Molecular Immunology 49 (2011) 107–114

Contents lists available at ScienceDirect

Molecular Immunology

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

Differential antiviral activity of Mx1, Mx2 and Mx3 proteins from gilthead seabream (*Sparus aurata*) against Infectious Pancreatic Necrosis Virus (IPNV)

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ARTICLE INFO

Article history:
Received 20 June 2011
Received in revised form 29 July 2011
Accepted 30 July 2011
Available online 27 August 2011

Keywords:
Mx proteins
Gilthead seabream
Sparus aurata
CHSE-214 cells
IPNV

ABSTRACT

Mx proteins are crucial effectors of the innate antiviral response mediated by the interferon type I signalling pathway. Recently, three Mx proteins, named SauMx1, SauMx2 and SauMx3, corresponding to three different genes, have been identified in the cultured marine species gilthead seabream (*Sparus aurata*). In this study, the three SauMx cDNAs were cloned into expression vectors and used for transfection of CHSE-214 cells. Monoclonal cell populations stably expressing each recombinant protein have been obtained and characterized. The protection conferred by each recombinant SauMx against Infectious Pancreatic Necrosis Virus (IPNV) infection has been *in vitro* evaluated, having found clear differences among them. According to the cytopathic effects and the virus yield reduction assays, only cells expressing SauMx2 and SauMx3 showed significant resistance to IPNV infection. Otherwise, quantitative RT real-time PCR assays suggested that each SauMx protein has a different target during the viral inhibition process. The differences observed among the three SauMx proteins are discussed in terms of their differential mechanism of action and antiviral specificity, suggesting, as a whole, to play a synergistic activity in the protection of gilthead seabream against IPNV.

1. Introduction

Mx proteins are key components of the antiviral state triggered by interferon (IFN) type I in response to viral infections. They belong to the dynamin superfamily of high molecular weight GTPases, which are involved in intracellular membrane remodelling and intracellular trafficking (Kochs et al., 2005). These proteins have been found in either nuclear or cytoplasmic locations, and are highly conserved in vertebrates (Horisberger and Gunst, 1991; Leong et al., 1998).

Mx proteins usually appear in different isoforms. Two Mx genes have been reported in amphioxus and in several species from mammals, including humans, whereas three have been described in rat (reviewed in Li et al., 2009). Interestingly, a large inter-specific variability in the number of Mx isoforms (from 1 to 7) has been disclosed in 12 fish species belonging to different taxa (reviewed in García-Rosado et al., 2010), which reveals the development of a variety of Mx functional strategies in this group to deal with viral infections. This large diversity supports the emerging role of the innate immune system variability in the defence strategies of fish and lower vertebrates against pathogens.

The direct antiviral activity of Mx proteins was demonstrated more than a decade ago (Haller et al., 1998) and, since then, functional studies, mostly in birds and mammals, have especially focused on host–virus interactions. Mx proteins may interfere viral replication at different stages depending on host cell–virus combinations, and although their precise antiviral mechanisms still remain to be elucidated, there are evidences indicating that they are mediated by the interaction between Mx proteins and specific viral components, thus impeding viral functions (reviewed in Haller et al., 2007a). In this way, human MxA specifically binds the nucleoprotein of La Crosse virus and Thogoto virus (*Orthomyxoviridae* family), which in turn affects the transcriptional activity of the viral ribonucleoprotein and compromises the assembly of nucleocapsids and the formation of new viral particles (Haller et al., 2007a; Kochs et al., 2002). However, for other Mx proteins, such as human MxB (Pavlovic et al., 1990), rat Mx3 (Meier et al., 1990) or duck Mx (Bazzigher et al., 1993), no antiviral function has been detected so far.

In fish, the Mx antiviral activity has been directly evidenced in several species and for various viruses. The interference of fish Mx proteins with viral RNA synthesis and/or viral protein expression has been repeatedly reported (Caipang et al., 2003; Larsen et al., 2004; Fernández-Trujillo et al., 2008; Wu and Chi, 2007; Wu et al., 2010). Also, the direct binding of fish Mx to several viral proteins has

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doi:10.1016/j.molimm.2011.07.023

been demonstrated (Chen et al., 2008; Wu et al., 2010). In contrast, similar to the Mx of higher vertebrates, no antiviral activity has been revealed for other fish Mx (Trobridge et al., 1997; Wu and Chi, 2007).

Our group has recently cloned and characterized three different Mx cDNAs from gilthead seabream (*Sparus aurata*), namely, SauMx1, SauMx2 and SauMx3 that correspond to three different genes (Fernández-Trujillo et al., 2011). In that study, the assignment of each Mx cDNA to a different gene, and the expression pattern in response to a viral challenge, have been related to the high resistance to viral infections shown by gilthead seabream, which is the most important species in Southern European fish aquaculture. Specifically, lymphocystis disease is the only natural viral infection reported to affect gilthead seabream (García-Rosado et al., 1999), while it could be an asymptomatic carrier of viral nervous necrosis virus (VNNV) (Castric et al., 2001) and Infectious Pancreatic Necrosis Virus (IPNV) (Pérez-Prieto et al., 2001).

IPNV (genus *Aquabirnavirus*, family *Birnaviridae*), is a non-enveloped, icosahedral virus with two segments of double-stranded RNA. Segment A encodes the polyprotein pVP2–VP4–VP3, which is cotranslationally cleaved by the VP4 protein, releasing pVP2 and VP3. pVP2 is further processed into a mature structural VP2, the external capsid protein. VP3 is the internal capsid protein, which is involved in the viral assembly (Pedersen et al., 2007). Segment A also contains a smaller overlapping ORF encoding the nonstructural protein VP5 (Pedersen et al., 2007). Segment B encodes the VP1 protein, the viral RNA-dependent RNA polymerase (RdRp).

IPNV is the aetiological agent of the Infectious Pancreatic Necrosis (IPN), which is an acute, highly contagious, and widespread disease affecting wild and cultured fish. It causes high mortality, and survivors may become asymptomatic carriers, and therefore, important foci of infection (Imajoh et al., 2005). Due to the economic importance of IPNV for the aquaculture industry, studies on the interaction between the host innate immune system and IPNV are needed to find out natural mechanisms to control viral infections and thus help to develop control measures.

To evaluate the antiviral activity of SauMx1, SauMx2 and SauMx3, three specific *in vitro* cellular systems expressing the corresponding protein were established and characterized. Additionally, the protective effect conferred to the cells by each SauMx protein against IPNV infection was evaluated. Results showed clear differences among the three recombinant SauMx proteins, which might correlate with differences in their antiviral specificity and/or mechanism of action.

2. Materials and methods

2.1. Plasmids

The cDNA sequences of SauMx1 (FJ490556), SauMx2 (FJ490555) and SauMx3 (FJ652200) were cloned into the pGEM-T vector (Promega) (Fernández-Trujillo et al., 2011). Each cDNA sequence was extracted by ApaI and NotI digestion and inserted into the polylinker site of the expression vector pcDNA4/HisMax (Invitrogen), which contains the CMV promoter. Once the correct assembly of the fragments was checked by sequencing, the 3.3-kb expression cassettes containing each SauMx cDNA under the control of the CMV promoter were obtained by PCR from the corresponding plasmids using the iProof High Fidelity DNA Polymerase (Bio-Rad) and the MxClaF and MxClaR primers, both carrying ClaI sites (Table 1). PCR conditions were 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 90 s, and a final step at 72 °C for 10 min. ClaI digested PCR products were cloned into the ClaI sites of the pCVpf plasmid (Gallardo-Gálvez et al., 2011), that contains the expression cassette of the fusion protein GFP–puro: Green Fluorescent Protein, used as a marker of transgenic cells, and the puromycin resistance gene, used as selectable marker. Thus, the three resulting plasmids had a final size of 8.6 kb, and included two independent expression cassettes: the GFP–puro fusion protein and the corresponding SauMx protein.

2.2. Cell culture and virus propagation

The CHSE-214 cell line, derived from Chinook salmon embryonic cells (*Oncorhynchus tshawytscha*) (Fryer et al., 1965), was grown at 20 °C in L-15 Leibovitz medium 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).

The IPNV Sp genotype used in this study, isolated from Senegalese sole, *Solea senegalensis* (Rodríguez et al., 1997), was propagated on CHSE-214 cells at 20 °C in L-15 medium supplemented with 2% FBS. Virus titres were calculated by the 50% tissue culture infective dose ($TCID_{50}$) method (Cunningham, 1973).

2.3. Transfection and clonal isolation

CHSE-214 cells were seeded in 6-well culture plates (5×10^5 cells per well), incubated for 24 h, and transfected with 500 ng of each plasmid added to 6 μl of Fugene Reagent (Roche) diluted in 100 μl of L-15 medium. The end-point dilution method was used for the clonal isolation. Briefly, GFP positive cells were counted at 5–6 d post-transfection using a fluorescence microscope (Olympus 1X50 SBF, equipped with an IX-FLA unit) and dilutions to seed 0.5 GFP-positive cells/well in 96-well culture plates were calculated. After an overnight incubation, all wells were examined for GFP expression, to ensure cloning and plating efficiency. Non-transfected cells were then added to help the growth of clonal cells. Then, clonal populations were selected using supplemented L-15 with 2 μg/ml of puromycin dihydrochloride (Sigma).

2.4. Indirect immunofluorescence staining

Cells expressing each SauMx protein were seeded on silane (aminoalkylsilane)-treated slides. Cell monolayers were washed with PBS, fixed with formalin at room temperature for 24 h, and permeabilized with 0.3% Triton X-100 at 4 °C for 30 min. Afterwards, slides were saturated with 5% skimmed milk in PBS (blocking solution). An Anti-HisG antibody (Invitrogen) against the poly-histidine tag (provided by the pcDNA4/HisMax vector) of the recombinant proteins was added to the blocking solution (1 μg/ml, final concentration). Incubation was carried out with shaking at room temperature for 30 min. A Qdot conjugate 605-goat anti-mouse IgG (Invitrogen) was used as secondary antibody (1:500 dilution in blocking solution). At this step, nuclei were stained with DAPI (1:2000 from a 1 mg/ml stock solution) and incubation was performed in darkness as described above. Finally, slides were washed with PBS for 1 h, mounted with 80% glycerol in PBS, and observed under a fluorescence microscope (Leica mod. TCS NT).

2.5. Cell growth curves

Control non-transfected CHSE-214 cells, and SauMx1-, SauMx2- and SauMx3-expressing cell clones were seeded in 24-well plates (2×10^5 cells/well) and cultured as described above. Three wells of each cell population were daily trypsinized and counted using a Beckman Coulter Z2 Counter. The experiment was repeated twice. Data obtained are presented as mean ± standard deviation. Differences between samples were tested by the two-tailed unpaired