Developmental and Comparative Immunology 40 (2013) 258–265

Contents lists available at SciVerse ScienceDirect

Developmental and Comparative Immunology

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

A NK-lysin from *Cynoglossus semilaevis* enhances antimicrobial defense against bacterial and viral pathogens

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ARTICLE INFO

Article history:
Received 19 December 2012
Revised 6 March 2013
Accepted 12 March 2013
Available online 19 March 2013

Keywords:
NK-lysin
Antimicrobial peptide
Cynoglossus semilaevis
Bacterial and viral infection

ABSTRACT

NK-lysin is an effector protein of cytotoxic T lymphocytes and natural killer cells. Mammalian NK-lysin is known to possess antibacterial property and antitumor activity. Homologues of NK-lysin have been identified in several teleost species, but the natural function of fish NK-lysin remains essentially unknown. In this study, we identified a NK-lysin, CsNKL1, from half-smooth tongue sole (*Cynoglossus semilaevis*) and analyzed its expression, genetic organization, and biological effect on pathogen infection. CsNKL1 is composed of 135 residues and shares 33.1–56.5% overall sequence identities with other teleost NK-lysin. CsNKL1 possesses a Saposin B domain and six conserved cysteine residues that in mammals are known to form three intrachain disulfide bonds essential to antimicrobial activity. The genomic sequence of the ORF region of *CsNKL1* is 1240 bp in length and, like human NK-lysin, contains five exons and four introns. Expression of *CsNKL1* occurred in multiple tissues and was upregulated by bacterial and viral infection in a time dependent manner. When CsNKL1 was overexpressed in tongue sole, significant upregulation of interleukin-1 and chemokines was observed in spleen and head kidney. Following bacterial and viral infection, the pathogen loads in the tissues of CsNKL1-overexpressing fish were significantly lower than those in control fish. These results indicate that CsNKL1 possesses the novel capacities of immunomodulation and enhancing antimicrobial defense against pathogens of both bacterial and viral nature.

1. Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are among the primary effector cells of the immune system. These cells possess cytolytic granules, which are specialized secretory lysosomes that contain an array of effector proteins with lytic property, notably granzymes, perforin, and granulysin (Lowin et al., 1995; Trapani and Smyth, 1993). Perforin is a pore-forming protein that mediates the entry of granzymes, a group of proteases involved in apoptosis, into the target cells, while granulysin is a molecule of the saposin-like family that is involved in lipid/membrane degradation (Clayberger and Krensky, 2003; Okada et al., 2003; Voskoboinik et al., 2010). Upon activation, CTLs and NK cells release the lytic molecules stored in the cytolytic granules, which in turn perturb cell membrane and induce cell apoptosis, whereby killing the target cells (Gamen et al., 1998; Pena and Krensky, 1997; Raja et al., 2003).

NK-lysin is a type of granulysin that was first isolated from pig small intestine as an effector peptide of CTLs and NK cells with antimicrobial property (Andersson et al., 1995a). It is present in CD8(+), CD2(+), and CD4(+) cells, and produced by CTLs and NK cells upon interleukin (IL)-2 stimulation. NK-lysin exhibits potent antibacterial activity against *Escherichia coli* and *Bacillus megaterium*, and moderate activity against *Acinetobacter calcoaceticus* and *Streptococcus pyogenes*. The peptide is also lytic against a number of different types of tumor cells but is not lytic for erythrocytes (Andersson et al., 1995a). NK-lysin binds to the lipopolysaccharides (LPS) of several bacterial species through interaction with the lipid part of LPS, and this binding inhibits LPS binding of mouse granulocytes and LPS-stimulated effects on mouse bone marrow cells (Andersson et al., 1999). Structural analysis showed that NK-lysin peptide is composed of 78 residues and contains six cysteine residues that form three intrachain disulfide bonds. The activity of the peptide is dependent on intact disulfides, and both the bactericidal effect and the cytolytic effect of the peptide were inhibited when NK-lysin was pre-treated with dithiothreitol (Andersson et al., 1996).

NK-lysin homologues have been identified in diverse organisms including chicken, horse, water buffalo, fish, and protozoa, and some of these proteins were found to possess antimicrobial and antitumor activity (Andr et al., 1996; Andr and Leippe, 1999; Endsley et al., 2004; Hong et al., 2006, 2008; Lee et al., 2012). In fish, eight NK-lysin genes have been identified from five species, i.e. Japanese flounder (*Paralichthys olivaceus*) (Hirono et al., 2007),

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http://dx.doi.org/10.1016/j.dci.2013.03.005

pufferfish (*Takifugu rubripes*) (GenBank accession number XP_003962755), Atlantic salmon (*Salmo salar*) (GenBank accession number NP_001134582), zebrafish (*Danio rerio*) (GenBank accession number AY184216), and channel catfish (*Ictalurus punctatus*) (Wang et al., 2006). A study of flounder NK-lysin showed that a synthesized 27-residue peptide based on the sequence of this protein displays antimicrobial activity against a number of Gram-negative bacteria (Hirono et al., 2007). The biological activity of other fish NK-lysin has not been reported.

Half-smooth tongue sole (*Cynoglossus semilaevis*) is a flat fish cultured widely in China as an economic species. In this study, we identified a NK-lysin gene from tongue sole and examined its expression pattern, genetic organization, and biological effect on pathogen infection. We found that NK-lysin exhibits tissue specific expression and, when overexpressed in fish, regulates immune gene expression and inhibits bacterial and viral infection.

2. Materials and methods

2.1. Fish

Half-smooth tongue sole (*C. semilaevis*) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Before experiment, fish were randomly sampled for the examination of the presence of bacteria and megalocytivirus in blood, kidney, and spleen as reported previously (Zhang et al., 2008, 2012). No bacteria or virus were detected from the examined fish. Before tissue collection, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) as reported previously (Wang et al., 2009).

2.2. Cloning of CsNKL1

A cDNA library of half-smooth tongue sole head kidney and spleen was constructed as reported previously (Wang et al., 2011). One of the clones was found to contain the full length cDNA of *CsNKL1* with 5′- and 3′-untranslated regions (UTRs). The nucleotide sequence of *CsNKL1* has been deposited in GenBank database under the accession number KC346974.

2.3. Sequence analysis

The cDNA and amino acid sequences of CsNKL1 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The molecular mass and theoretical isoelectric point (pI) were predicted using EditSeq in DNASTAR software package (DNASTAR Inc. Madison, WI, USA). Signal peptide search and subcellular localization prediction were performed with SignalP 3.0 and WoLF PSORT respectively. Multiple sequence alignment was created with the ClustalX program.

2.4. Quantitative real time reverse transcription-PCR (qRT-PCR) analysis of CsNKL1 expression

2.4.1. CsNKL1 expression in fish tissues under normal physiological conditions

Spleen, heart, gill, brain, kidney, liver, muscle, and intestine were taken aseptically from three tongue sole (average 5.1 g) and used for total RNA extraction with the RNAprep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously (Zheng and Sun, 2011). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of *CsNKL1* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with β-actin as the control. All data are given in terms of mRNA levels relative to that of β-actin and expressed as means plus or minus standard errors of the means (SEM).

2.4.2. CsNKL1 expression during pathogen infection

The fish bacterial pathogen *Vibrio anguillarum* C312 was cultured in Luria–Bertani broth (LB) medium at 28 °C as reported previously (Zheng et al., 2010) to an OD_{600} of 0.8. The cells were washed with PBS and resuspended in PBS to 2×10^7 colony forming units (CFU)/ml. The fish viral pathogen megalocytivirus RBIV-C1 (Zhang et al., 2012) was suspended in PBS to 5×10^4 copies/ml. Tongue sole were divided randomly into three groups and injected intraperitoneally (i.p.) with 100 μl *V. anguillarum*, megalocytivirus, or PBS. Fish (three at each time point) were euthanized at 1, 4, 12, 24, and 48 h post-bacterial infection and at 1, 2, 3, and 4 days post-viral infection. Tissues were collected under aseptic conditions. Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above.

2.5. Plasmid construction

pIG1, which was the backbone plasmid for the construction of CsNKL1-expressing plasmid, was constructed by inserting linker EEHE (5′-AATTCGATATCCATCATCACCATCACCATTGAG-3′) into the EcoRI site of the mammalian expression plasmid pIRES2-EGFP (Clontech, Mountain View, CA, USA). To construct pCsNKL1, which expresses CsNKL1, the coding sequence of CsNKL1 was amplified by PCR with primers F1 (5′-GATATCGCCACCATGAACAAATCTCCAATCC-3′, underlined sequence, EcoRV site) and R1 (5′-GATATCGTTGTTTGGATAGAAGAGGAGAT-3′, underlined sequence, EcoRV site); the PCR products were ligated with the T-A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the *CsNKL1*-containing fragment, which was inserted into pIG1 at the EcoRV site, resulting in pCsNKL1. In pCsNKL1, *CsNKL1* and the reporter *EGFP* are co-transcribed (in the order of *CsNKL1* and *EGFP*) as a single bicistronic mRNA, from which CsNKL1 and EGFP are synthesized as two independent proteins. Hence, production of EGFP can serve as an indicator of CsNKL1 expression.

2.6. Overexpression of CsNKL1 in tongue sole

pCsNKL1 and pIG1 were diluted in PBS to 200 μg/ml. Tongue sole were divided randomly into three groups and injected intramuscularly with 50 μl of pCsNKL1, pIG1, or PBS. Muscle, kidney, and spleen were taken from the fish at 7 days post-plasmid administration and used for examination of the presence of plasmid and CsNKL1 expression. For plasmid detection, total DNA was extracted from the tissues with Trizol (Tiangen, Beijing, China), and PCR detection of pCsNKL1 and pIG1 was performed with the primers F2 (5′-CTACTTGGCAGTACATCTACGT-3′) and R2 (5′-ATGGTGATGGTGATGATGGAT-3′). To examine *CsNKL1* expression, total RNA was extracted from the tissues and used for RT-PCR with primers F3 (5′-AAATCTCCAATCCTGCTCTTCTG-3′) and R2. To examine EGFP production, the muscle tissues were fixed in 10% buffered formalin, and the fixed tissues were processed according to standard histological techniques. The tissue sections were observed with a fluorescence microscope (Nikon E800, Japan). Immunocolloidal gold