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Endogenous grouper and barramundi Mx proteins facilitated the clearance of betanodavirus RNA-dependent RNA polymerase

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ABSTRACT

This study confirmed that the infection of nervous necrosis virus (NNV), belonging to the betanodavirus, can induce the expression of endogenous Mx in grouper fin-3 (GF-3), grouper brain (cGB), and barramundi brain (cBB) cells, but not in grouper fin-1 (GF-1) cells. In a co-sedimentation assay, RdRp appeared in the mitochondrial pellet of GF-1 cells without endogenous Mx expression. However, in GF-3, cGB, and cBB cells, RdRp was detected in the nuclear pellet accompanied by endogenous Mx. By immunostaining, RdRp was found to colocalize with not only endogenous Mx but also lysosomes and monodansylcadaverine (MDC)-labeled autophagic vacuoles. In GF-1 cells, the RdRp level continuously increased during 24–72 h post infection (hpi). When endogenous Mx expressed during 24–72 hpi in virus-infected GF-3, cGB, and cBB cells, the RdRp level peaked at 24 hpi but decreased at 48–72 hpi. The degradation of RdRp could be suppressed by treatment with 3-methyladenine (3MA), NH_4Cl, and Mx-specific siRNA respectively. After poly I:C transfection, the endogenous Mx level peaked at 3 days post transfection (dpt) and then spontaneously decreased at 5–7 dpt. The poly I:C-indued Mx also colocalized with MDC-labeled autophagic vacuoles at 3 dpt, and its degradation could be inhibited by 3MA or NH_4Cl treatments. Therefore, the anti-NNV mechanism of endogenous grouper and barramundi Mx is suggested to sequester RdRp for degradation through autophagy and lysosomes.

1. Introduction

Nervous necrosis virus (NNV) is the causative agent of viral nervous necrosis (VNN) disease, which has caused mass mortality of cultured marine fish at the larval stage, resulting in severe economic loss (Chi et al., 2003; Munday et al., 2002). NNV belongs to the betanodavirus genus of the *Nodaviridae* family. The target organ of NNV is the nervous system, and the pathological characteristics of VNN disease is the vacuolation of the brain and retina (Chi et al., 1997; Munday et al., 2002). NNV is a non-enveloped icosahedral virus with a diameter of 20–34 nm, and its viral genome contains two-segmented, single-stranded, positive-sense RNAs without a poly A tail (Chi et al., 2001; Mori et al., 1992). RNA1 encodes the RNA-dependent RNA polymerase (RdRp), and RNA2 encodes the capsid protein. During NNV replication, a subgenomic RNA derived from RNA1—termed RNA3—encodes B2 protein, which antagonizes cellular RNA interference (Fenner et al., 2006).

The type I interferon (IFN) response is a crucial host defense against virus infection. Mx protein is one of the IFN-inducible proteins that exhibit antiviral activity against viruses (Verhelst et al., 2013). The GTP-binding domain at the N-terminal of Mx protein is vital for antiviral activity, and the leucine-zipper motif at the C-terminal can interact with viral proteins and determines antiviral specificity (Frese et al., 1995; Zurcher et al., 1992). Mx proteins belong to the dynamin-like superfamily that associate with intracellular membranes and are involved in a wide range of intracellular transport processes such as endocytosis, intracellular vesicle transport, organelle maturation, and cell division (Danino and Hinshaw, 2001; Schmid et al., 1998; Sever et al., 2000; van der Bliek, 1999). Mx proteins exhibit biophysical features similar to dynamin, including the propensity to self-assemble into ring-like and helical structures, the ability to tubulate lipids, and the possession of mechanochemical function (Accola et al., 2002; Danino and Hinshaw, 2001; McNiven et al., 2000; Stowell et al., 1999; Sweitzer and Hinshaw, 1998).

Groupers and barramundi are major cultured fish species in Taiwan and have suffered from VNN disease for decades. Both

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grouper and barramundi Mx proteins were reported to exhibit anti-NNV activity. Three forms of Mx genes (MxI, MxII, and MxIII) were cloned from orange-spotted grouper (*Epinephelus coioides*) and transfected to grouper brain 3 (GB3) cells, respectively. The propagation of NNV was inhibited in the stable clones of GB3 cells expressing grouper MxI, MxII, and MxIII respectively (Lin et al., 2006). The grouper fin-1 (GF-1) cell line can produce a high yield of NNV (Chi et al., 1999), and it is deficient in expressing endogenous grouper Mx, suggesting that endogenous grouper Mx may play a crucial role in repressing NNV replication. Chen et al. (2008) cloned an orange-spotted grouper Mx gene which belongs to grouper MxII. Over-expression of the grouper Mx in GF-1 cells resulted in a reduction of NNV yield, and the grouper Mx was shown to interact with NNV capsid protein through Far-Western analysis (Chen et al., 2008). The mechanism of how grouper Mx antagonizes NNV replication in cells remains unknown. However, endogenous barramundi Mx (BMx) protein in cBB cells can be induced by NNV infection and interferes with NNV RNA synthesis by interacting with viral RdRp (Wu et al., 2010). In NNV-infected cBB cells, the NNV RdRp level peaked at 24 hpi, but subsequently decreased, whereas the BMx level continuously increased during 48–72 hpi. BMx can interact with NNV RdRp to form NNV RdRp/BMx complexes, and viral RdRp was found to colocalize with both BMx and lysosomes. It is thus speculated that BMx inhibits NNV RNA synthesis by redistributing NNV RdRp to lysosomes for degradation (Wu et al., 2010). However, how BMx facilitates the degradation of NNV RdRp remains unclear, which is one aim of this study. Furthermore, to realize whether the anti-NNV mechanism of endogenous BMx only exists in the cBB cell line or is a common biological characteristic of fish endogenous Mx protein, the mechanism of endogenous grouper Mx (GMx) against NNV is investigated, which is the second aim of this study.

We established GF-3 and cGB cell lines separately derived from orange-spotted grouper fin and brain, and confirmed that the endogenous GMx expression in GF-3 and cGB cells could be induced by NNV infection or poly I:C transfection. The anti-NNV mechanism of endogenous GMx were investigated, and how NNV RdRp was eliminated by endogenous Mx proteins was revealed in this study.

2. Materials and methods

2.1. Cell lines and viruses

The cBB cells (Wu and Chi, 2006) were derived from the brain tissue of barramundi (*Lates calcarifer*), and cultured in Leibovitz's L15 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (GIBCO) at 28 °C. GF-1 cells were derived from the grouper fin tissues and cultured in L-15 medium supplemented with 5% FBS at 28 °C (Chi et al., 1999). Grouper fin-3 (GF-3) and grouper brain (GB) cell lines were respectively derived from the fin and brain tissues of orange-spotted grouper (*E. coioides*) and maintained in L-15 medium with 10% FBS at 28 °C. GB cells were found to be persistently infected with NNV, but cured to become an NNV-free cGB cells by using serial treatments with polyclonal anti-NNV antibodies as the method described in our previous study (Wu and Chi, 2006). The NNV strain B00GD, isolated from NNV-infected barramundi (Chi et al., 2003), was used in this study.

2.2. Cloning of grouper Mx cDNA in GF-3 and cGB cells

The NNV-infected GF-3 and cGB cells (MOI = 10) were harvested at 24 hpi, and the total RNA was respectively extracted by acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Reverse transcription was carried out by incubating 1 μg total RNA at 42 °C for 1 h in 30 μl 1 × reaction buffer containing 0.3 μM oligo$(dT)_{20}$, 0.4 mM dNTP, 11.7 mM DTT, 40 U ribonuclease inhibitor rRNasin (Promega), and 60 U MMLV reverse transcriptase (Promega).

In both cDNA derived from GF-3 and cGB cells, a fragment of grouper Mx gene with 956 bp was amplified by the primer set, forward primer 5′-ctgcctgctatcgccgtgatagg-3′ and reverse primer 5′-ctgatggcatcctgagt-3′. For the PCR reaction, an aliquot (5 μl) of the cDNA was amplified in final volume of 25 μl standard 1 × PCR buffer containing 0.4 μM forward and reverse primer, 0.25 mM dNTP, and 0.5 U *Taq* DNA polymerase (NEB). The PCR was carried out in GeneAmp PCR System 2700 (Applied Biosystems) with a denaturing step of 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 1 min, with a final extension of 68 °C for 5 min. The PCR product (956 bp) was cloned into pGEM-T easy vector (Promega), sequenced, and identified as grouper MxII gene (Lin et al., 2006). Afterward, the open reading frame (ORF) of grouper MxII gene in GF-3 and cGB cells and BMx gene in cBB cells was amplified by the primer set, forward primer 5′-ccgaattcatgaacaccctgaaccaaca-3′ and reverse primer 5′-ccccgggctagaactccaccaggtatg-3′ containing the stop codon. The PCR was carried out in GeneAmp PCR System 2700 (Applied Biosystems) with a denaturing step of 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 68 °C for 2 min, with a final extension of 68 °C for 5 min. The PCR products of GMx (1881 bp) from GF-3 and cGB cells and that of BMx (1875 bp) were cut by EcoRI and XmaI (NEB) respectively, cloned into pEGFP-N_2 vector (Biosciences Clontech), and then sequenced. The three constructed plasmids are named pGMx-GF3, pGMx-cGB, and pBMx and can express GMx or BMx without GFP protein due to a stop codon at the terminal of Mx ORF. The deduced amino acid sequences of grouper MxII 12-2 (ABD95982), GF-3 Mx, cGB Mx, and cBB Mx (AAW22002) were aligned by MEGA 6.

2.3. Western blot

For co-sedimentation assay, the GF-1, GF-3, cGB and cBB cells seeded in 75-T flask (1.5×10^6 cells per flask) were respectively transfected with poly I:C (1 μg ml^{-1}) or infected with NNV (NOI = 100). The cells were incubated for 24 h, washed with PBS for 3 times, re-suspended in 500 μl lysis buffer containing 50 mM Tris (pH 8.0), 1 mM DTT, 1 × protease inhibitor (Roche). After the lysates were homogenized and centrifuged at 1000 × *g* for 15 min at 4 °C (P1, the nuclear pellet), 10 000 × *g* for 15 min at 4 °C (P10, the mitochondrial pellet), and 100 000 × *g* for 60 min at 4 °C (P100, the microsomal pellet), the postmicrosomal supernatant (S100) was harvested. Each pellet (P1, P10 and P100) was re-suspended in 500 μl lysis buffer, and 10 μl of each sample was conducted by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The Mx protein and NNV proteins in P1, P10, P100 and S100 were analyzed by Western blot.

For analyzing the NNV RdRp expression and degradation, GF-1, GF-3, cGB and cBB cells seeded in 3-cm dish (4×10^5 cells per dish) were infected with NNV (MOI = 100). The infected cells were treated with 3-methyladenine (3MA) or NH_4Cl at 24 hpi. The concentration of 3MA for treating GF-1 cells was 0.625 mM and for treating GF-3, cGB and cBB cell was 2.5 mM. The concentration of NH_4Cl for treating GF-1 cells was 6.25 mM and for treating GF-3, cGB and cBB cell was 25 mM. The cells were harvested at 24, 48 and 72 hpi, washed with PBS for 3 times, and lyzed in 40 μl lysis buffer containing 50 mM Tris (pH 8.0), 1 mM DTT, 1 × protease inhibitor (Roche), 1% NP-40.

For down-regulating the expression of endogenous Mx protein, GF-3 and cBB cells seeded in 3-cm dish (1×10^5 cells per dish) were respectively transfected with siRNA specific to GMx gene 5′-