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Zebrafish larvae are unable to mount a protective antiviral response against waterborne infection by spring viremia of carp virus

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ABSTRACT

Interferons (IFNs) and their receptors exist in all classes of vertebrates, where they represent early elements in innate and adaptive immunity. Both types I and II IFNs have been discovered in fish and type I IFN has recently been classified into two groups based on their primary protein sequences and biological activities. Thus, although groups I and II zebrafish IFN show powerful antiviral activities, only group I (IFNϕ1) is able to protect the fish against bacterial infection. In addition, group II IFNs (IFNϕ2 and IFNϕ3) induce a rapid and transient expression of antiviral genes, while group I IFN exerts a slow but more powerful induction of several antiviral and pro-inflammatory genes. To gain further insight into the IFN system of fish, we have developed a waterborne infection model of zebrafish larvae with the spring viremia of carp virus (SVCV). Larvae were challenged 3 days post-fertilization by immersion, which considerably reduces the manipulation of fish and represents a more natural route of infection. Using this infection model, we unexpectedly found an inability on the part of zebrafish larvae to mount a protecting antiviral response to waterborne SVCV. Nevertheless, zebrafish larvae showed a functional antiviral system since ectopic expression of the cDNA of both groups I and II IFN was able to protect them against SVCV via the induction of IFN-stimulated genes (ISGs). Interestingly, group II IFNs also induced group I IFN, suggesting crosstalk between these two kinds of antiviral IFN. These results further confirm the antiviral activities of type I IFN in the zebrafish and provide the first viral infection model for zebrafish larvae using a natural route of infection. This model, in combination with the powerful gene overexpression and morpholino-mediated knockdown techniques, will help to illuminate the IFN system of teleost fish.

1. Introduction

IFNs and their receptors represent a subset of class 2 α-helical cytokines that exist in all classes of vertebrates and represent early elements in innate and adaptive immunity [1]. About 10 mammalian IFN species have been discovered and they are classified into type I (IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ, IFNτ, IFNν and IFNζ), type II (IFNγ) and type III (IFNλ) [1,2]. Types I and III IFN play a major role in protection against viral infection and their induction is governed by several toll-like receptors [3]. In contrast, type II IFNγ is secreted by T lymphocytes under specific activation conditions and by NK cells [4]. Although originally defined as a cytokine with direct antiviral activity, IFNγ regulates several aspects of the innate and adaptive immune responses [4,5].

IFNs are restricted to vertebrates and have been studied mainly in birds and mammals [2]. More recently, type II IFNγ has been sequenced in several teleost fish, where it has been found to be relatively well-conserved and interestingly, some teleosts have more than one IFNγ gene [6–8]. On the other hand, virus-induced IFNs have also been identified in teleost fish with an ambiguous nomenclature (reviewed by [9,10]). While some authors have designated these virus-induced IFNs as type III IFNλ, based on the genomic organization of the zebrafish IFN gene [11] and on the protein structure of its receptor components [12], others have designated them as type I IFN on the basis of the primary nucleotide and protein homology, their cysteine pattern and the presence of a CAWE motif, typical of mammalian IFNαs [13]. Therefore, as fish virus-induced IFNs show a combination of features observed in mammalian type I and type III IFNs, Stein et al. [10] proposed that they should be named IFNϕ. In addition, fish virus-induced IFNs have recently been classified into two groups based on their primary protein sequences: group I, which contains two cysteine residues and is universally present in teleosts; and group II containing four cysteine residues and found only in more primitive species such as the rainbow trout and zebrafish (*Danio rerio*, Cypriniformes) [13]. These two groups differ in their expression pattern and biological activities. Notably, group I IFN

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from zebrafish [14], rainbow trout [13], Atlantic salmon (*Salmo salar*, Salmoniformes) [15] and the channel catfish (*Ictalurus punctatus*, Siluriformes) [16] were potent inducers of the expression of antiviral genes, such as the myxovirus (influenza) resistance gene (Mx) and elicited antiviral responses in cell lines, whereas group II IFN from the rainbow trout showed poor ability in this respect [13]. In addition, it has also been shown that zebrafish IFNϕ1 (belonging to group I) and IFNϕ2 (belonging to group II) increase the survival of embryos infected intravenously (i.v.) with SVCV [12] and IHNV [17]. More interestingly, groups I and II IFN show non-redundant, complementary roles in the zebrafish even though both groups show strong *in vivo* antiviral activities [18]. Thus, zebrafish group II IFNs induced a rapid and transient expression of antiviral genes, while zebrafish group I IFN exerted a slow but more powerful induction of several antiviral and pro-inflammatory genes and was also able to protect the fish against bacterial infection [18].

To gain further insight into the functions of zebrafish IFNs, we set up a waterborne viral infection model of zebrafish larvae and tested the bioactivities of zebrafish group I (IFNϕ1) and group II (IFNϕ2 and IFNϕ3) IFN. Our results revealed the unexpected inability of zebrafish larvae to mount an immune response to waterborne SVCV despite the presence of a functional antiviral system, which was able to eliminate the virus when appropriately induced. Thus, overexpression of group I and II IFNs protected zebrafish larvae against SVCV via the induction of several ISGs. These results further support the involvement of type I IFN in the protection of zebrafish against viral infections and provide the first waterborne viral infection model for zebrafish larvae.

2. Materials and methods

2.1. Animals

Wild-type zebrafish (*Danio rerio* H. Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center (ZIRC) and maintained as described in the zebrafish handbook [19]. All animal studies were carried out in accordance with the European Union regulations for animal experimentation.

2.2. Expression constructs and microinjection

All the zebrafish IFNϕ1, IFNϕ2 and IFNϕ3 expression constructs were in pcDNA3.1/V5-His backbone (Invitrogen) and have been previously described [18]. Plasmid DNA (50–100 pg/egg) were mixed in microinjection buffer (0.5× Tango buffer and 0.05% phenol red solution) and microinjected (0.5–1 nl) into the yolk sac of one-cell-stage embryos using a Narishige IM300 microinjector.

2.3. Infection assays

The SVCV isolate 56/70 was kindly provided by Dr. P. Fernández-Somalo (Laboratorio Central de Veterinaria, MARM). The virus stock was propagated in EPC cells and titrated into 96-well plates according to Reed and Muench [20]. Virus was inactivated at 65 °C for 1 h followed by UV irradiation at 254 nm for 1 h. Groups of 25–30 zebrafish larvae were challenged 3 days post-fertilization (dpf) in 5 ml egg water (60 μg/ml sea salts in distilled water) containing $\sim 10^8$ 50% tissue culture infectious

Table 1
Primers used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html).

Gene	Accession number	Name	Nucleotide sequence (5′ → 3′)	Use
Zebrafish primers				
il1b	NM_212844	F5	GGCTGTGTGTTTGGGAATCT	gene expression
		R5	TGATAAACCAACCGGGACA	
lta	AB183467	F2	AAGCCAAACGAAGGTCA	gene expression
		R2	AACCCATTTCAGCGATTGTC	
ifnphi1	NM_207640	F3	GAGCACATGAACTCGGTGAA	gene expression
		R3	TGCGTATCTTGCCACACATT	
ifnphi2	NC_007114	F1	CCTCTTTGCCAACGACAGTT	gene expression
		R1	CGGTTCCTTGAGCTCTCATC	
ifnphi3	NC_007114	F1	TTCTGCTTTGTGCAGGTTTG	gene expression
		R1	GGTATAGAAACGCGGTCGTC	
ifng1-1	NM_001020793	F2	CTTCAGACAACCAGCGCATA	gene expression
		R2	TTTTCCAACCCAATCCTTTG	
ifng1-2	AB158361	F5	CTATGGGCGATCAAGGAAAA	gene expression
		R3	CTTTAGCCTGCCGTCTCTTG	
mavs	NM_001080584	F	GTTCCCGGTCCAAGACACTA	gene expression
		R	TTGTCGCCTGAGTTGTTCTG	
mxb	XM_693985	F	AATGGTGATCCGCTATCTGC	gene expression
		R	TCTGGCGGCTCAGTAAGTTT	
mxc	NM_001007284	F	GAGGCTTCACTTGGCAACTC	gene expression
		R	TTGTTCCAATAAGGCCAAGC	
rig1	ENSDART00000058176	F	TTGAGGAGCTGCATGAACAC	gene expression
		R	CCGCTTGAATCTCCTCAGAC	
rsad2	EF014961	F1	AGCAGATCACCGCTCTCAAT	gene expression
		R1	CCAGACACTGGATGCTCTGA	
pkz	NM_001013299	F1	GGAGCACCGTACAGGACATT	gene expression
		R1	CTCGGGCTTTATTTGCTCTG	
tnfa	NM_212859	F2	GCGCTTTTCTGAATCCTACG	gene expression
		R2	TGCCCAGTCTGTCTCCTTCT	
tlr3	NM_001013269	F1	AAAGGGCTACGTTTGGTGTG	gene expression
		R1	GTTGGTGGAGTTCAGCCATT	
tlr22	NM_001128675	F1	TGGGCCAAGAAGAATGAATC	gene expression
		R1	ATGACAACAGGAGGGTGAGG	
SVCV primers				
SVCV N protein	U18101	F2	TGAGGTGAGTGCTGAGGATG	gene expression
		R2	CCATCAGCAAAGTCCGGTAT	