Developmental and Comparative Immunology 33 (2009) 991–999

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

NOD-like subfamily of the nucleotide-binding domain and leucine-rich repeat containing family receptors and their expression in channel catfish

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ARTICLE INFO

Article history:
Received 30 January 2009
Received in revised form 3 April 2009
Accepted 27 April 2009
Available online 14 May 2009

Keywords:
NLR
Receptor
Catfish
Fish
Ictalurus punctatus
Immunity
Disease resistance
NACHT
NOD
CARD
Pathogen recognition

ABSTRACT

The NLRs (nucleotide-binding domain and leucine-rich repeat containing family receptors) are a recently identified family of pattern recognition receptors in vertebrates. Several subfamilies of NLRs have been characterized in human, mouse, and zebrafish, but studies of NLRs in other species, especially teleost species, have been lacking. Here we report characterization of five NLRs from channel catfish: NOD1, NOD2, NLRC3, NLRC5, and NLRX1. Structural analysis indicated that the genes were organized in a similar fashion as in the mammals and in zebrafish. Phylogenetic analysis suggested that they were orthologous to the NOD-like subfamily of NLRs. All five NOD-like genes exist as a single copy gene in the catfish genome. Hybridization of gene-specific probes allowed mapping of three NLR genes to the catfish physical map, laying a foundation for genome characterization and for establishing orthologies with NLR genes from other species. These genes are widely expressed in various tissues and leukocyte cell lines. While the majority of the NLR genes appeared to be constitutively expressed, NOD1 was induced after infection with a bacterial pathogen, *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), suggesting its involvement in immunity against the intracellular pathogen.

1. Introduction

In lower vertebrates, the innate immune response is the primary line of defense against many acute infections of pathogens [1]. The innate immune system provides an immediate and rapid response to infection by recognition of foreign molecules that have invaded the host. Specifically, pattern recognition receptors (PRRs) are proficient at detecting exogenous microbial components. These microbial components, termed pathogen-associated molecular patterns (PAMPs), are typically conserved molecular signatures crucial to the survival of the pathogen. Microbial PAMPs include flagellin, lipopolysaccharides, lipoproteins, peptidoglycan, and nucleic acids. Once a PAMP is detected by the host innate immune system, a vigorous response to eliminate the pathogen ensues. This response involves induction of inflammation and further immune signaling cascades including phagocyte recruitment, production of secretory anti-microbial peptides, and stimulation of the adaptive immune system, along with other co-regulatory physiological responses [2,3].

PRRs can be found in the extracellular space, membrane-associated to various host cell types, or in the cytosol. Of the PRRs that have been identified to-date, the Toll-like receptors were the earliest characterized and have since become the focus of many studies. These receptors have been demonstrated to recognize various PAMPs of bacteria, viruses, fungi, and parasites at the cell membrane or the endosomal region [2]. The Toll-like receptors, however, could not account for all host pattern recognition due to

Abbreviations: BIR, baculovirus inhibitor of apoptosis protein repeat; CARD, caspase recruitment domain; CIITA, class IImajor histocompatibility complex, transactivator; HET-E, bacterial nucleotide triphosphatase protein; IPAF, ice protease-activating factor; LRR, leucine-rich repeat; NACHT, NAIPCIITA, HET-E, and TP-1 proteins; NAIP, neuronal apoptosis inhibitory protein; NALP, NACHT domain-leucine-rich repeat-, and PYD-containing protein; NLR, nucleotide-binding domain and leucine-rich repeat containing family receptor; NLRC3, NLR familyCARD domain containing 3; NLRC5, NLR familyCARD domain containing 5; NLRX1, NLR family member X1; NOD, nucleotide-binding oligomerization domain; NOD1, nucleotide-binding oligomerization domain containing 1; NOD2, nucleotide-binding oligomerization domain containing 2; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; PYD, pyrin; TP-1, telomerase-associated protein.

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doi:10.1016/j.dci.2009.04.004

their incapability to recognize some intracellular pathogens. This led to studies suggestive of other PRRs involved in recognition of intracellular pathogens in early host defense [4–6]. The nucleotide-binding domain and leucine-rich repeat containing family receptors (NLRs) were established as a set of proteins capable of inducing inflammation after infection with an invasive form of *Shigella flexneri* in human epithelial cells [4,6]. The NLRs, best characterized in mammals, have since been categorized as cytosolic surveillance molecules in both Gram-positive and Gram-negative intracellular bacterial infections. The NLRs have neither signal peptides nor transmembrane domains, indicative of their localization to the cytosol. Recent studies have also demonstrated NLR responsiveness to extracellular pathogens, which occurs once the specific immunogen is internalized by the host cell and reaches the cytosol [7].

The NLR family was found to share a distinct structural motif similarity to the disease resistance superfamily of proteins (R-proteins) in plants [8]. The NLR and R-proteins share not only structural similarities but also some functional and regulatory similarities, suggesting a common evolution route in the NLR pathway [9]. The typical characteristics of the NLR family include a structure with three domains: (1) an N-terminal effector binding domain, (2) a central NACHT domain (named after NAIP, CIITA, HET-E, and TP-1 proteins), and (3) a leucine-rich repeat (LRR) C-terminus. The C-terminal LRR domain possibly serves as the pattern recognition or ligand-binding site. The centralized NACHT domain is responsible for oligomerization and autoactivation of the molecule. The N-terminus is responsible for protein–protein interaction, signal transduction, and initiation of immune cascades [10]. The NLRs contain either a caspase recruitment domain (CARD), a baculovirus inhibitor of apoptosis protein repeat (BIR) domain, or a pyrin (PYD) domain at the N-terminus.

The presence of the different N-terminal domains divides the NLRs into subfamilies: the NODs (nucleotide-binding oligomerization domain) and IPAF (ICE protease-activating factor) (CARD), the NAIPs (neuronal apoptosis inhibitory proteins) (BIR), and the NALPs (NACHT domain-, leucine-rich repeat-, and PYD-containing proteins) (PYD) [7,10]. An examination of the zebrafish genome suggests that most of the innate components in mammals possess orthologs in fish [11]. This was demonstrated for the NLRs recently, where zebrafish may contain three subfamilies of NLRs: one that resembles mammalian NODs, one that resembles mammalian NALPs, and one unique subfamily of genes with portions that resemble both mammalian NOD3 and NALPs [12]. Obviously, studies in other fishes are needed to elucidate the identity and functions of this family of receptors, particularly considering whole genome duplication events that have occurred during teleost evolution, and various levels of gene duplication resulting from random gene loss after whole genome duplication.

Channel catfish (*Ictalurus punctatus*) is the predominant aquaculture species in the United States (USDA-NASS, 2008; http://www.nass.usda.gov/QuickStats/index2.jsp). The catfish aquaculture industry continues to suffer serious economic losses due to diseases, including enteric septicemia of catfish (ESC) caused by the Gram-negative intracellular bacterium *Edwardsiella ictaluri*. The closely related blue catfish (*I. furcatus*) possesses several superior performance traits over channel catfish, including disease resistance to ESC [13–15]. This makes blue catfish and channel catfish excellent candidates for the investigation of the teleost immune system. The catfish immune system is one of the best characterized from fish species, and it is the only fish species where clonal functionally distinct lymphocyte lines have been established [16–21]. Further, catfish represent a diverse and ancient lineage of fishes [22], and are a natural choice for the study for evolutionary and comparative genomics as well as genome duplication.

A number of innate immune genes have been characterized in catfish including a large number of chemokines [13,23–29] and anti-microbial peptides [23,30–33]. Currently, a few innate PRRs in catfish have been characterized including several Toll-like receptors [34–37], and a few members of the lectin family of proteins [38,39]. However, research on intracellular PRRs in the catfish has been lacking. Here we report the identification and characterization of catfish NLRs homologous to the NOD zebrafish/mammalian subfamily and investigate their expression after the bacterial infection.

2. Materials and methods

2.1. Sequencing and identification of NOD-like genes

Catfish ESTs were downloaded from GenBank dbEST to search for NLR sequences. Sequences were clustered using CAP3 [40] and putative identities assigned using BLASTX against the non-redundant databases at the NCBI. Additional EST sequences recently sequenced from the Joint Genome Institute (clones CBPO016765 and CBPO016765 for NOD1; CBPO1077 and CBPO831 for NOD4; CBZC4133, CBZC23554, and CBCZ12005 for NOD5) were identified using BLAST and included in the CAP3 clustering. NOD-like sequences were identified and partial sequences for NOD1, NLRC5, and NLRX1 homologues were found. In zebrafish, NOD1-5 receptors are referred to as NLR subfamily A: NLR-A1 to NLR-A5 [12]. The NOD1-5 subfamily in mammals has many names in the literature; however, a standard nomenclature was recently recommended by the HUGO Gene Nomenclature Committee (HGNC) for humans and mouse [41]. We will use this nomenclature to refer the catfish homologues for this study. The NOD1-5 subfamily of NLRs in catfish will be named as NOD1, NOD2, NLRC3, NLRC5, and NLRX1, respectively, for standardization (Table 1).

After clustering and BLAST analysis, degenerate primers designed from NLR alignments as well as primers designed from zebrafish sequences were used to amplify NLRs that were absent from the EST clusters (NOD2 and NLRC3, Supplemental Table 1). Total RNA was extracted from a healthy adult catfish liver and spleen using TRIzol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's recommendations. The RNA was pooled (250 ng

Table 1
Nomenclature of the NOD subfamily of NLRs.

Common name	Zebrafish subfamily designation	Zebrafish symbol designation	Catfish subfamily designation	Catfish symbol	HGNC approved human subfamily	HGNC approved human symbol	HGNC human domain organization[a]	Human orthologous sequence
NOD1	NLR-A	NLR-A1	NLRC	NOD1	NLRC	NOD1	C-N-L	NP_006083
NOD2	NLR-A	NLR-A2	NLRC	NOD2	NLRC	NOD2	C-C-N-L	NP_071445
NOD3	NLR-A	NLR-A3	NLRC	NLRC3	NLRC	NLRC3	C-N-L	NP_849172
NOD4	NLR-A	NLR-A4	NLRC	NLRC5	NLRC	NLRC5	C-N-L	NP_115582
NOD5	NLR-A	NLR-A5	NLRX	NLRX1	NLRX	NLRX1	X-N-L	NP_078894

[a] The following domain abbreviations are used: C = CARD; N = NACHT; L = LRR, leucine-rich repeat; X = unknown effector domain.