Fish & Shellfish Immunology 55 (2016) 173–185

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

NOD2 in zebrafish functions in antibacterial and also antiviral responses via NF-κB, and also MDA5, RIG-I and MAVS

Peng Fei Zou [a, b, 1], Ming Xian Chang [b, 1], Ying Li [c], Na Na Xue [b], Jun Hua Li [b], Shan Nan Chen [b], Pin Nie [a, b, *]

[a] College of Fisheries, Jimei University, 43 Yindou Road, Xiamen, Fujian Province, 361021, China
[b] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, 430072, China
[c] Department of Environmental Science and Engineering, Xiamen University, Tan Kah Kee College, Zhangzhou, Fujian Province, 363105, China

ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form
21 May 2016
Accepted 22 May 2016
Available online 24 May 2016

Keywords:
Nod-like receptor
NOD2
RLR
NF-kappaB
Zebrafish

ABSTRACT

NOD2/RIPK2 signalling plays essential role in the modulation of innate and adaptive immunity in mammals. In this study, NOD2 was functionally characterized in zebrafish (*Danio rerio*), and its interaction with a receptor-interaction protein, RIPK2, and RLRs such as MDA5 and RIG-I, as well as the adaptor, MAVS was revealed in fish innate immunity. The expression of NOD2 and RIPK2 in ZF4 cells has been constitutive and can be induced by the infection of *Edwardsiella tarda* and SVCV. The NOD2 can sense MDP in PGN from Gram-negative and -positive bacteria. It is further revealed that the NOD2 and RIPK2 can activate NF-κB and IFN promoters, inducing significantly antiviral defense against SVCV infection. As observed in the reduced bacterial burden in RIPK2 overexpressed cells, RIPK2 also has a role in inhibiting the bacterial replication. The overexpression of NOD2 in zebrafish embryos resulted in the increase of immune gene expression, especially those encoding PRRs and cytokines involved in antiviral response such as MDA5, RIG-I, and type I IFNs, etc. Luciferase reporter assays and co-immunoprecipitation assays demonstrated that zebrafish NOD2 is associated with MDA5 and RIG-I in signalling pathway. In addition, it is further demonstrated that RIPK2 and MAVS in combination with NOD2 have an enhanced role in NOD2-mediated NF-κB and type I IFN activation. It is concluded that teleost fish NOD2 can not only sense MDP for activating innate immunity as reported in mammals, but can also interact with other PRRs to form a network in antiviral innate response.

1. Introduction

Innate immune system plays as the first defense against invading pathogens on the basis of PRRs which recognize PAMPs [1]. Several classes of PRRs are well characterized, including Toll-like receptors (TLRs) which are transmembrane proteins functioning in the recognition of distinct PAMPs derived from various microbial pathogens [2,3], and RLRs and NLRs, which are two classes of major cytosolic PRRs detecting cytosolic PAMPs to activate various signalling cascades [3]. RLRs, including RIG-I, MDA5 and laboratory of genetics and physiology 2 (LGP2), sense viral RNA and activate antiviral innate immune response with typical production of type I IFNs and inflammatory cytokines [4,5]. NLRs, which contain more than 20 members in mammals [6], sense a variety of characteristic bacterial products for stimulating NF-κB pathway and inflammasome [6,7].

NOD2, also known as CARD15, is a member of the NLR receptor

Abbreviations used are: NOD, nucleotide oligomerization domain; NF-κB, nuclear transcription factor-κB; MDA5, melanoma differentiation-associated gene 5; RIG-I, retinoic acid-inducible gene I; MAVS, mitochondrial antiviral signaling protein; RIPK2, receptor-interacting serine-threonine kinase 2; RLR, retinoic acid-inducible gene I-like receptor; ZF4, zebrafish embryonic fibroblast cell line; SVCV, Spring Viremia of Carp Virus; PGN, peptidoglycan; MDP, muramyl dipeptide; NLR, NOD-like receptor; MAPK, mitogen-activated protein kinase; PRR, pattern recognition receptor; PAMP, pathogen associated molecular pattern; CARD, caspase recruitment domain; LRR, leucine-rich repeat; ORF, open reading frame; hpi, hours post-infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hpt, hours post-transfection; hpf, hours post-fertilization.

* Corresponding author. State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, 430072, China.
E-mail addresses: pniefish@hotmail.com, pinnie@ihb.ac.cn (P. Nie).
[1] The two first authors contributing equally to this research.

http://dx.doi.org/10.1016/j.fsi.2016.05.031
1050-4648/

family, and is composed of three main domains: an N-terminal domain which contains two adjacent CARDs and is involved in the interaction with downstream factors [7,8], a central NACHT domain (also designed as a NOD domain), which facilitates self-oligomerization and promotes activation of effector molecules [9], and multiple C-terminal LRRs which are essential for the detection and recognition of PAMPs [7,10]. In mammals, it was demonstrated that NOD2 can be activated primarily by the constituent of PGN, i.e. MDP [11,12]. Upon ligand sensing, NOD2 promotes the recruitment of RIPK2 through CARD-CARD interaction [8,13,14], and the recognition of MDP by NOD2 may lead to the activation of NF-κB and MAPK pathways, which include p38, extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) [15]. Through NF-κB and MAPK pathways, the NOD2-mediated inflammatory response may have important medical significance as genetic mutations in NACHT and LRRs of NOD2 are associated with autoinflammatory diseases, such as Crohn disease [12,16], Blau syndrome [17] and early-onset sarcoidosis [18]. Recent studies have shown that NOD2 can recognize viral ssRNA and activate the expression of interferon-regulatory factor 3 (IRF3) through the adaptor protein MAVS for the production of IFN-β [19,20]. Furthermore, NOD2-deficient mice failed to produce interferon efficiently with enhanced susceptibility to virus-induced pathogenesis. It was also shown that NOD2 can interact with oligoadenylate synthetases (OAS), and is involved in the antiviral signalling pathway mediated by OAS2-RNase-L [21].

The RIPK2, also known as RICK, CARDIAK, RIP2, was originally identified as a protein implicated in NF-κB activation and apoptosis induction [22–24]. It is further demonstrated that RIPK2 is a critical downstream mediator of NOD1 and NOD2 signalling [8,25,26], and NOD2/RIPK2 signalling plays essential role in the modulation of innate and adaptive immunity [27,28]. However, it was reported that the role of NOD2/RIPK2 in innate immunity was confined to the mediation of antibacterial responses [27,29], despite that NOD2 has been shown to activate the induction of type I IFN via MAVS-IRF3-IFN-β cascade, and whether NOD2/RIPK2 signalling is implicated in antiviral immune response is still unknown.

In teleost fish, orthologues of human NOD2 have been identified in zebrafish *Danio rerio* [30–32], channel catfish *Ictalurus punctatus* [33], grass carp *Ctenopharyngodon idella* [34] and rainbow trout *Oncorhynchus mykiss* [35], and orthologues of human RIPK2 in zebrafish [30,32]. It is bioinformatically revealed that NOD2/RIPK2 signalling is conserved in teleost fish [30,32]. It was shown experimentally that silencing of NOD2 impaired the ability of zebrafish larvae in reducing intracellular bacterial burden and then in controlling systemic infection [36], and the over-expression of trout NOD2 effector domains resulted in induced expression of proinflammatory cytokines, and antibacterial peptides, as well as a variety of caspases and type I and type II IFNs [35]. However, the function of NOD2 in relation with RIPK2 remains poorly understood in teleost fish. In the present study, the role of NOD2/RIPK2 signalling was investigated in antibacterial as well as in antiviral immune responses in zebrafish. The functional interaction between NOD2 and other RLRs, i.e. MDA5 and RIG-I was also investigated in zebrafish in the present study to examine if NOD2 interacts with other PRRs and if these two RLRs have other immune roles in addition to their antiviral immunity in fish [37–40]. The antibacterial and antiviral function of NOD2/RIPK2 was therefore demonstrated in zebrafish in the present study. The interaction between NOD2 and MDA5, RIG-I was also demonstrated in the modulation of NOD2-mediated NF-κB activation. These findings provide a new and essential perspective on NOD2/RIPK2-mediated signalling, and the cross-talk between NOD2 and MDA5, RIG-I in innate immune response in fish.

2. Materials and methods

2.1. Maintenance of zebrafish, bacteria, virus, and cell lines

Zebrafish (*Danio rerio*) breeders were maintained at 28 °C on a 14 h (h) light/10 h dark cycle [41] in a flow-through system. All zebrafish embryos were collected from artificial fertilization and cultured in petri dishes at 28 °C.

Edwardsiella tarda PPD130/91 strain was used as an intracellular pathogen [42] in this study. *E. tarda* was grown in tryptic soy broth (TSB, BD Biosciences) liquid culture at 28 °C. For virus infection, SVCV, a negative ssRNA virus, which is infective in *Epithelioma papulosum cyprini* (EPC) cells [39], was cultured at 25 °C, with the virus titre determined by plaque assay under methylcellulose overlay (0.5% in Dulbecco modified Eagle medium (DMEM)).

Zebrafish embryonic fibroblast cells (ZF4) [43] were grown at 28 °C in a mixture of 90% 1:1 mixed DMEM and Ham F12 medium (Invitrogen-Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen-Gibco), 100 U/ml penicillin (P), and 100 μg/ml streptomycin (S) as described previously [44]. EPC cells were purchased from China Center for Type Culture Collection (GDC174) and maintained in DMEM supplemented with 10% FBS and 100 U/ml P/S at 25 °C. Human embryonic kidney cells (HEK 293T) (ATCC CRL-11268TM) were maintained at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS.

2.2. Gene cloning, plasmid construction and transfection

The full-length coding sequences of NOD2, MAVS, RIPK2, MDA5a, MDA5b [39], RIG-Ia, RIG-Ib [40] in zebrafish were amplified from the cDNA synthesized from total RNA extracted from zebrafish larvae by PCR using Ex-Taq (Takara, Dalian, China). The primers used for the amplification were designed according to the data in NCBI GenBank database (XM_692832.4 for NOD2, FN178460.1 for MAVS and NM_194411.2 for RIPK2) or to our previous sequences submitted also in the GenBank (JX462556 for MDA5a, JX462557 for MDA5b, JX462558 for RIG-Ia and JX462559 for RIG-Ib) (Supplemental Table 1). Total RNA was extracted from 20 zebrafish larvae using TRIzol reagent (Invitrogen-Gibco) according to the manufacturer's recommendation. The cDNA template was synthesized from the RNA by using the Superscript reverse transcriptase (Fermentas Life Sciences, Germany). The PCR was performed under the following conditions: 1 cycle of 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min; and 1 cycle of 72 °C for 10 min. The PCR fragments were cloned into pMD18-T vector (Takara, Dalian, China) and sequenced subsequently by Sangon Biotech Co., Ltd (Shanghai, China).

For overexpression studies, the ORF of zebrafish NOD2, MAVS, RIPK2, MDA5a, MDA5b, RIG-Ia and RIG-Ib was inserted respectively into ptGFP1 vector which was modified from the pTurboGFP-N vector and contains two sets of CMV promoter and SV40 3′UTR to drive the expression of target gene product and GFP as separate proteins rather than as a fusion protein [37]. In addition, the ORF of RIPK2 was also inserted into pTurboGFP-N vector (Evrogen, Moscow, Russia), the ORFs of MDA5a and MDA5b were subcloned into pcDNA3.1/myc-His (−) A vector (Invitrogen), and the ORF of NOD2 was inserted into p3xFLAG-CMV™-14 expression vector (Sigma Aldrich, St Louis, MO). To understand the role of different domains of NOD2 in zebrafish, various NOD2 mutants, ΔCARD1 without CARD1 (residues 101–980), ΔCARDs without CARDs (residues 263–980), ΔLRRs (residues 1–726), or containing only CARD1 (residues 1–99), CARD2 (residues 101–279), CARDs (residues 1–279), NACHT (residues 263–441), LRRs (residues 727–980) respectively were cloned and constructed into ptGFP1 vector. The primers including the restricted enzyme sites used for gene cloning