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RIG-I specifically mediates group II type I IFN activation in nervous necrosis virus infected zebrafish cells

Hsu-Yu Chen [a], Wangta Liu [b], Sung-Yu Wu [a], Pinwen Peter Chiou [a], Yen-Hsing Li [c], Yen-Chun Chen [b], Gen-Hwa Lin [a], Ming-Wei Lu [d, *], Jen-Leih Wu [a, *]

[a] Institute of Cellular and Organismic Biology, Academia Sinica, Taipei 115, Taiwan
[b] Department of Biotechnology, Kaohsiung Medical University, Kaohsiung 807, Taiwan
[c] Department of Basic Medical Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA
[d] Department of Aquaculture, The College of Life and Resource Science, National Taiwan Ocean University, Keelung 202, Taiwan

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ABSTRACT

The type I interferon (IFN) response has been shown to be crucial for the survival of zebrafish larvae infected with nervous necrosis virus (NNV). Teleost type I IFNs can be divided into two groups, based on their cysteine content. While teleost group I IFNs have been extensively studied in terms of their regulation and anti-viral properties, the characteristics of teleost group II IFNs have been relatively unexplored. In this study, we describe the mechanism by which group II IFNs are activated in response to NNV infection in a zebrafish cell line, by focusing on the relationship between type I IFNs and pattern recognition receptors. Expression profile analysis of infected cells by microarray and qPCR revealed signaling activation of two pattern recognition receptors (PRRs): RIG-I like receptors (RLRs) and MyD88-dependent Toll-like receptors (TLRs). Knockdown of retinoic acid-inducible gene I (RIG-I) specifically repressed induction of group II IFNs (IFNϕ2, IFNϕ3) by NNV infection. Furthermore, Ingenuity Pathway Analysis (IPA) was used to demonstrate that RIG-I knockdown results in down-regulation of the inflammatory response in NNV-infected cells. Taken together, our results indicate that RIG-I plays an essential role in zebrafish group II type I IFN induction and the inflammatory response to NNV infection.

1. Introduction

Type I interferons (IFNs) play an important role in the innate immune response to virus infection in mammals. The infected cells produce IFNα/β upon recognition of viral nucleic acid by pattern recognition receptors (PRRs). The secreted IFNs then bind to interferon receptors, thereby triggering downstream activation of antiviral proteins or pro-inflammatory responses. Recent studies have shown that the type I IFN system of teleost fish has similar features with that of mammals [1,2].

Type I IFNs of vertebrates have been categorized into three groups, based on their cysteine content: a 4 cysteine (4C)-containing group, and two 2 cysteine (2C)-containing groups [3]. The 4C group is present in all vertebrates from cartilaginous fish to mammals, while the 2C group is divided into teleost-specific (C2 and C3) and mammalian (C2 and C4) groups [4]. The two fish IFNs are referred to as group I type I IFNs (2C group) and group II type II IFNs (4C group). Each group of IFNs exhibits unique expression patterns in different cells or tissues [4–6], suggesting they might be differentially regulated. The two mammalian type I IFN groups share a common receptor complex composed of two chains, interferon (alpha, beta, and omega) receptor 1 and 2 (IFNAR1 and IFNAR2) [7,8]. In contrast, teleost group I and II type I IFNs bind to two distinct receptor complexes, composed of three different chains: cytokine receptor family member b1, b2, and b5 (CRFB1, CRFB2, and CRFB5) [9,10]. The differences between the receptor systems suggest that the function and regulation of fish type I IFNs are more complicated than those of mammals. In zebrafish, four functional IFNs have been found: IFNϕ1 [11], IFNϕ2, IFNϕ3 [4], and IFNϕ4 [12]. Zebrafish IFNϕ1 and IFNϕ4 belong to group I, while IFNϕ2 and IFNϕ3 belong to the group II type I IFN family [3,4]. To date, most published studies have focused on the antiviral function and activation of group I type I IFNs [10,13]. Conversely, the biofunction and regulation of fish group II type I IFNs remain unclear.

PRRs play a crucial role in regulating type I IFN activation. Three major PRR signaling pathways are involved in the type I IFN

* Corresponding authors. Tel./fax: +886 2 27899534.
E-mail addresses: mingwei@mail.ntou.edu.tw (M.-W. Lu), jlwu@gate.sinica.edu.tw (J.-L. Wu).

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response: RLR, TLR3, and TLR7(8). Different receptors recognize different molecular patterns of virus RNA genomes, and activate the appropriate downstream anti-viral signaling pathway [14]. TLR3 specially recognizes double-stranded RNA (dsRNA) of the virus genome, or the replication intermediate and activate downstream signaling through TRIF signaling. TLR7 and TLR8, MyD88-dependent TLRs signaling, can detect single-stranded RNA (ssRNA) of RNA viruses [11,16]. TLR22, a fish specific TLR, also recognizes viral dsRNA, and activates type I IFN through TRIF signaling [17]. The RLR pathway consists of RIG-I, MDA5, and LGP2, which are essential RNA virus sensors in the cytoplasm. LGP2 also functions as a negative regulator of RIG-I and MDA5 [15]. Interestingly, RIG-I and MDA5 play different roles in activating the host antiviral response. RIG-I preferentially binds to viral RNA bearing a triphosphate at the 5′ end [18–20] and requires a short, blunt double-stranded structure for binding [21,22]. On the other hand, MDA5 prefers long, double-stranded replication intermediates [21,23].

Nervous necrosis virus (NNV) is a single-stranded RNA virus, with a genome composed of two positive sense single-stranded RNAs, RNA1 and RNA2. NNV RNA2 was predicted to form a stem loop structure near its 3′ end [24]. NNV can infect more than 34 species of fish, including both marine and freshwater species; such infection causes necrosis in brain and retina, with a mortality rate of up to 98% at the larvae and juvenile stage. The type I IFN response has been reported to play a crucial role in protection against NNV infection in fish [25]. While adult zebrafish exhibit up-regulation of IFNϕ1 upon infection, infected larvae are unable to produce a protective level of IFNϕ1. Treatment of zebrafish larvae with type I IFN prior to infection can effectively increase their survival rate. Furthermore, *Mx*, a downstream gene of IFN signaling, was induced earlier than *IFNϕ1* up-regulation in infected fish, and *Mx* levels continued to increase while *IFNϕ1* expression decreased [26]. These results suggest that there may be other type I IFNs involved in the innate immune response to NNV. The regulation and activation of the type I IFN response to NNV infection is of great interest since most of the host fish are major aquaculture species. However, the involvement of PRR pathways and different groups of type I IFN in NNV infection is poorly understood.

To obtain insights into the interactions between PRRs and different groups of type I IFNs in fish, we set up an infection model using a zebrafish cell line (ZF-4). The availability of the zebrafish genome sequence facilitates the use of microarray analysis for genome-wide expression profiling. In addition, it has previously been shown that IFNs in these cells significantly inhibit virus infection, making it an ideal model for the study of IFN regulation upon virus infection [11]. In the present study, we demonstrate that RLR and MyD88 signaling are activated during NNV infection, and that two groups of type I IFN are induced. One of the most interesting findings reported here is that RIG-I specifically regulates expression of group II, but not group I IFNs. Moreover, knockdown of RIG-I expression repressed the antiviral and inflammatory responses. The findings herein enhance our understanding of the regulation of group II type I IFNs during NNV infection.

2. Materials and methods

2.1. Cells and viruses

ZF-4 cells, an embryonic-derived cell line, were previously reported to respond to virus infection through IFN signaling. Cells were grown at 28 °C in RPMI 1640 medium (containing RPMI1640 powder, 10% FBS, 2 g sodium bicarbonate, 10 ml Penicillin Streptomycin, 10 ml sodium pyruvate, and 10 ml GlutaMAX). Red-spotted grouper nervous necrosis virus (RGNVV) [27] was prepared from the infected GF-1 cells [28]. The culture medium from infected GF-1 was harvested on the 5th day after infection, and virus titers were determined based on $TCID_{50}$ [29] using GF-1 cells.

2.2. Real-time cellular analysis

This study used xCELLigence (Roche Applied Science) to monitor virus-induced cytopathogenicity. ZF-4 cells were seeded in E16 plates at a density of 10,000 cells per well. The wells are coated by a gold electrode array, which can directly measure changes in electrical impedance resulting from cell adhesion across the well. Independent recordings were taken from each well, and these data were converted into Cell Index using the software provided by the manufacturer. Only living adherent cells are detected. The greater the Cell Index (CI), the greater the extent of cell adhesion. Once the CI reached the stationary phase, the cells were washed with PBS and infected with NNV at three different multiplicities of infection (MOI = 1, 5, or 10). The mock infection group was treated with medium only (negative control). The CI values were measured every 30 min.

2.3. Virus infection and replication assay

ZF-4 cells were seeded in 12 well plates, and then inoculated with NNV. Cells were incubated with NNV at different MOIs (1–10) for 1 h. The cells were then washed to remove the unbound viral particles, and further cultured at 28 °C. The culture supernatant and the cells were collected at various time points after infection. The cell pellets were subjected to total RNA extraction and gene expression analysis. The viral titer of the culture supernatant was

Table 1
Sequences of primers used in the present study.

Gene name	Accession number	Primer sequence
ifnphi1	NM_207640	F: AAGTTTTTAGTCCTGACATTGGATCA
		R: TCCCAGTTCACCGAGTTCATG
ifnphi2	NC_007114	F: CCTCTTTGCCAACGACAGTT
		R: CGGTTCCTTGAGCTCTCATC
ifnphi3	NM_001111083	F: TTCTGCTTTGTGCAGGTT TG
		R: GGTATAGAA ACGCGGTCGTC
mxa	NM_182942	F: AAGGCCACAACCGAAAATAAAA
		R: CGCAAGGTGGCATGATTG
mxb	XM_693985	F: AATGGTGATCCGCTATCTGC
		R: TCTGGCGGCTCAGTAAGTTT
mxc	NM_001007284	F: GGACCTGAACCTGCCTGCTATTG
		R: AGGGCTTCCAGCACT
mxe	NM_182867	F: GGATTCCAAAGCCATTAAGACGG
		R: TGACAATGTTCTCGAAGGTTCTGTAGT
pkr	NM_001040376	F: CCAGAGCAAATAAAGCCCGAG
		R: TCACCTTTCTCCATCAAACCGT
isg15	NM_001204169	F: AACTCGGTGACGATGCAGC
		R: TGGTGATCGATGGTTCGATTCT
il1b	NM_212844	F: GAAGCACATCAAACCCCAATCC
		R: AGACGGCACTGAATCCACCAC
tlr3	NM_001013269	F: AAAGGGCTACGTTTGGTGTG
		R: GTTGGTGGAGTTCAGCCATT
myd88	NM_212814	F: GCCCAGGAGCTCGCACTA
		R: CGGCCTCTTCATGGATTTGT
trif	NM_001044759	F: CACCACAAGCTGAACTCCGTTA
		R:TTTTGCGTGACAATCGATTCTC
rig1	ENSDART00000058176	F: TTGAGGAGCTGCATGAACAC
		R: CCGCTTGAATCTCCTCAGAC
rig-moe3i3[a]	ENSDART00000058176	F: CCA GAG GGA GTC TGA GGA GA
		R: ACCTTAAACCAGTTTGATTTATCTGAG
mda5	XM_689032	F: GAATCAGAATGTTCGCGTGTGT
		R:CCTCGTCAGGGCTAGATTTGG
lgp2	XM_003198080	F: GCTCAGACTCAGGC ATATCA
		R: AGCCAGATGATGCTGTTCTCT
ef-1a	XM_005173785	F: TGCCTTCGTCCCAATTTCAG
		R: TACCCTCCTTGCGCTCAATC

[a] Primers designed specifically to examine the splicing blocking by morpholinos.