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Characterization of three Nod-like receptors and their role in antimicrobial responses of goldfish (*Carassius auratus* L.) macrophages to *Aeromonas salmonicida* and *Mycobacterium marinum*

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ABSTRACT

The nucleotide-binding oligomerization domain proteins Nod1, Nod2 and Nlrx1 are cytoplasmic pathogen recognition receptors (PRRs) of the Nod-like receptor (NLR) family. In this report, goldfish Nod1 (gfNod1), Nod2 (gfNod2) and Nlrx1 (gfNlrx1) genes were cloned and characterized. The full length of gfNod1, gfNod2 and gfNlrx1 were 3234 bp, 3129 bp and 4900 bp, encoding 937, 982 and 1008 amino acids, respectively. The three Nod-like receptors have a NACHT domain and C-terminal leucine rich repeat (LRR) domains. In addition to these, gfNod1 and gfNod2 also had an N-terminal CARD domain (two in gfNod2). Phylogenetic analysis showed that the three NLRs are highly conserved. Quantitative gene expression analysis of the three receptors revealed the greatest mRNA levels in the spleen, and in isolated neutrophils and splenocytes. Furthermore, treatment of goldfish macrophages with LPS, Poly I:C, MDP, PGN, heat-killed *Aeromonas salmonicida* or *Mycobacterium marinum* differentially altered the expression of the Nod-like receptors. Our results indicate that Nod-like receptors are functionally highly conserved and that they play a pivotal role in recognition of fish pathogens such as *A. salmonicida* and *M. marinum*.

1. Introduction

The conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) are recognized by germ line-encoded innate immune receptors (PRRs), which exist as four major classes, including the Toll-like receptors (TLRs), the Nod-like receptors (NLRs), the retinoid acid-inducible gene-1 (RIG-1)-like receptors (RLRs) and the C-type lectin receptors (CLRs). PAMPs include lipopolysaccharides (LPS), polyinosinic: polycytidylin acid (Poly: IC), lipopeptide, peptidoglycan (PGN), flagellin, dsRNA, ssRNA and CpG DNA, among others. Recognition of these PAMPs by PRRs triggers the activation of signaling pathways including nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs) and type I interferons (IFN), which promote inflammatory and antimicrobial responses.

The TLRs are the best known group of the innate immune receptors whose function has been reasonably well characterized in different infectious diseases. TLRs play a fundamental role in innate immune responses by sensing the molecular signatures of microbial pathogens that recognize structural components shared by many bacteria, viruses and fungi (Takeda et al., 2003). Structurally, TLRs are type I membrane proteins characterized by an ectodomain composed of leucine rich repeats (LRR) that are responsible for recognition of PAMPs, and a cytoplasmic domain known as the TIR domain homologous to the cytoplasmic region of the IL-1 receptor, which is required for the activation of defense responses against an invading organism (Uematsu and Akira, 2007). To date, 10 members of TLRs have been identified in humans, and 13 in mice. A number of studies have identified their respective ligands that include LPS (TLR4), lipoproteins (TLR2), flagellin (TLR5), non-methylated CpG motifs of DNA (TLR9), double-stranded RNA (TLR3), and single-stranded RNA (TLR7 and TLR8) (Akira et al., 2001; Takeda and Akira, 2005). After recognition of microbial pathogens, TLRs trigger intracellular signaling pathways that result in the induction of type I interferons (IFN) and chemokines, as well as other signaling pathways that play a role in the generation of antimicrobial

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responses against different pathogens (Iwasaki and Medzhitov, 2004). In contrast, NLRs and RLRs are intracellular cytosolic sensors (Uematsu and Akira, 2007; Takeda and Akira, 2005) that are not very well characterized, especially in lower vertebrates.

The NLRs appear to be primarily involved in bacterial recognition. Until now, several Nod-like receptors have been identified in different bony fishes including zebrafish (Laing et al., 2008), Japanese flounder (Unajak et al., 2011), olive flounder (Park et al., 2011), trout (Chang et al., 2011), rohu (Swain et al., 2012a,b), grass carp (Chen et al., 2010) and catfish (Rajendran et al., 2012). The Nod1 and Nod2 in these fish were differentially expressed in all the tissues with notable differences in the expression of these receptors in different fish. For example, Nod1 was highly expressed in the spleen of rohu (Swain et al., 2012a) and kidney of oliver flounder (Park et al., 2011), while the highest mRNA levels of Nod2 were detected in muscle (Swain et al., 2012b) and liver (Chang et al., 2011) of trout. The injection of rohu with LPS, Poly I:C, *Aeromonas hydrophila*, *Edwardsiela tarda* or *Shigelle flexneri* resulted in the up-regulation of Nod1 expression (Swain et al., 2012a). Similarly, the expression of Nod2 was enhanced in response to PGN, LTA, Poly I:C, *A. hydrophila* and *E. tarda* (Swain et al., 2012b). The NLRs appear to play an important role in viral infections of fish (Chen et al., 2010) which is perhaps not surprising since Nod1, Nod2 and Nlrx1 of grass carp shared high identity based on the phylogenetic analysis (Park et al., 2011; Chang et al., 2011; Swain et al., 2012a,b; Chen et al., 2010; Rajendran et al., 2012). To date, relatively few studies have identified and characterized the ligands of NLRs in bony fish.

In this study, we report on the cloning and characterization of goldfish Nod1, Nod2 and Nlrx1. Comprehensive Q-PCR analysis revealed that these three NLRs were differentially expressed in tissues and different immune cell populations. Treatment of macrophages with different chemical stimuli or exposure to *Aeromonas salmonicida* or *Mycobacterium marinum* increased the expression of these NLRs.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus* L.) were obtained from Aquatic Imports (Calgary, AB) and maintained at the Aquatic Facility of Department of Biological Sciences, University of Alberta. The fish were kept in tanks with a continuous water flow system at 17 °C on a simulated natural photoperiod, and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least 3 weeks prior to use in the experiments. All fish ranged from 10 to 15 cm in length and whenever possible an equal number of both sexes were used. Prior to handling, fish were sedated using a TMS (tricaine methane sulfonate) solution of 40–50 mg/L in water. The animals in the aquatic facility were maintained according to the guidelines of the Canadian Council of Animal Care (CCAC-Canada).

2.2. Bacteria

The *M. marinum* strain ATCC 927 (fish isolate) was a kind gift from Dr. Lourens Robberts, School of Public Health, University of Alberta. Bacteria were generated as previously described (Grayfer et al., 2011). Briefly, *M. marinum* were grown with shaking at 30 °C as a dispersed culture in 7H9 broth (Difco, USA) supplemented with 0.5% glycerol and 10% albumin-dextrose complex for 7–10 days. The number of colony forming unites (cfu)/mL was determined by plating on Middlebrook 7H10 agar (Difco). Before use in the experiments, bacterial cultures were dispersed by 10–15 passages though a 25-gauge needle. When required, enumerated bacterial cultures were heat-killed by incubation in an 80 °C water bath for 30 min. Heat killing efficiency (loss of bacterial viability) was confirmed by plating heat killed *M. marinum* on Middlebrook 7H10 agar (Difco), and failure of bacteria to grow after 5 days of incubation.

A. salmonicida A449 was a kind gift from Dr. Jessica Boyd (NRC Institute, Halifax, Canada) and is a virulent strain of *Aeromonas* that possesses an A layer and is auto-aggregating as previously described (Katzenback and Belosevic, 2009). Glycerol stocks of *A. salmonicida* A449 stored at −80 °C were used to streak Tryptic Soy Agar (TSA) + 20 μg/mL chloramphenicol (Sigma) plates and incubated at 18 °C for 72 h. A single colony was used to inoculate 5 mL of tryptic soy broth (TSB) + 20 μg/mL chloramphenicol that was grown for 24 h at 18 °C with shaking to stationary phase. A 1:100 dilution of this culture was used to inoculate 100 mL of TSB + chloramphenicol and cultured at 18 °C until mid-log phase. Bacteria were harvested and washed twice in sterile 1× PBS. Due to the auto-aggregating nature of the bacteria, a sample of bacteria was serially diluted in TSB containing 1% SDS prior to plating on TSA + chloramphenicol plates which mitigated clumping and allowed for the enumeration of individual colony forming units (CFUs). Plates were incubated for 48 h at 18 °C and colonies counted. *A. salmonicida* A449 was heat-killed by incubating at 60 °C for 45 min in a circulating water bath. Following heat killing, a sample of the bacteria was plated on TSB + chloramphenicol plates to ensure bacteria were non-viable. Heat-killed *A. salmonicida* A449 was stored at −20 °C until used.

2.3. Identification of goldfish Nod1, Nod2 and Nlrx1

To clone Nod1, Nod2 and Nlrx1, primers were designed based on the nucleotide sequences of Nod1 gene of grass carp (Accession No. FJ937972.1) and zebrafish (Accession No. XM_002665060.2), Nod2 gene of rohu (Accession No. JF923468.1) and zebrafish (Accession No. XM_692832.4) and Nlrx1 gene of catfish (Accession No. FJ004848.1) and zebrafish (Accession No. XM_680389.5). All primers used for expression and cloning of goldfish Nod1, Nod2 and Nlrx1 are listed in Supplementary Table 1. RACE PCR (Clonetech, USA) was performed following the manufacturer's specifications to obtain the full open reading frame and untranslated 5′ and 3′ sequences of goldfish Nod1, Nod2 and Nlrx1. Sequencing was performed by first cloning the Nod1, Nod2 and Nlrx1 amplicons into the pJET1.2/blunt cloning vector (Fermantas) and then identifying the colonies that contained positive inserts by colony PCR using the pJET1.2 sequencing primers (Fermantas). Positive clones were isolated using the QIAquick PCR purification kit (Qiagen), and sequenced using the DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia) and a PE Applied Biosystems 377 automated sequencer. Subsequent gene annotations were conducted using BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Conserved domains of NLRs were identified by Pfam (http://pfam.sanger.ac.uk/search/sequence). Phylogenetic analysis of Nod1, Nod2 and Nlrx1 was done by neighbor joining method of MEGA4 program and bootstraped 10,000 times (Tamura et al., 2007).

2.4. Analysis of goldfish NLRs tissue gene expression

Total RNA was isolated from kidney, spleen, liver, heart, muscle, intestine, and gill of healthy goldfish using TRIzol (Gibco) and reverse transcribed into cDNA using Superscript III cDNA synthesis kit (Invitrogen, USA) in accordance to manufacturer's directions. Primers specific for the goldfish NLRs were designed using Primer Express software (Applied Biosystems, USA) and the expression of