for sequence analysis. The MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTALW.

Channel catfish were reared at the hatchery of the Auburn University Fish Genetics Research Unit. Challenge experiments were conducted as previously described [31] with modifications [32,33]. Briefly, catfish were challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *Edwardsiella ictaluri*.

Eight tissues were collected from healthy channel catfish including gill, head kidney, intestine, liver, muscle, skin, spleen, and trunk kidney. Head kidney was collected from challenged fish. Samples were collected from 10 fish at each time point including control (before challenge), and 4 h, 24 h, 3 days, and 7 days after challenge. Samples of each tissue from 10 fish were pooled. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powder and thoroughly mixed. A fraction of the tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method [34] using the Trizol reagents kit from Invitrogen following the manufacturer's instructions. Extracted RNA was stored in a −70 °C freezer until used as template for reverse transcriptase PCR (RT-PCR).

RT-PCR reactions were conducted using the SuperScript™ III One Step RT-PCR System (Invitrogen). Sequences of the RT-PCR primers are shown in Table 1 and their positions within NK-lysin cDNA are shown in Fig. 1. The RT-PCR reaction was conducted using the SuperScript™ III One Step RT-PCR System (Invitrogen) according to the manufacturer's instructions. The reaction also included the primers for β-actin amplification (Table 1), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: 45 °C for 15 min for one cycle (reverse transcription reaction), at 94 °C for 2 min (denaturation) and 40 cycles (for the analysis of tissue expression and developmental expression), or 28 cycles (for the analysis of expression after infection) of 94 °C for 15 s, 53 °C for 30 s, 72 °C for 30 s. Forty cycles of PCR were used for expression analysis in various tissues and development to assure detection of RT-PCR products in tissues with minimal level of expression. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel and documented with a Gel Documentation System (Nucleotech Corp., San Mateo, CA).

The sequence of the channel catfish NK-lysin cDNA is shown in Fig. 1 and has been deposited to GenBank with an accession number of (AY934592). The channel catfish NK-lysin cDNA has an open reading frame of 447 bp encoding a protein of 148 amino acids. It contains a 5′-untranslated region (UTR) of 46 bp and a 3′-UTR of 117 bp. A typical poly $(A)^+$ signal AATAAA is located 13 bp upstream of the poly A tail. The channel catfish NK-lysin sequence is moderately conserved with NK-lysin sequences from other species. Multiple sequence alignments (Fig. 2) reveal that it shares 44.6% amino acid identity with a 121 amino acid unpublished zebrafish NK-lysin sequence (GenBank accession number (AY184216)). Known mammalian NK-lysins and the two human granulysin splicing variants, NKG5 and 519, were also

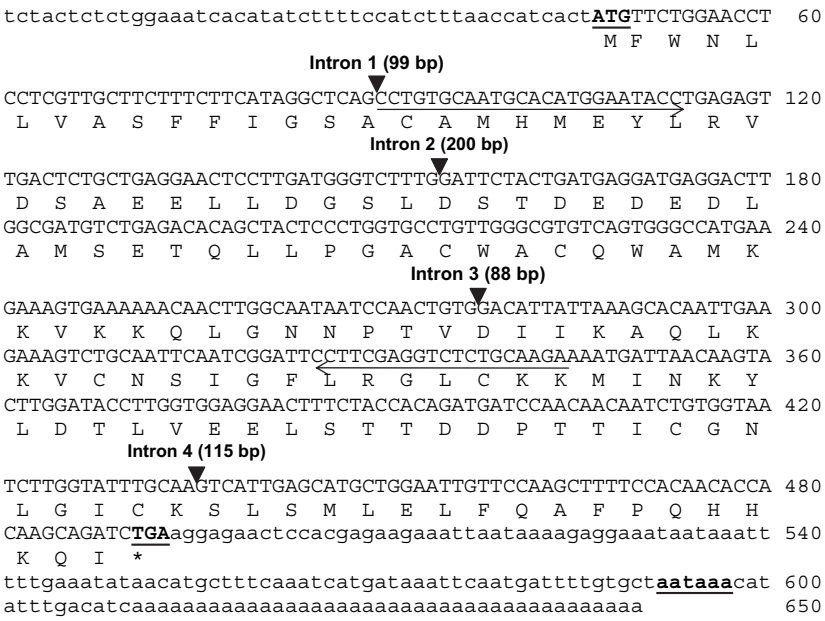

Fig. 1. The nucleotide and deduced amino acid sequences of channel catfish NK-lysin cDNA. 5′- and 3′-UTR are shown as small letters. The translation start codon ATG, the termination codon TGA, and the poly $(A)^+$ signal sequence AATAAA are in bold font and underlined. The termination is labeled by asterisk. The locations of the RT-PCR primers are indicated by underline arrows. The locations of introns and their sizes are indicated by solid arrow heads.

included in the alignment (Fig. 2). While the mammalian NK-lysins are highly similar, for example sharing 67.4% amino acid identity between the horse [35] and porcine NK-lysin [10], sequence similarity between the fish and mammalian NK-lysins is low. The catfish NK-lysin shared less than 25% amino acid identity with any mammalian sequence. The pattern of six conserved cysteine residues (with the exception of granulysin) typical of SAPLIP domains was conserved. The processing sites used to form the mature lytic 9 kDa protein in humans are also shown in Fig. 2.

The complete gene sequence of the NK-lysin gene was deposited in GenBank with the accession number of (AY934593). The channel catfish NK-lysin gene has five exons and four introns, indicating conservation of granulysin/NK-lysin gene organization. To our best knowledge, gene structures from NK-lysin sequences from pig and cow are not known. The five exons/four introns structural feature is conserved between the catfish NK-lysin gene and the NKG5 splicing variant of human granulysin (Fig. 3) [36].

RT-PCR analysis using total RNA from various normal tissues of channel catfish indicated that the NK-lysin gene exhibits strong tissue-specific expression. Gene expression was detected in high levels in gill, head kidney, intestine, spleen, and trunk kidney, at very low levels in the skin and liver, and not detected at all in the muscle (Fig. 4). These results are consistent with known tissue expression of porcine NK-lysin which has been found in spleen, bone marrow, colon, and small intestine [10,37]. In both cases, expression seems to be limited to tissues that harbor lymphocyte populations. The particularly high expression level in gill is notable, as that is a primary site of pathogen entry in fish as well as the focus of powerful host defense. Antimicrobial peptides including moronecidin, hepcidin, and chrysophsin [38–40] have been commonly isolated from the gill. Hwang et al. [41] reported the expression of another cytolytic granule protein, perforin, in the gill of Japanese flounder.

In order to assess potential roles of NK-lysin during bacterial infection, RT-PCR was conducted using RNA isolated from head kidney tissues of channel catfish at several time points after bacterial challenge. As

Fig. 2. Alignment of the deduced amino acid sequences of catfish NK-lysin with those of human, bovine, horse, porcine, and zebrafish. Large arrows indicate the six conserved cysteine residues. The two arrows indicate the processing sites of the 9 kDa granulysin as reported [4].

shown in Fig. 5, NK-lysin expression was gradually increased after challenge with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC). After the bacterial challenge, the catfish NK-lysin was initially reduced in its expression at 4 h after challenge, but elevated NK-lysin expression was detected 3 days after challenge and continued to increase until 7 days after challenge (Fig. 5). This pattern of expression profiles after ESC challenge differs from those of many genes involved in the innate immune responses of catfish such as chemokines [32,33], and several other antimicrobial peptides [22–24]. Granulysin and NK-lysin belong to a group of genes, including other cytolytic granules members such as perforin and granzyme, which are induced late after T-cell activation, usually between 3 and 5 days [5,10]. Catfish NK-lysin exhibits, therefore, a conserved expression pattern with mammalian orthologues suggesting conservation of gene function.

In this work, we report the identification, sequencing analysis, and expression of an NK-lysin antimicrobial peptide in channel catfish. This important cytolytic granule protein has not been previously

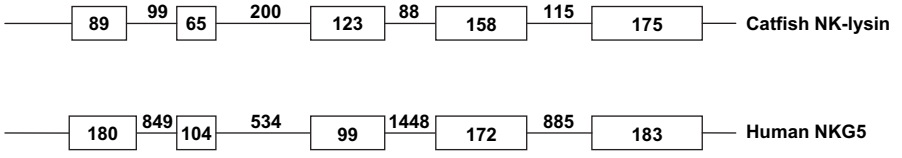

Fig. 3. Comparison of genomic structure and organization of the channel catfish NK-lysin gene with that of the human granulysin. The exons are shown as rectangles with their sizes indicated by numbers within the rectangles; introns are indicated by lines and their sizes are indicated above the lines.

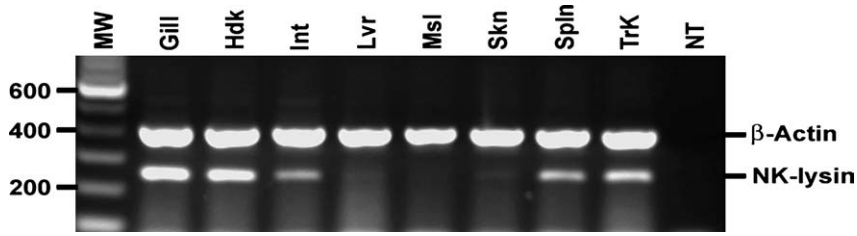

Fig. 4. RT-PCR analysis of NK-lysin gene expression in various tissues. RT-PCR products were analyzed on an agarose gel. Tissues were specified on the top of the gel, gill (Gill), head kidney (Hdk), intestine (Int), liver (Lvr), muscle (Msl), skin (Skn), spleen (Spln), trunk kidney (Trk), and no RNA control (NT). Molecular weight markers (MW) are specified on the left margin. The positions of the RT-PCR amplified bands of beta-actin and NK-lysin are indicated on the right margin.

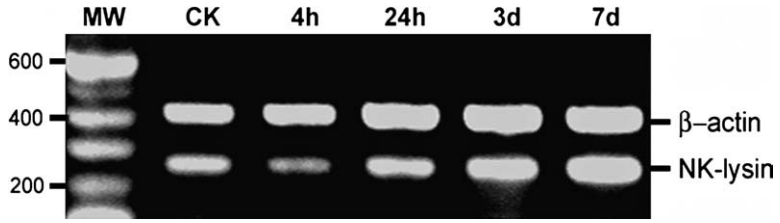

Fig. 5. RT-PCR analysis of NK-lysin gene expression after bacterial challenge with *Edwardsiella ictaluri*. Samples of head kidney were collected at 0 h (CK), 4 h, 24 h, 3 days, and 7 days after challenge. RT-PCR products were analyzed on agarose gels. The positions of the RT-PCR amplified bands of beta-actin and NK-lysin are indicated on the right margin, and the molecular weight markers (MW) are indicated on the left margin.

reported from lower vertebrate species. Sequence analysis revealed that the putative protein sequence contained the six conserved cysteine residues characteristic of the saposin gene family. Gene structure was conserved between catfish NK-lysin and human granulysin NKG5. Additionally, the catfish NK-lysin gene exhibited strong tissue-specific expression in tissues known to harbor lymphocyte populations, consistent with its function in NK cells and cytotoxic T lymphocytes. Following challenge with *E. ictaluri*, catfish NK-lysin was not upregulated until day 3, a response typical of genes induced late after T-cell activation.

While the antimicrobial activities of catfish NK-lysin remain to be determined, our observation of conservation of protein features, gene structure, and expression patterns all suggest that NK-lysin is an important component of the immune response of teleost fish. As antimicrobial peptides have been shown to increase resistance of fish against their pathogens [42–44], and are useful as indicators for stresses [45], future work identifying similar cytolytic proteins in catfish, combined with further characterization of NK-lysin, will provide us with valuable molecular resources for the development of strategies toward the control of severe diseases in commercial aquaculture.

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