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Long Double-stranded RNA Induces Sequence-specific RNA Interference and Type I Interferon Responses in Rock Bream (*Oplegnathus fasciatus*)

Kosuke Zenke and Ki Hong Kim*

Department of Aquatic Life Medicine, Pukyong National University, Busan 608-737, Korea

To determine whether long double-stranded RNA (dsRNA) induces RNA interference and type I interferon (IFN) responses in fish, long dsRNAs encoding enhanced green fluorescent protein (EGFP), GFPuv, and polyinosinic-polycytidylic acid sequences were co-injected with an EGFP expressing plasmid, into rock bream (*Oplegnathus fasciatus*). We investigated the EGFP mRNA and protein levels, and the transcriptional responses of dsRNA-dependent protein kinase and Mx1 genes. Long dsRNAs were strong inducers of a type I IFN response in rock bream, resulting in nonspecific suppression of exogenous gene expression. Furthermore, sequence-specific knockdown of exogenous gene expression at the mRNA level was detected at an early phase (24 h). These results suggested that long dsRNA may inhibit exogenous gene expression through an early mRNA interference response and a later type I IFN response in fish.

Key words: Double-stranded RNA, RNA interference, Type I interferon response, Rock bream (*Oplegnathus fasciatus*)

Introduction

Double-stranded RNA (dsRNA) derived from the replication intermediates of many RNA viruses is one of the major pathogen-associated molecular patterns (PAMPs) that trigger innate immune responses in vertebrates. In mammalian cells, endosomal toll-like receptor 3 (TLR3) recognizes dsRNA (Alexopoulou et al., 2001; Wang et al., 2004; Schulz et al., 2005) and recruits Toll/IL-1 receptor (TIR) domain-containing adaptor protein, which activates TIR domain-containing adapter-inducing interferon-β (TRIF) by interacting with its TIR domain (Yamamoto et al., 2002). Subsequently, TRIF indirectly activates several transcription factors such as interferon (IFN) regulatory factor 3, nuclear factor-κB, and activator protein 1, which modulate the transcription of IFN-β and inflammatory cytokines (Sharma et al., 2003; Yamamoto et al., 2003; Hoebe et al., 2003; Meylan et al., 2004). The dsRNA-dependent protein kinase R (PKR) is also a key player in the type I IFN response. Upon activation of PKR through recognition of dsRNA, PKR phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α) (Meurs et al., 1990), resulting in the termination of general protein synthesis (Hershey, 1991; Williams, 1999; Sudhakar et al., 2000). A similar dsRNA recognition mechanism occurring through TLR3 and PKR was recently reported in fish species (Matsuo et al., 2008; Rothenburg et al., 2008; Zhu et al., 2008; Zenke et al., 2009). Secreted IFN regulates the expression of numerous genes that encode antiviral proteins such as Mx proteins (de Veer et al., 2001).

Within the last decade, much attention has been focused on understanding dsRNA as a powerful inducer of RNA interference (RNAi). In eukaryotic cells, dsRNA is degraded by an RNase III-like enzyme, Dicer, into small dsRNA fragments of about 21 bp, which are called small interfering RNAs (siRNAs). Together with the RNA-induced silencing complex, siRNAs can mediate sequence-specific degradation of target mRNAs (Hannon, 2002). However, unlike in lower eukaryotes (Fire et al., 1998; Timmons and Fire, 1998), the introduction of long dsRNA in vertebrates can cause severe side effects owing to the dsRNA-induced type I IFN response (Elbashir et al., 2001). To circumvent this problem, chemically synthesized or *in vitro* trans-

*Corresponding author: khkim@pknu.ac.kr

cribed siRNAs have been used for RNAi-mediated silencing of mRNAs (Elbashir et al., 2001; Tuschl, 2002; Dykxhoorn et al., 2003). However, recent studies have reported that even short RNAs (i.e., siRNAs) can induce a type I IFN response in a sequence-dependent or cell type-dependent manner (Sledz et al., 2003; Kariko et al., 2004; Reynolds et al., 2006; Svoboda, 2007).

In fish, artificially synthesized long dsRNAs such as polyinosinic-polycytidylic acid (poly I:C) sequences are known to induce a type I IFN response and have been used experimentally to prevent viral diseases in vivo (Jensen et al., 2002a; Plant et al., 2005; Fernandez-Trujillo et al., 2008) and in vitro (Saint-Jean and Pérez-Prieto, 2006; Jensen and Robertsen, 2002; Jensen et al., 2002b). However, it is not known whether long dsRNAs encoding mRNAs elicit the sequence-specific knockdown of target genes in fish. If long dsRNA were to induce a RNAi response independent of a type I IFN response, this technology may be applicable as an antiviral measure to silence the expression of target viral genes and to induce a type I IFN response.

In the present study, to determine whether long dsRNA induces both RNAi and type I IFN responses in fish, long dsRNAs encoding enhanced green fluorescent protein (EGFP), GFPuv, and poly I:C were co-injected with an EGFP expression vector into rock bream (*Oplegnathus fasciatus*), a commercially important marine species in South Korea. We investigated the EGFP mRNA and protein expression levels as well as the transcriptional responses of the PKR and M×1 genes at 24 and 72 h after injection.

Materials and Methods

Fish

Rock bream fingerlings (body weight, approximately 2-3 g each) were obtained from a commercial hatchery in South Korea. The fish were fed with commercial pellets, and the water was replaced every day. The fish were acclimatized for at least 1 week before the experiments were performed.

Preparation of dsRNA

dsRNA was synthesized *in vitro* using a Megascript RNAi kit (Ambion) following the manufacturer's instructions. Briefly, DNA fragments encoding the EGFP and GFPuv genes were amplified by PCR using primers that included the T7 RNA polymerase promoter sequence (Table 1). Then, the sense and antisense single-stranded RNAs were simultaneously transcribed using T7 RNA polymerase and 1 μg of the PCR product as a substrate, followed by annealing to produce dsRNA. Any template DNA and single-stranded RNA remaining in the reaction was digested using DNase I and RNase A, respectively, and the dsRNA was precipitated with ethanol precipitation. The integrity and quantity of dsRNA were evaluated by agarose gel electrophoresis and spectrophotometry, respectively. The sizes of the dsRNAs were 500 bp for EGFP (dsRNA-EGFP) and 540 bp for GFPuv (dsRNA-GFPuv).

Co-injection of dsRNA and EGFP-expressing plasmid

We used the EGFP expression plasmid pβA-EGFP, which contains the β-actin promoter of rock bream, as described previously (Zenke and Kim, 2009). A total of 50 fish were randomly divided into five groups, which were kept separately in small net cages. The fish in each group were intramuscularly injected near the upper right side, just beneath the mid-dorsal fin, with 2.5 μg pβA-EGFP plasmid alone (positive control) or mixed with 5 μg of dsRNA-EGFP, dsRNA-GFPuv, or poly I:C; or with PBS alone (negative

Table 1. Oligonucleotides used in this study

Name	Sequence (5' to 3')	Purpose
EGFP-T7 F	TAATACGACTCACTATAGGGGTGGTGCCCATCCTGGTCGAG	dsRNA-EGFP preparation
EGFP-T7 R	TAATACGACTCACTATAGGGTGCACGCTGCCGTCCTCG	dsRNA-EGFP preparation
GFPuv-T7 F	TAATACGACTCACTATAGGGCAACATACGGAAAAC	dsRNA-GFPuv preparation
GFPuv-T7 R	TAATACGACTCACTATAGGGTGTCGACAGGTAATG	dsRNA-GFPuv preparation
RbPKR F	CAAAGCAGGCCAGTGGATAC	Detection of PKR
RbPKR R	ACTTTGCCAATGGATTGTCTG	Detection of PKR
RbMx1 F	CAGCTGAAACTCAAGGAAGTGGC	Detection of Mx1
RbMx1 R	CTGGTGCTTTTCTTGGCTGG	Detection of Mx1
EGFP F	CAACTACAACAGCCACAACG	Detection of EGFP
EGFP R	TTACTTGTACAGCTCGTCCATG	Detection of EGFP
RbβA F	GCCCCAGGCATCAGGGAGTG	Detection of β-actin
RbβA R	ACCGTGCTCGATGGGGTACT	Detection of β-actin

*T7 promoter sequences were underlined.

control). At 24 and 72 h after injection, the five groups were sampled, and a portion of muscle (about 5×5×5 mm) near the injection site was excised from the fish. For fluorescence quantification, half of the excised tissue was homogenized in 0.1 M Tris-Cl (pH 7.4) buffer containing 0.1% Triton X-100. The lysate was centrifuged at 8,000×*g* for 10 min, and the supernatant containing the soluble fraction was collected. The quantity of total protein in the supernatant was measured using the BCA method. Fluorescence was measured using a Polarion fluorescence plate reader (Tecan). For each sample, fluorescence intensity was calculated as fluorescence per μg of protein.

To prepare samples for semi-quantitative RT-PCR analysis, total RNA was isolated from the remaining half of the muscle and spleen tissues, and treated with RQ1 RNase-free DNase (Promega) according to the manufacturer's instruction. For cDNA synthesis, 0.5 μg of purified RNA was incubated with 0.5 μL of random primer (0.5 μg/mL; Promega) at 70°C for 10 min; the template/primer was mixed with 2 μL of 5× reaction buffer, 2 μL of 10 mM dNTP mix (Takara), 0.5 μL of M-MLV reverse transcriptase (Promega), and 0.25 μL of RNase inhibitor (Promega) in a final reaction volume of 10 μL, followed by incubation at 42°C for 60 min.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was carried out to determine the relative mRNA expression levels of EGFP and the type I IFN-inducible genes PKR and Mx1. PCR was performed in a reaction volume of 10 μl using 2× Prime Taq Premix (Genet Bio) and 1 μl of cDNA template diluted to 10^{-1}. The oligonucleotide primer pairs for the target genes (PKR, Mx1, and EGFP) and internal control gene (β-actin) are listed in Table 1. The primers for Mx1 were designed to detect the Mx1-specific isoform found in rock bream (Zenke and Kim, 2009). The reaction conditions consisted of 95°C for 4 min (initial denaturation), followed by 25 cycles (for β-actin), 26 cycles (for PKR and EGFP), or 30 cycles (Mx1) of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 7 min. PCR amplicons were electrophoresed in a 1% agarose gel and visualized using ethidium bromide staining. Images of the stained gels were imported into Quantity-One image analysis software using a Gel Doc XR imaging device (Bio-Rad). Band intensities of the target genes were normalized to that of β-actin to estimate the relative mRNA levels.

Statistical analysis

One-way ANOVA followed by Turkey's HSD test was used for statistical analysis.

Results

Effect of dsRNA injection on the expression of the EGFP reporter gene

At 24 h post injection (p.i.), there was no significant difference in fluorescence intensity among the groups. The fluorescence intensity was similar between the group injected with EGFP expression vector (positive control) and the group injected with PBS (negative control), and no fluorescence level exceeded the background level (autofluorescence of muscle tissue) (Fig. 1A). However, unlike EGFP protein expression, EGFP mRNA expression was significantly lower in the dsRNA-EGFP-injected group compared with the positive control group injected with EGFP vector alone (Fig. 1B). In addition, although not statistically significant, the EGFP transcript level in the group injected with dsRNA-GFPuv was reduced by approximately 40% compared with the level in the positive control group.

At 72 h p.i., significant fluorescence was observed in the group injected with EGFP vector alone. There was a slight increase in fluorescence in the groups co-injected with EGFP expression vector and dsRNA, but the level was not significantly different from the autofluorescence observed in the negative control group injected with PBS. There was no difference in the EGFP mRNA level among the groups (Fig. 1B).

Transcriptional responses of PKR and Mx1 to dsRNA injection

To examine whether dsRNA delivery can cause a type I IFN response in rock bream, the mRNA expression levels of PKR and Mx1 were assessed at 24 and 72 h following dsRNA injection (Fig. 2). At 24 h p.i., PKR mRNA expression was significantly upregulated, 5 to 6 fold in muscle and 7 to 8 fold in spleen, in individuals injected with dsRNA species or poly I:C, compared with fish injected with EGFP or PBS. However, at 72 h p.i., there was no significant difference in PKR mRNA expression among any of the groups or tissues. The expression pattern of Mx1 was similar to that of PKR.

Discussion

In mammals, it has been reported that long dsRNA-mediated RNAi effects can be significantly masked by a nonspecific type I IFN response (Elbashir et al., 2001). In the present study, we investigated whether this phenomenon also occurs in

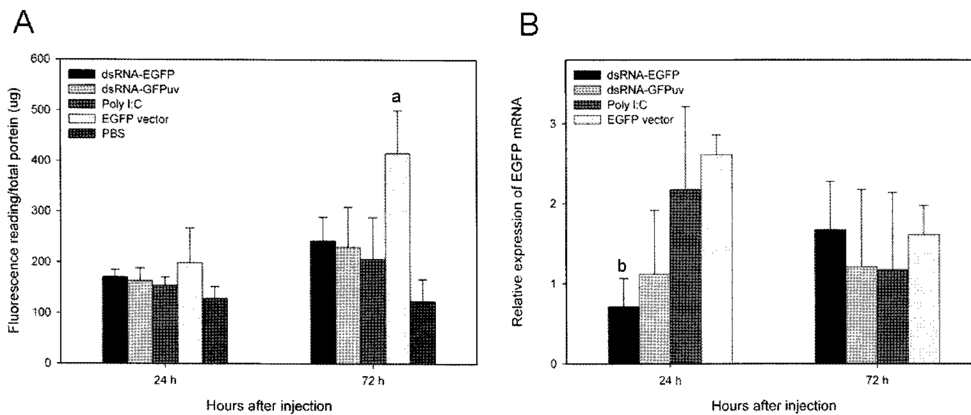

Fig. 1. Effects of co-injection of EGFP expression plasmid and dsRNA on EGFP protein and transcripts levels. Each group of rock bream was injected with EGFP expression plasmid and dsRNA encoding EGFP (dsRNA-EGFP), GFPuv (dsRNA-GFPuv), or poly I:C. One group was only injected with EGFP expression plasmid (Vector), and control group was injected with PBS. After 24 h and 72 h injection, a portion of muscle was excised from the site of injection and subjected to quantification of fluorescence or semi-quantitative RT-PCR analysis. (A) Quantification of fluorescence. (B) A semi-quantitative RT-PCR analysis of EGFP mRNA expression. All data was presented as mean value with standard deviation (n=5). [a]Significantly different from the group injected with PBS (P<0.01), [b]significantly different from the group injected with vector only (P<0.0001).

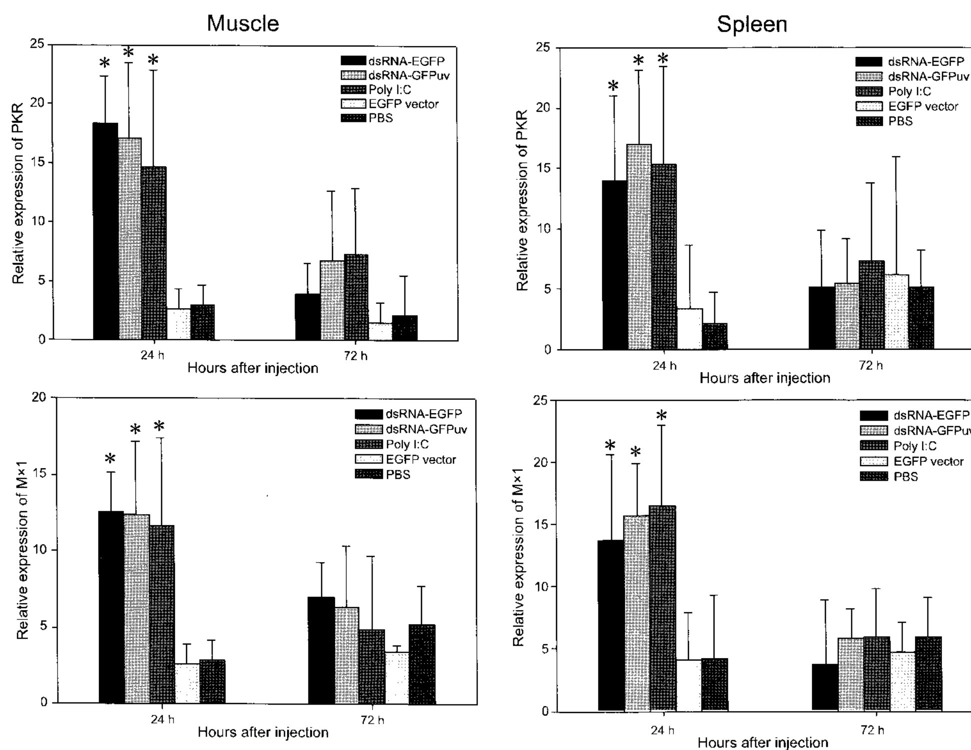

Fig. 2. Up-regulation of PKR and Mx1 after co-injection of EGFP expression plasmid and dsRNA. After co-injection of EGFP expression plasmid and dsRNA, semi-quantitative RT-PCR analyses of PKR and Mx1 expression were carried out using muscle and spleen cDNAs. All data was presented as mean value with standard deviation (n=5). *Significantly different from the group injected with vector only (P<0.05).

fish.

At 24 h p.i., no EGFP protein expression was observed, but EGFP mRNA expression was detected. Interestingly, while there was no apparent reduction in EGFP expression in rock bream co-injected with poly I:C, fish co-injected with dsRNA-EGFP

exhibited significantly reduced EGFP mRNA levels compared with the levels in fish injected with EGFP expression vector alone. Furthermore, the group co-injected with dsRNA-GFPuv, which has 47.6% sequence identity with EGFP, exhibited a moderate, but not significant, reduction in EGFP mRNA. It appears that the reduction in EGFP mRNA expression in each group was dependent on the identity between the dsRNA and EGFP mRNA sequences; this suggests the induction of RNAi via dsRNA.

It is probable that some of the siRNAs produced from dsRNA-GFPuv shared 100% nucleotide sequence identity with regions of the EGFP sequence, resulting in moderate inhibition of EGFP mRNA levels in the dsRNA-GFPuv-injected group. In mammalian cells, long dsRNAs were processed into siRNAs independent of their apparent RNAi activity (Yang et al., 2001). RNAi activity was induced via long dsRNAs in mammalian cells in which the type I IFN system was lacking or had not developed, as in cells of mouse pre-implantation embryos (Wianny and Zernicka-Goetz, 2000), oocytes (Stein et al., 2005), or undifferentiated embryonic stem cells (Yang et al., 2001; Kunath et al., 2003). However, the RNAi activity in differentiated cells may be masked by a type I IFN response (Yang et al., 2001). The lack of detectable EGFP protein expression at 24 h p.i. in the groups co-injected with dsRNAs as well as in the group injected with EGFP expression vector alone indicates a low level of mRNA translation into protein, as opposed to a nonspecific type I IFN response, despite the high PKR and Mx1 mRNA levels which are indicative of a strong type I IFN response.

A sequence-dependent reduction of the EGFP mRNA level was observed at 24 h p.i., aside from nonspecific inhibition of expression; however, at 72 h p.i., there was no difference in mRNA expression. EGFP protein expression was detected at 72 h, but only in the group injected with EGFP expression vector alone. No EGFP protein expression was observed in the groups injected with dsRNA; this indicates that EGFP protein expression was suppressed in these groups, although the results from the present study do not confirm that the suppression of EGFP expression was attributable to RNAi. The present results suggest that nonspecific inhibition through PKR activation is also a dominant mechanism for the inhibition of exogenous gene expression, as has been reported in mammals (Yang et al., 2001).

In summary, this study is the first to report that in fish, long dsRNA encoding a specific gene may inhibit target gene expression through both an early RNAi response and a later type I IFN response. Further studies investigating the effects of dsRNA dosage, treatment time, and sequence length on the induction of RNAi and IFN responses in fish are needed to evaluate the efficacy of using long dsRNAs to control fish diseases.

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