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Research paper

Synergistic effects in the antiviral activity of the three Mx proteins from gilthead seabream (*Sparus aurata*)

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ABSTRACT

Due to their direct antiviral activity, Mx proteins play a main role in the response mediated by type I interferon against viral infections. The study on gilthead seabream Mx proteins is especially interesting, since this species is unusually resistant to viral diseases, being asymptomatic carrier of several viruses pathogenic to other fish species. Gilthead seabream has three Mx proteins (Mx1, Mx2 and Mx3) that, separately, display antiviral activity against a wide range of viruses, showing interesting differences in their antiviral specificities. In this work, the possible synergy between the three Mx isoforms has been studied using *in vitro* systems consisting of CHSE-214 cells stably expressing two or the three gilthead seabream Mx proteins. The antiviral activity of these Mx combinations has been tested against the Infectious Pancreatic Necrosis Virus (IPNV), the Viral Haemorrhagic Septicaemia Virus (VHSV), the European Sheatfish Virus (ESV) and the Lymphocystis Disease Virus (LCDV). A synergistic effect of the Mx proteins was only detected against ESV, no synergy was observed against LCDV, and a negative interference was detected against the two RNA viruses tested, IPNV and VHSV, as viral replication was higher in cells expressing certain Mx combinations than in cells expressing these proteins separately. These results suggest a functional interaction between gilthead seabream Mx isoforms, which results in a higher or lower antiviral activity depending on the virus tested, thus supporting the idea of complex virus-host interactions and finely tuned mechanisms controlling the antiviral activity of Mx proteins.

1. Introduction

Type I interferon (IFN I) response is a key component of the innate immune system against viral infections in vertebrates (Samuel, 2001). IFN I induces hundreds of genes, some of which encode direct antiviral effectors, such as the Mx proteins. Mx proteins belong to the dynamin superfamily of high molecular weight GTPases, which are involved in intracellular membrane remodelling and intracellular trafficking (Kochs et al., 2005). They form homo-oligomers and self-assemble into ring-like and helical structures, which are critical for GTPase activity, protein stability, and viral recognition (Haller et al., 1998, 2007). Mx proteins interfere with viral replication at different stages, and, although their precise antiviral mechanisms are still unknown, there are evidences indicating that they rely on a direct interaction between the Mx protein and a specific viral target, that needs to be identified in each case (Haller and Kochs, 2011).

Mx proteins usually appear in different isoforms. Two Mx genes have been reported in amphioxus and in several mammalian species, including humans, whereas three genes have been described in rat (Li et al., 2009). Interestingly, a large variability in the number of Mx isoforms (from 1 to 7) has been disclosed in 15 fish species (Novel et al., 2013). Furthermore, Mx isoforms from the same fish species can differ in their antiviral activity mechanisms and/or antiviral specificity range (Fernández-Trujillo et al., 2011a, 2013; Tafalla et al., 2007; Zenke and Kim, 2009). However, the putative synergy in the antiviral activity of Mx isoforms from the same species has never been addressed.

Three Mx proteins, Mx1, Mx2, and Mx3, corresponding to three different genes, have been identified in gilthead seabream (*Sparus aurata*) (Fernández-Trujillo et al., 2011a), which is one of the most important species in Southern Europe aquaculture. This fish species is highly resistant to viral diseases, being asymptomatic carrier of several viruses pathogenic to other fish species such as Viral Nervous Necrosis Virus (VNNV), Infectious Pancreatic Necrosis Virus (IPNV), and Viral Haemorrhagic Septicaemia Virus (VHSV) (Castric

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et al., 2001; Esteban et al., 2008; Pérez-Prieto et al., 2001). Actually, the only viral disease affecting gilthead seabream is the lymphocystis disease (LCD), caused by the Lymphocystis Disease Virus (LCDV) (García-Rosado et al., 1999).

The antiviral activity of each gilthead seabream Mx protein has been tested against IPNV, VHSV, LDCV and the European sheatfish virus (ESV), having shown that Mx1 presents activity against IPNV, VHSV and LCDV; Mx2 against IPNV, LCDV and ESV; and Mx3 against IPNV and VHSV (Fernández-Trujillo et al., 2011b, 2013). Thus, in order to get more insight into the antiviral activity of gilthead seabream Mx proteins, the aim of the present study was to test if these three Mx proteins show synergy in their antiviral activity. For that, four *in vitro* experimental systems stably expressing combinations of the Mx proteins have been developed (Mx1+2, Mx1+3, Mx2+3 and Mx1+2+3 expressing cells). The antiviral activity in these cell lines was tested against IPNV, VHSV, ESV and LCDV, and compared with the antiviral activity in cells expressing each Mx separately.

2. Material and methods

2.1. Cell culture and viral isolates

The CHSE-214 cell line (Fryer et al., 1965) was grown at 20 °C in L-15 Leibovitz (L-15) medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS, Invitrogen), 2% L-glutamine, and 1% antibiotic/antimycotic solution (100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL fungizone, Invitrogen). The stable Mx expression systems were cultured under the same conditions.

The fish viruses used in this study were: (i) IPNV III-1 genotype (reference strain IPNV Sp), *Birnaviridae* family, with bisegmented double-stranded RNA (dsRNA) genome. This isolate was obtained from Senegalese sole (*Solea senegalensis*) (Rodríguez et al., 1997); (ii) VHSV Ia genotype, *Rhabdoviridae* family, with one single-stranded negative sense RNA (ssRNA) segment; (iii) ESV, *Iridoviridae* family, with dsDNA genome and (iv) LCDV (SA9 isolate, obtained from diseased gilthead seabream (Cano et al., 2010), also member of the *Iridoviridae* family.

IPNV, VHSV and ESV were propagated and titrated using the BF-2 cell line (Wolf et al., 1966). Inoculated cells were cultured at 20 °C in L-15 medium supplemented with 2% FBS, 4 mM L-glutamine, 1% antibiotic/antimycotic solution. LCDV was propagated and titrated on the SAF-1 cell line (Béjar et al., 1997), under the same culture conditions. The 50% tissue culture infective dose ($TCID_{50}$) was estimated by the method of Reed and Muench (1938).

2.2. Plasmids

The three double expression systems were generated with the pMx2-Z and pMx3-Z expression plasmids (Fernández-Trujillo et al., 2011b), containing the zeocin resistance gene and the Mx2 and Mx3 cDNAs, respectively, under the control of the citomegalovirus (CMV) promoter.

For the triple expression system, the complete CMV-Mx1 expression cassette was extracted from the pMx1-Z vector (Fernández-Trujillo et al., 2011b) by *BglII-ApaI* digestion and subsequently subcloned into the pcDNA5/TO vector (Invitrogen), which contains the hygromycin B resistance gene. For that, the enhancer sequence of this plasmid was previously removed by *BglII-ApaI* digestion. This vector, named pMx1-H, was confirmed by sequencing.

2.3. Transfection and isolation of clonal populations

For transfections, cells were seeded onto six-well culture plates and transfected 24 h later with 500 ng of the corresponding plasmid added to 6 μL of Fugene reagent (Roche) diluted in 100 μL of L-15 medium. Mx1-expressing cells were transfected with pMx2-Z and pMx3-Z to obtain the Mx1+2 and Mx1+3 systems. Mx2-expressing cells were transfected with pMx3-Z to obtain the Mx2+3 system. Transfected cells were selected with zeocin (Invitrogen, 1.25 mg/mL, final concentration). To obtain the triple system, the polyclonal population expressing Mx2+Mx3 was transfected with the pMx1-H construction and selected with hygromycin B (75 μg/mL, Invitrogen).

After several weeks of selection, several monoclonal populations of each expression system were obtained using the end-point dilution method as previously described (Fernández-Trujillo et al., 2011b). To select one clone of each expression system, the Mx genes transcriptional level and the growth rate of the lines obtained were evaluated.

Table 1
Primers used in this study.

PCR product	Primer name	Primer sequence (5′–3′)
Mx1 FJ490556 (86 bp)	Mx1-F	GAGGGAGAGGAGTGGTC
	Mx1-R	CGAATCTTTTTCTCCACATCC
Mx2 FJ490555 (187 bp)	Mx2-F	CCATAAAGCAAGAAAAGGAATG
	Mx2-R	GGTGCCTTTTTAAGATGTACG
Mx3 FJ652200 (178 bp)	Mx3-F	ATCTATGCTGAGATCCCAGTTC
	Mx3-R	ATGTTGAAGAAAATACCATTCC
CHSE eF1α FJ890356.1 (105 bp)	CHSEeF-F	GGCAAGGGCTCTTTCAAGTATG
	CHSEeF-R	TGCCGGTCTCAAACTTCCA

2.4. Characterisation of clonal populations

To quantify the expression of the recombinant Mx genes in each clonal population, quantitative RT real-time PCRs were carried out in several consecutive passages. Cells were grown in 25-cm^2 flasks, washed with PBS, and harvested with a cell scrapper at 95% confluence. Total RNA was extracted with TRIzol (Invitrogen) and treated with RNase-free DNase (Takara) at 37 °C for 30 min. RNA (2 μg) was reverse transcribed with the SuperScript II Reverse Transcriptase (Invitrogen) using oligo dT primers in a 40-μL reaction mixture.

Quantitative real-time PCRs were carried out using a 7500 Real-Time PCR system (Applied Biosystems). The elongation factor 1α (eF1α) was used as reference gene. The primer sequences and the size of each PCR product are shown in Table 1. Reactions were conducted in a volume of 20 μL containing 10 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems), 1 μL of each primer (15 μM), 6 μL of nuclease-free water, and 2 μL of cDNA. Cycling parameters were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 57 °C for 1 min. All samples were run in triplicate with non-template controls on the same plate. Fluorescence was measured at each cycle during the 57 °C step.

Data were analysed with the comparative Ct method and presented as mean ± standard deviation (SD) of relative transcription with respect to the eF1α transcription. The differences between samples were tested by the two-tailed unpaired Student's *t*-test. Differences of $p < 0.05$ were considered statistically significant.

To evaluate the growth of each Mx expression system, cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assays (Mosmann, 1983). Cells were seeded onto 96-well plates (2×10^2 cells per well and 12 wells per system) and one plate per day was measured for 6 d by adding 10 μL of MTT (5 mg/mL) to each well. After 4 h at 20 °C, a volume of 150 μL of acid isopropyl alcohol was added, and the optical density (OD) was measured at 550 nm.