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Cloning of two rainbow trout nucleotide-binding oligomerization domain containing 2 (NOD2) splice variants and functional characterization of the NOD2 effector domains

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ABSTRACT

Nucleotide-binding oligomerization domain 2 (NOD2) is a cytoplasmic pattern recognition receptor (PRR), which is involved in innate antibacterial and antiviral responses. Here, two NOD2 splice variants, trNOD2a and trNOD2b, are reported in rainbow trout *Oncorhynchus mykiss*, that share 63% and 61% similarity with human NOD2, respectively. These two trout NOD2 splice variants were shown to be constitutively expressed in thymus, gills, skin, muscle, liver, spleen, head kidney, intestine, heart, and brain, with the expression of trout NOD2 (trNOD2) mainly contributed by trNOD2a in all the examined tissues. PolyI:C transfection up-regulated the expression of trNOD2a and trNOD2b in RTG-2 cells. The expression of trNOD2a/b was modulated by the inflammatory stimulant interferon-γ (IFN-γ) or interleukin-1β (IL-1β). Overexpression of trout NOD2 effector domains resulted in induced expression of proinflammatory cytokines including IL-1β, tumor necrosis factor-α (TNF-α), IL-6 and IL-8, the antibacterial peptide cathelicidin-2, a variety of caspases including caspase-2, -6, -7, -8, -9, and type I and type II IFN. These results suggest that fish NOD2 functions in inflammatory events, possibly via NF-κB activation, regulation of apoptosis, and triggering of antibacterial and antiviral defences.

1. Introduction

The innate immune system, the first line of defence against infection, relies on host germline-encoded pathogen recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs). After sensing the presence of a PAMP, host innate immune cells initiate a broad spectrum of defence mechanisms that result in the development of inflammation and host resistance to infection [1]. PRRs comprise an array of sensors present in the extracellular, membrane, and cytoplasmic compartments. When pathogens bypass the membrane-associated PRRs by entering the cytosol of cells directly, or are actively transported into host cells by type III or type IV secretion systems from microbes residing extracellularly, cytoplasmic PRRs are required to detect PAMPs [2]. Cytoplasmic PRRs can be grouped into three families: interferon (IFN)-inducible proteins, the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). RLRs are helicases that sense primarily viruses [3]. In contrast, NLRs are mainly involved in antibacterial immune responses [4].

In mammals, NLRs are multi-domain proteins composed of an amino-terminal effector-binding domain (EBDs) such as a caspase recruitment domain (CARD), pyrin domain (PYD), acidic domain, or baculovirus inhibitor repeats (BIRs), a central nucleotide-binding domain (NOD, also designated a NACHT domain) and carboxy-terminal leucine-rich repeats (LRRs) [5]. The N-terminal domain of the NLRs mediates signaling through its interaction with downstream factors. The NOD domain is closely related to the oligomerization module, which has been shown to be required for the activation of downstream effector molecules [6]. The LRRs are essential for pathogen detection and recognition [7].

The NOD2 gene belongs to the subfamily of NLRs characterized by CARD-containing effector-binding domains. Vertebrate NOD2 is composed of two adjacent N-terminal CARDs, a centrally located NOD domain and multiple C-terminal LRRs [8–10]. In mammals, NOD2 induces multiple effector signaling pathways to resist microbial pathogens. The best characteristic of these is the activation of NF-κB and MAPKs through interactions with the CARD

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domain of receptor-interacting protein 2 (RIP2) [11]. NOD2 signaling by activating NF-κB and MAPKs leads to the induction of proinflammatory cytokines and chemokines [12], and anti-microbial peptides [13], which mediate the antimicrobial response. Besides the function in antibacterial defence, new studies show that NOD2 also has an important role in recognizing viruses and inducing type I IFN production through association with the mitochondrial antiviral signaling protein (MAVS) [14].

In teleost fish, orthologs of human NOD2 have been reported in zebrafish [15,16], channel catfish [17], and grass carp [10]. In the present study, our main aims were to assess whether fish NOD2 can undergo alternative splicing, as well as whether NOD2 domains have different effects in inflammatory events. The results we present clearly show that a region of the NOD2 RNA transcript that encodes the N-terminal CARD domains is alternatively spliced, and that overexpression of the trout NOD2 different domains has a similar role in the production of proinflammatory cytokines, an antibacterial peptide, a variety of caspases, and type I and type II IFN. The present study provides evidence that the role of NOD2 in NF-κB activation, regulation of apoptosis, and triggering of antibacterial and antiviral defences is conserved in teleost fish.

2. Materials and methods

2.1. Cloning and sequencing of trout NOD2

The expressed sequence tag (EST) sequences in the Computational Biology and Functional Genomic Laboratory (http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi) were searched using the grass carp NOD2 protein sequence [10] as a bait sequence for TBLASTX analysis. Several candidate ESTs were found with high sequence homology to known NODs, among which EST sequences TC74620 (*Salmo salar*) and CX244678 (*Oncorhynchus mykiss*) aligned well with the C terminal and middle region of NOD2 respectively. Based on the sequence of a partial trout NOD2 (CX244678), nested primers trNOD2GSP1 and trNOD2GSP2 (Table 1) were designed and used in 5′ RACE using a GeneRacer™

Table 1
Primer sequences used in this study.

The first run PCR for 5′RACE	GeneRacer™ 5′ Primer	CGACTGGAGCACGAGGACACTGA
The nested PCR for 5′ RACE	GeneRacer™ 5′ Nested Primer	GGACACTGACATGGACTGAAGGAGTA
The first run PCR for 5′ RACE	trNOD2GSP1	GGTGGAGCGGTCCGAGTTATTGTT
The nested PCR for 5′ RACE	trNOD2GSP2	ACCGTCGGGTCGCGATGATGCT
The first run PCR for 3′ coding region	trNOD2Fout	GGACCGCTCCACCATCCCTAA
	trNOD2Rout	TGTAACTGCTGAATCAACCC
The nested PCR for 3′ coding region	trNOD2Fin	GCAAGACAGGCAGCAACAGC
	trNOD2Rin	GCACTTGGCATCATCAGAAT
Primers used to confirm alternative splice	trNOD2F1	GGACCATTCTGTTTCGCACTCG
	trNOD2R1	CAGGTCTCATCTCCCTTGGTGTACAC
NOD2a CARD domain	NOD2aCARDF	GCGAAGCTTTCCTACATCAGTATGAGTG
	NOD2abCARDR	GTTCCGCGGTCATTGGTTAGATGGTGAGGG
NOD2b CARD domain	NOD2bCARDF	GCCAAGCTTTCCTGGGGCATGCTGTTC
	NOD2abCARDR	GTTCCGCGGTCATTGGTTAGATGGTGAGGG
NACHT domain	NACHTF	GCCAAGCTTAGTATGGACACTGTGCTAGTCTCT
	NACHTR	GTTCCGCGGTCAGCGATGATGCTTCCTCAC
LRR domain	LRRF	GCGAAGCTTGCAATGGCCTACGTGCTCC
	LRRR	GTTCCGCGGTCAGAATGTAAGCCTGGGT
Real-time PCR	trNOD2aF	GCTGTAGCAAACTGGCTCAAA
	trNOD2abR	CAGGTCTCATCTCCCTTGGTG
	trNOD2bF	CTCCCGCTGAAACCTGGTCC
	trNOD2abR	CAGGTCTCATCTCCCTTGGTG
	trNOD2F	TCTCTCTCTAAGGCTGGGAAAC
	trNOD2R	TTGCCAACACCATTGTCTACCA
	IRF3F	ACTGGTCATGGTCGAGGTGGT
	IRF3R	CACAAGTCCATCATCTCCTGCAG
	IRF7F	GACCGTAAAATATTCAGGGCATGG
	IRF7R	CTTCTCTGTGCGGGCTCTCATA
	IFNg2F	CAAACTGAAAGTCCACTATAAGATCTCCA
	IFNg2R	GGTCCAGCCTCTCCCTCAC
	IL-6F	CCTTGCGGAACCAACAGTTTG
	IL-6R	CCTCAGCAACCTTCATCTGGTC
	TNFαF	CTGTGTGGCGTTCTCTTAATAGCAGCTT
	TNFαR	CATTCCGTCCTGCATCGTTGC
	CATH2F	ACATGGAGGCAGAAGTTCAGAAGA
	CATH2R	GAGCCAAACCCAGGACGAGA
	caspase1F	AAACCCATTCAACTAGTGCTG
	caspase1R	CTTCACTGACTCCATTTCCTCC
	caspase2F	CTGGGTGTTTGAGGCATTTGA
	caspase2R	TCTCCTTACAGCGATGGTGGG
	caspase3F	GCTGCTCCGCTTCGTTCGTG
	caspase3R	CCAGGAGCCAGTCTGAGTGTT
	caspase6F	CCAATGCTGACCGCTCCAATCT
	caspase6R	CCCACGGCACGCCTGTAGTATGA
	caspase7F	CTCTTCTTCATCCAGGCTTGTC
	caspase7R	TCTTCTCGCTGAAACGTGGGT
	caspase8F	GGAGGGCGGAGTCACATACA
	caspase8R	GCGTCAGCACCGACAGAAT
	caspase9F	CTTTGCCTACGCCTACCACT
	caspase9R	GCTCCTGCCTTATTTGCTT