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NK-lysin of channel catfish: Gene triplication, sequence variation, and expression analysis

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Abstract

Antimicrobial peptides (AMPs) are important components of the host innate immune response against microbial invasion. In addition to the previously known four classes of antimicrobial peptides, a fifth class of antimicrobial peptides has been recently identified to include NK-lysins that have a globular three-dimensional structure and are larger with 74–78 amino acid residues. NK-lysin has been shown to harbor antimicrobial activities against a wide spectrum of microorganisms including bacteria, fungi, protozoa, and parasites. To date, NK-lysin genes have been reported from only a limited number of organisms. We previously identified a NK-lysin cDNA in channel catfish. Here we report the identification of two novel types of NK-lysin transcripts in channel catfish. Altogether, three distinct NK-lysin transcripts exist in channel catfish. In this work, their encoding genes were identified, sequenced, and characterized. We provide strong evidence that the catfish NK-lysin gene is tripled in the same genomic neighborhood. All three catfish NK-lysin genes are present in the same genomic region and are tightly linked on the same chromosome, as the same BAC clones harbor all three copies of the NK-lysin genes. All three NK-lysin genes are expressed, but exhibit distinct expression profiles in various tissues. In spite of the existence of a single copy of NK-lysin gene in the human genome, and only a single hit from the pufferfish genome, there are two tripled clusters of NK-lysin genes on chromosome 17 of zebrafish in addition to one more copy on its chromosome 5. The similarity in the genomic arrangement of the tripled NK-lysin genes in channel catfish and zebrafish suggest similar evolution of NK-lysin genes.

Keywords: Antimicrobial peptide; Fish; Catfish; Disease; NK-lysin; Innate immunity; Gene

1. Introduction

The NK-lysin gene was initially cloned and characterized from humans, but its significance was unrealized until its orthologue was identified from porcine natural killer cells and cytotoxic T lymphocytes as an antimicrobial peptide (Manning et al., 1992; Houchins et al., 1993; Andersson et al., 1995). In recent years, extensive research has been conducted for the analysis of structure and antimicrobial activities of NK-lysin (Stenger et al., 1998; Ernst et al., 2000; Gansert et al., 2003; Jacobs et al., 2003). NK-lysin genes share sequence similarities to the pore-forming proteins of *Entamoeba histolytica*, termed amoebapores (Leippe, 1995). They have been reported to have antimicrobial activities against a wide spectrum of microorganisms including bacteria, fungi, protozoa, and parasites (Stenger et al., 1998; Ernst et al., 2000; Gansert et al., 2003; Jacobs et al., 2003), and therefore, have been widely regarded as antimicrobial peptides. In contrast to classical antibiotics, these peptides act by direct physical destabilization of the target cell membrane with a high specificity for bacteria (Schroder-Borm et al., 2003). 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 from various species for the development of alternative therapeutants (for review, see Patrzykat and Douglas, 2003).

Antimicrobial peptides are widespread in nature as defense mechanisms of plants and animals. Over 800 AMP sequences have been deposited in the Antimicrobial Peptide Database (http://bbcm1.univ.trieste.it/~tossi/pag1.htm). Increasing numbers of antimicrobial peptides have been identified from teleost fishes in recent years (e.g., Cole et al., 1997; Douglas et al., 2001,

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2003a,b; Lauth et al., 2002; Shike et al., 2002, 2004; Noga and Silphaduang, 2003; Zhang et al., 2004; Bao et al., 2005, in press; Xu et al., 2005). As the largest vertebrate group containing over 23,000 species (Helfman et al., 1997), teleosts should be a rich source of antimicrobial peptides. Channel catfish has played an important role as a model species for the study of comparative immunology (Clem et al., 1990; Vallejo et al., 1991a,b). It is also the primary aquaculture species in the United States. Studies of its innate immune components should have both scientific and practical implications.

Antimicrobial peptides can be divided into four different classes according to their structural features. The first class comprises mainly linear, helical, and amphipathic peptides without disulphide bonds. Well-known examples of this class are cecropins (Steiner et al., 1981). The second class includes linear peptides with an over-representation of a single amino acid, such as histatin 5 (Oppenheim et al., 1988). The third class comprises peptides with loop structures connected by one disulphide bridge, such as the brevinins from frog skin (Simmaco et al., 1998). The fourth class includes peptides with two or more disulphide bonds. These peptides usually possess a defined β-sheet structure, as found in the well-characterized mammalian defensins (Lehrer and Ganz, 2002).

Recently, with the discovery of porcine NK-lysin and human granulysin (Andersson et al., 1995; Pena et al., 1997), a fifth class of antimicrobial polypeptides has been recognized in natural killer and T cells (Bruhn et al., 2003). Compared with the classical antimicrobial peptides, NK-lysin and granulysin are much larger with 78 and 74 amino acid residues and a globular three-dimensional structure. Since the initial identification of NK-lysin in porcine and granulysin in humans a few years ago, a bovine homolog was recently identified (Endsley et al., 2004).

The NK-lysin genes characterized to date are highly conserved in gene structure, organization, and sequences. All the NK-lysin genes identified to date have a structure of five exons and four introns. Their primary sequences are rich in positively charged amino acids and the sulfite bond-forming cysteines. We previously reported a NK-lysin cDNA from channel catfish (Wang et al., 2006). BLAST searches of the draft genome sequences of the closely related zebrafish species reveal multiple copies of the NK-lysin gene. Questions remain, therefore, as to how many NK-lysin genes exist in the catfish genome and how they are organized. Here we report the identification of novel forms of NK-lysin genes, provide evidence for the presence of three tandem copies of NK-lysin genes in the catfish genome, and discuss the evolutionary implications of these findings.

2. Materials and methods

2.1. DNA sequencing and sequence analysis

Plasmid DNA containing the NK-lysin cDNA was prepared using the alkaline lysis method (Sambrook et al., 1989) using the Qiagen Spin Column Mini-plasmid kits (Qiagen, Valencia, CA). Three microliters of plasmid DNA (about 0.5–1.0 μg) were used in sequencing reactions. Chain termination sequencing was performed using the BigDye sequencing kits (ABI, Foster City, CA). The PCR profiles were: 95 °C for 30 s, 54 °C for 15 s, 60 °C for 4 min for 60 cycles. An initial 3 min denaturation at 95 °C and a 5 min extension at 60 °C were used. Sequences were analyzed on an ABI Prism 3100 automatic sequencer.

Bioinformatic analysis of sequences was conducted by using BLAST and DNASTAR software package (Serapion et al., 2004). BLAST searches were conducted to determine gene identities, and to determine if the cDNA contained a full open reading frame. DNASTAR software package was used for sequence analysis. MegAlign program of the DNASTAR package was used for sequence alignment using CLUSTAL W.

2.2. Tissue sampling and RNA extraction

Eleven tissues were collected from healthy channel catfish including brain, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, stomach, and trunk kidney. Samples of each tissue from 10 fish were pooled. The experimental fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg/l before tissues were collected. Tissues were quick frozen in liquid nitrogen and kept in a −80 °C ultra-low freezer until preparation of RNA. 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 (Chomczynski and Sacchi, 1987) using the Trizol reagents kit from Invitrogen following manufacturer's instructions. Extracted RNA was stored in a −70 °C freezer until used as template for reverse transcriptase-PCR (RT-PCR).

2.3. Assigning NK-lysin gene to BAC clones

We previously reported a NK-lysin cDNA (Wang et al., 2006). The cDNA sequences were used to design overgo probes (Bao et al., 2005, in press; Xu et al., 2005) for hybridization to BAC filters. High-density filters of 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). Sequences of the overgo primers are shown in Table 1 and their positions within NK-lysin cDNA are shown in Figs. 1 and 2. Overgo hybridization method was adapted from a web protocol (http://www.tree.caltech.edu/). Briefly, overgo primers were selected following a BLAST search against GenBank to screen out repeated sequences and then purchased from Sigma Genosys (Woodlands, Texas). Two hundred nanograms of overgo primers each were labeled with 40 μl of a freshly prepared master mix composed of 14.0 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.02 mM dGTP, 0.02 mM dTTP, 20 μCi [α-^{33}P]dCTP, 20 μCi [α-^{33}P]dATP (3000 Ci/mmol, Amersham, Piscataway, NJ), and five units of Klenow enzyme (Invitrogen). Labeling reactions were carried out at room temperature for 2 h. After removal of unincorporated nucleotides using a Sephadex G50 spin column, probes were denatured at 95 °C for 10 min and added