Cloning and analysis of antiviral activity of a barramundi (*Lates calcarifer*) Mx gene[☆]

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Abstract

We obtained a full-length cDNA clone for the Mx gene of barramundi (*Lates calcarifer*), using RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR) amplification of RNA extracted from a barramundi brain cell line cBB. The Mx cDNA of 2.2 kb contains an open reading frame (ORF) of 1875 nucleotides encoding a protein of 624 amino acids. The predicted barramundi Mx protein is 71.4 kDa and contains a tripartite guanosinetriphosphate (GTP)-binding motif at the amino terminal and a leucine zipper at the carboxyl terminal, characteristic of all known Mx proteins. Poly I:C-transfection induced the expression of Mx gene in cBB cells, and the induction level at 28 °C was higher than that at 20 °C. Moreover, Mx gene expression was also induced by viral infection, including fish nodavirus, birnavirus, and iridovirus. Among these, nodavirus was a stronger inducer than the other two viruses. Using an antiviral activity assay, we revealed that poly I:C-transfected cBB cells had antiviral activity against fish nodavirus and birnavirus, but not iridovirus. Furthermore, the replication of nodavirus and birnavirus could be restored after the expression of Mx gene was down-regulated by siRNA. Therefore, these results indicated that the expression of barramundi Mx gene was able to inhibit the proliferation of fish nodavirus and birnavirus.

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1. Introduction

The type I interferon (IFN) system is one of the most important mechanisms for antiviral defense. In the type I IFN response, many IFN-related proteins are induced for establishing an antiviral state, such as double-stranded RNA-activated protein kinase (PKR), the 2', 5'-oligoadenylate synthetase, and Mx proteins [1]. Mx proteins belong to the dynamin superfamily [2] and contain a tripartite GTP-binding domain essential for the antiviral activity [3]. The first Mx protein was found in mice and named because of its resistance to orthomyxovirus influenza A [4]. Mx genes were subsequently found in higher vertebrates, including human [5], some livestock species [6–8], and birds [9,10]. Moreover, some Mx proteins have been shown to inhibit virus replication [11–14].

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The lack of information on fish IFN and the difficulty of IFN detection due to its small production and short half-life, led to the use of Mx gene expression as an indicator of IFN activity. Until now, Mx remains useful as a sensitive and more stable marker of the IFN response. In fish, the first Mx gene was identified from perch (*Perca fluviatilis* L.) [15]. Following, piscine Mx genes have been cloned and characterized in rainbow trout (*Oncorhynchus mykiss* Walbaum) [16], Atlantic salmon (*Salmo salar* L.) [17], Atlantic halibut (*Hippoglossus hippoglossus* L.) [18], Japanese flounder (*Paralichthys olivaceus*) [19], fugu (*Takifugu rubripes*) [20], gilthead sea bream (*Sparus aurata*) [21], channel catfish (*Ictalurus punctatus*) [22], and orange-spotted grouper (*Epinephelus coioides*) [23]. However, only few papers have demonstrated the role of fish Mx proteins in IFN response. For example, the Japanese flounder Mx can inhibit the replication of hirame rhabdovirus and viral hemorrhagic septicemia virus (VHSV) [24], while the Atlantic salmon Mx1 can inhibit the replication of infectious pancreatic necrosis virus (IPNV) [25].

Fish nodavirus, nervous necrosis virus (NNV), and fish iridovirus are two major viral pathogens among many species of cultured marine fish in Taiwan [26,27]. The mortality of NNV-infected fish at the larval stage could be as high as 80–100%, and most of the survivors became persistently infected [28]. The information about the mechanism of NNV persistent infection has been very limited until the NNV-persistently infected cell line BB was established [29], and a negative control cell line cBB was obtained by treating BB cells with NNV-specific antiserum [30]. Mx mRNA was detected in the BB cells, but not in the NNV-free cBB cells. In the present study, a barramundi Mx cDNA was cloned from the cBB cells, and the expression of Mx gene in cBB cells was analyzed after poly I:C transfection and virus infection. The antiviral activities against fish nodavirus, birnavirus and iridovirus in poly I:C-transfected cBB cells were examined, and the level of viral replication was further analyzed in cBB cells whose Mx gene expression was down-regulated by siRNA.

2. Materials and methods

2.1. Cell lines and viruses

The cBB cells were cultured with L-15 medium supplemented with 10% fetal bovine serum (FBS), and incubated at 28 °C. Three fish viruses used in the study were: (1) fish nodavirus, B00GD, an NNV isolate of infected barramundi [26]; (2) fish birnavirus, IPNV-SP; and (3) fish iridovirus, TGIV, an isolate from infected grouper [27]. The GF-1 (Grouper Fin-1) cell line [31], the RTG-2 cell line, and the GF-3 (Grouper Fin-3) cell line were respectively used for nodavirus, birnavirus, and iridovirus proliferation and titration.

2.2. Cloning of a barramundi Mx cDNA fragment

A primer set, which was designed from the rainbow trout Mx [16] and channel catfish Mx [22] was chosen to amplify a fragment of the barramundi Mx gene. The sequence of forward primer was 5'-TGAGGAGAAGGTGCGTCC-3' and that of reverse primer was 5'-CTTCTGACCCCTGCACCTGACGA-3'. The acid guanidinium thiocyanate-phenol-chloroform extraction method [32] was used to extract the total RNA from the cBB cells, which were infected with NNV (B00GD) (MOI = 5) for 2 days. Reverse transcription was carried out by incubating 1 µg total RNA at 42 °C for 1 h in 30 µl 1X 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). For PCR reaction, an aliquot (5 µl) of the cDNA was amplified in final volume of 25 µl standard 1X PCR buffer containing 0.4 µM forward and reverse primer, 0.25 mM dNTP, and 0.5 U DyNazyme II DNA polymerase (Finnzymes). The PCR was carried out in GeneAmp PCR System 2400 (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 72 °C for 45 s, with a final extension of 72 °C for 5 min. A 718 bp PCR product was cloned into pGEM-T easy vector (Promega) and sequenced.

2.3. RACE cloning of the full-length barramundi Mx cDNA

RACE was carried out using the Generacer™ primers (Invitrogen), including 5' RACE Abridged Anchor Primer (AAP), 3' RACE Adapter Primer (AP) and Abridged Universal Amplification Primer (AUAP). The gene-specific primers (gsp) used in the RACE were designed from the 718 bp fragment sequence of barramundi Mx obtained in Section 2.2, including 5' RACE first gsp (5'-GCGCCTCCAACACGGAGCTC-3'), 5' RACE second gsp

(5'-CAGCGCCAGGTCCTTCTCCAC-3'), 3' RACE first gsp (5'-CAGGATGAAATGGCCGGGGTTGGTGTGG-3'), 3' RACE second gsp (5'-CACAGAGGCTTTGAAGATGGCACAGGAG-3'). Both 5' and 3' RACE were performed on the total RNA extracted from cBB cells, which were infected with NNV (B00GD) (MOI = 1) for 2 day.

For 5' RACE, 24 µg total RNA of NNV-infected cBB cells were transcribed into cDNA using 5' RACE first gsp. The cDNA was then purified by PCR-M Clean Up System (Viogene) and eluted with 25 µl deionized-distilled water. A poly (C) tail was synthesized onto the ends of the cDNA (9 µl) with 5 U terminal transferase (Biolabs) in 25 µl reaction buffer (1× NEBuffer 4, 0.25 mM CoCl₂, 0.2 mM dCTP) incubating at 37 °C for 30 min, and the reaction was stopped by heating at 95 °C for 5 min. An aliquot (5 µl) of the poly (C) tailed cDNA was amplified by PCR using 5' RACE AAP and 5' RACE first gsp. The nested PCR was amplified with AUAP and 5' RACE second gsp. The PCR program was as same as that in Section 2.2. A 224 bp PCR product was cloned into the pGEM-T easy vector (Invitrogen) and sequenced.

For 3' RACE, 6 µg total RNA was transcribed into cDNA with 3' RACE AP. An aliquot (5 µl) of the cDNA was amplified by PCR with 3' RACE first gsp and AUAP. The PCR reaction was performed by an initial denaturing step of 94 °C for 3 min, followed by 5 cycles of 94 °C for 30 s, 72 °C for 2 min 30 s, and 5 cycles of 94 °C for 30 s, 70 °C for 30 s, 72 °C for 2 min, and 25 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min, with a final extension of 72 °C for 5 min. The nested PCR amplified with 3' RACE second gsp and AUAP. The nested PCR reaction was performed by an initial denaturing step of 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min, with a final extension of 72 °C for 5 min. A 1526 bp PCR product was cloned into the pGEM-T easy vector (Invitrogen) and sequenced. The 5' and 3' RACE sequence overlapped the 718 bp fragment sequence of barramundi Mx by 76 bp and 178 bp, respectively.

Mx amino acid sequences of different species were aligned using Clustal W, and a phylogenetic tree (neighbor-joining) was carried out using MEGA3 [33], with bootstrapping values taken from 1000 replicates.

2.4. Mx expression in cBB cells induced by poly I:C transfection or fish virus infection

The cBB cells were seeded in 30 mm cultured dishes (2×10^5 cells per dish). On the second day, cells were transfected with poly I:C ($1 \mu\text{g ml}^{-1}$) using lipofectin (Invitrogen), or individually infected with fish nodavirus (B00GD), fish birnavirus (IPNV-SP), and fish iridovirus (TGIV) (MOI = 10). Total cellular RNA was extracted at 0, 6, 12, 24, 48, 72 h post transfection or infection, and 1 µg of total RNA was then transcribed into cDNA with oligo(dT)₂₀ described as above.

Real-time PCR was conducted to determine the level of Mx gene expression in the poly I:C-transfected or virus-infected cells over the control. Primers for Mx were forward primer 5'-TGAGGAGAAGGTGCGTCC-3' and reverse primer 5'-GCGCCTCCAACACGGAGCTC-3'. Actin was used as an internal control, and primers of actin were forward primer 5'-CACTCAACCCCAAGCCAACAGG-3' and reverse primer 5'-AAAGTCCAGCGCCACGTAGCACAG-3' [25]. An aliquot (0.5 µl) of the cDNA was analyzed with PCR mixture in a total volume of 20 µl containing 0.5 µM forward primer and reverse primer in 1X iQ SYBR Green SuperMix (Bio-Rad). The amplification was carried out in iCycler iQ Real-Time PCR Detection System (Bio-Rad) with an initial denaturing step of 94 °C for 3 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, and fluorescence detection at 85 °C for 20 s. All samples were analyzed in triplicate. The Mx expression levels were normalized with internal control (actin), and the normalized Mx expression value of the negative control without poly I:C transfection or virus infection was regarded as 1.

2.5. Antiviral activity assay

The optimal multiplicity of infection (MOI) of each virus capable of inducing complete cytopathic effect (CPE) of cBB cells was determined and applied in the following experiment.

The cBB cells were pre-seeded in a 96-well tissue culture plate (1×10^4 cells per well), and transfected with poly I:C ($1 \mu\text{g ml}^{-1}$) on the second day. Control cells were mock-transfected. After 24 h incubation, supernatants were removed from cBB cells, and the cells were washed 3 times in phosphate buffered saline (PBS). Then, every 4 wells as a group was infected with fish nodavirus (B00GD) (MOI = 100), fish birnavirus (IPNV-SP) (MOI = 0.1), and fish iridovirus (TGIV) (MOI = 10), respectively.

After the appearance of complete CPE in the wells of control cells, the entire 96-well plate was stained by crystal violet (0.5% in methanol) for 30 min. Then, the plate was washed in tap-water and air-dried. The optical density (OD) of each well was measured at 595 nm using an ELISA reader (MRX_{plus}, DYNEX Technologies). The highest OD₅₉₅ values appeared in the wells of cBB cells without viral infection (non-infected control). The relative survival rate of cBB cells after each virus infection was calculated with the formula: (the average OD₅₉₅ value from 4 wells of virus-infected cells/the average OD₅₉₅ value from 4 wells of the non-infected cells) × 100%.

2.6. The influence of Mx gene expression on the replication of fish virus

The cBB cells pre-seeded in 24-well-plates (4×10^4 cells per well) were transfected with a mixture of four barramundi Mx-specific siRNAs (100 nM) or 100 nM non-silencing control siRNA (QIAGEN) as negative control, and then transfected with poly I:C (25 ng ml⁻¹) 4 h later. After 18 h, total RNA of the cells was extracted for real-time PCR and analyzed for the Mx gene expression. The treated-cells were also infected with nodavirus, birnavirus, and iridovirus, respectively. The cells were washed with PBS 1 h post virus adsorption, and incubated at 28 °C for nodavirus and iridovirus replication or 20 °C for birnavirus replication. After 24 h, the infected cells were harvested and frozen-thawed 3 times for progeny virus titration. The sense sequences of the Mx-specific siRNAs are Mx-1 r(AGAUGGAGAUGAUCGUUUA)dTdT, Mx-2 r(GCUUCAUUGGAUUUCCUAA)dTdT, Mx-3 r(GACAAAGAU CGAAGCCAUA)dTdT, Mx-4 r(AGCUGAUGUUACACCUAA)dTdT, and the sense sequence of the non-silencing control siRNA is r(UUCUCCGAACGUGUCACGU)dTdT.

3. Results

3.1. Sequence analysis of the barramundi Mx gene

The 5' and 3' RACE sequence overlapped the 718 bp fragment of barramundi Mx gene sequence by 76 bp and 178 bp respectively. The three parts of the sequences were aligned to give a full length cDNA sequence of 2214 bp, which contains an ORF of 1875 bp, encoding a 624 amino acid polypeptide with a predicted molecular weight of 71.4 kDa. The amino terminal contains a tripartite GTP-binding motif and a dynamin family signature. The carboxyl terminal contains a putative leucine zipper motif conserved in all other Mx proteins (Fig. 1). The GeneBank accession number of barramundi Mx sequence is [AY821518](#).

3.2. Comparison of barramundi Mx protein with other Mx proteins

The results of the BLAST analysis using the deduced amino acid sequence of the barramundi Mx protein are shown in Table 1. Barramundi Mx showed highest homology with grouper Mx (87% identity), followed by Japanese flounder Mx (85% identity). Of the non-teleost Mx, barramundi Mx showed 56% identity with human MxA. The Clustal W alignment of the deduced barramundi Mx amino acid sequence with other known fish Mx is shown in Fig. 1, and the highest similarity was located in the amino terminals of the Mx proteins. Subsequently, a neighbor-joining tree comparing all the fish Mx, using human MxA and duck Mx as outgroups, revealed that the barramundi Mx belongs to the cluster of fugu, sea bream, grouper, Japanese flounder, and Atlantic halibut Mx (Fig. 2).

3.3. Induction of Mx expression by poly I:C transfection

Poly I:C-transfected cBB cells were incubated either at 28 or 20 °C, and the time course expression of Mx was detected by real-time PCR. When poly I:C-transfected cBB cells were incubated at both 28 and 20 °C, Mx gene expression peaked at 24 h post-transfection, and decreased thereafter. Moreover, the overall Mx gene expression at 28 °C was higher than that at 20 °C. At the point of their highest expression (24 h post-transfection), the level of Mx gene expression at 28 °C was 4-fold higher than that at 20 °C (Fig. 3).

Barramundi	1	---MN-TLNQ	QYEEKVRPCI	DLIDSLRSLG	VEKDLALP	AI	AVIGDQSSGK	SSVLEAL	SGV	AL	PRGSGIVT	R	PLELKMKR	KKEGEWEYVK	ISYQ	---	D	HEEIEDPAH	VEKKIREAQD
AS_Mx1	1	---	N.....	H.....										H.....	---	---	---	SD	---
AS_Mx2	1	---	N.....	H.....										H.....	---	---	---	SD	---
AS_Mx3	1	---	N.....	H.....										H.....	---	---	---	SD	---
RBT_Mx1	1	---	N.....	H.....										H.....	---	---	---	SD	---
RBT_Mx2	1	---	Y.....	H.....	S.....									H.....	---	---	R.....	SD	N...K...
RBT_Mx3	1	---	N.....	H.....										R.....	H.....	---	---	SD	---
Jp_Flounder	1	---	---	S..E							K.....	A.NV.....	R.....	Y.....	D.....	D.....			---
At_Halibut	1	---	---	S..E							S...K.....	RS.DD.....	R.....	Y.....	H.....	D.....	M.....		---
Fugu	1	---	---	S...								R.V...P...N	L.....	E...K...V.....	D.....	K.....			---
Grouper	1	---	---	---								---	R.....	G...C...K...L.....	SD	N.....			---
Sea_Bream	1	---	---	---	R.....							---	---	---	LD.....	E.....			---
Catfish	1	---	SAS	SE			A.....					---	---	---	SR.EDF.H...K.KKDHD	E.....	Y.....	QN...D.....	---
Zebrafish	1	MEKLSY	FS		I.....	T...N.....						P.....	I...T.DQDR.H.R	K.....	T...C...D.FD.....	E.....	Q.....		---
Barramundi	112	EMAGVGVGIS	DLISLEIAS	PDVPLTL	D	LP	GL	ARVAVK	QOPENIGEIQ	KRLIQKFIKR	QETISLVVPP	CNDVIATTEA	LMAQGEVDPD	GERTLIGL	IK	PK	LV	VDKGTEE	TVVDIVHNEV
AS_Mx1	113			G.....															
AS_Mx2	113			G.....															
AS_Mx3	113			G.....															
RBT_Mx1	113			G.....															
RBT_Mx2	113			G.....															
RBT_Mx3	113			G.....															
Jp_Flounder	112	K.....				T.....	S.....												
At_Halibut	112	K.....	A.....			T.....	L.....												
Fugu	112			E.....		T.....	L.....												
Grouper	112					D...K...	G...R...T...												
Sea_Bream	112		M.....																
Catfish	118	H.....		E.....	VT	A.....													
Zebrafish	116		A.....	EE.....	Q.T	A.....													
Barramundi	232	IHLKGMVIM	KRCQGKEITE	KVSLTEAMER	EKAFFQDHVH	FHTLYNDGHA	TVPKLAEKIT	LELVHIIERS	LPRLEEQIEE	KLAQTQAE	RYNGPPTDA	AERLYVLIDK	VTAFTQDAIN						
AS_Mx1	233	...	T.....	M.....	R...S...T...		KE.A	LS...DE...	I.....										
AS_Mx2	233	...	T.....	M.....	R...S...T...		KE.A	LS...DE...	I.....										
AS_Mx3	233	...	T.....	M.....	R...S...T...		KE.A	LS...DE...	I.....	I...K...									
RBT_Mx1	233	...	T.....	M.....	R...S...T...		KE.A	LS...DE...	I.....										
RBT_Mx2	233	...	Q.T.....	M.....	R...S...T...		KE.A	LS...DE...	I.....	Q...K...	M...K...	EE.RTAL	KC.T...	E.P	K...Y...	...	L.....		
RBT_Mx3	233	...	T.....	M.....	R...S...T...		KE.A	LS...DE...	I.....										
Jp_Flounder	232	...	R...Q.....	L.....	A.....	A.....				I.....									
At_Halibut	232	...	P.T.....	R...Q.....	E.....	L...T...TE.A	Y...E.L			I...K...									
Fugu	232	...	R.....	ID.....	A...T.T	T...R.A	Q...D.Q	IL.....		N...K...									
Grouper	232	...	R.....	---	L...S	---	R.....	E.Q											
Sea_Bream	232	...	---	A.....	---	---	K.....	---		---	---	---	---	---	---	---	---	---	---
Catfish	238	Y.T.....	R.....	MD	R...H...T.K	D...K.P	S...EE.M	I.N.....		---	---	---	---	---	---	---	---	---	---
Zebrafish	236	...	T.....	R.....	MD	Q.T.N.T.T	S...K.P	SK.EE.F	I.....	I...K...	---	---	---	---	---	---	---	---	---
Barramundi	352	LAAGEELQCG	DKLVFSTLR	REFGKWYSQL	DHSGQFNRR	IDREVEEYEE	RYRGRELPGF	INNYTFEVMV	KEQIKQLEEP	AVKKLKDIDG	AVRKAQVQLA	HSSGIFPNPL	MKTAKTKIEA						
AS_Mx1	353	ST...KS	VR.....	K.....	KLH	E...E...Q	EG.AD.K	T.....											
AS_Mx2	353	ST...KS	VR.....	K.....	KLH	E...E...Q	EG.AD.K	TS.....											
AS_Mx3	353	ST...KN	VR.....	K.....	KLH	E...E...Q	EG.AD.K	T.....											
RBT_Mx1	353	ST...MKS	VR.....	K.....	KLH	ER.EI.Q	EG.DD.K	T.....											
RBT_Mx2	353	ST...KS	---	T.....	KATV	R...K...KK	EK.AD.K	T.....		I...D...									
RBT_Mx3	353	ST...KS	VR.....	K.....	KLH	R...E...Q	EG.SN.K	T.....											
Jp_Flounder	352	...	R.....	M.....	K...NTH	RT.H...MK	KK.D...			N...M.AG	S.....								
At_Halibut	352	...	R.....	H.....	M...S.R.SNC	KT.H...IK	EKK.D...			S...M...									
Fugu	352	TT...DIC	ENI.L		QK.E	IRHL	I...VK.E	LE...V.Q	K.....										
Grouper	352	TT...DK	---	A.....	AR.NAH	L...EK.K	EK.N...K	---	---	---	---	---	---	---	---	---	---	---	---
Sea_Bream	352	...	RS...	---	KL.M	QV.AA.KESI	QY.VK	WN	VE.QA...R	K.....									
Catfish	358	TT...KNI	QH...I.S	---	Q.AL	KMH	D...ET.KS	EK.N...K	---	---	---	---	---	---	---	---	---	---	---
Zebrafish	356	...	TVK	T	TD.LL	PE...	Q.A...SHI	R.DS.KK	EK.DN.V	K.....									
Barramundi	472	IKQEKESTAE	SLTRTKQME	MIVYSQDRTY	SSSLDRKRE	EDEDE	---	---	LKCP	KFNTRKSIHV	CMNHNALQE	LMLHLKSYYS	IASQRLADQI	PMVIRYQMLQ	ESAVQLQREM				
AS_Mx1	473	...	VN.....	M.....		T...S...H...E...	ED	---	---	RPL	PTIKI	TIF	ST.....	M.....	R...S.....	LV.....	F.S.....		
AS_Mx2	473	...	VN.....	M.....		T...S...H...E...	ED	---	---	RPL	PTIKI	TIF	ST.....	M.....	R...S.....	LV.....	F.S.....		
AS_Mx3	473	...	VN.....	M.....		L...T...S...H...E...	E	---	---	EDK	P.SEL	TIF	T.....	M.....		LV.....	F.S.....		
RBT_Mx1	473	...	VN.....	M.....		L...T...S...H...E...	---	---	---	QPL	T-EL	TIF	ST.....	M.....	W...S.....	LV.....	F.S.....		
RBT_Mx2	472	...	VN.....	M.....		L...T...S...KK...	E.L	EGELV	KNLGSW	GL	PVSV	T.N	GL.T...	R...	M.....	H.....	LV.....	F.S.....	
RBT_Mx3	473	...	VN.....	M.....		T...S...H...E...	ED	---	---	RPL	PTPKI	TIF	ST.....	M.....	R...S.....	LV.....	F.S.....		
Jp_Flounder	472	...	I.....	M.....	---	---	E.N.E	---	---	---	---	---	---	---	---	---	---	---	---
At_Halibut	472	...	PI.....	M.....	TM.....	N...E...K	RE.S.S	---	---	---	---	---	---	---	---	---	---	---	---
Fugu	472	...	RM.KD	KL.....	LL.T.....	DES.K	Q.KCVQ	---	---	TKI	VRPPAPRFPF	KQ.....	T.....						
Grouper	472	...	---	M.....	L.T.....	N.....	E.DT	---	---	R.G.V	AKQ...	Y.R							
Sea_Bream	469	RK...I...	AL.....	LL.....	---	S.K	E	---	---	---	---	---	---	---	---	---	---	---	---
Catfish	478	...	V...E...	TM.....	L.I.T	SM	DT...TL	VK	E.G	RQKVG	---	ILPN	SYSISC	LYN	HSN	R...E			
Zebrafish	476	...	LN...L...	M.K		L.....	G...	Q.KHA	DK	LE.M	KERPQ	---	PKI	LPLLS	FDL	GT.....	R	MR.....	ML...A
Barramundi	581	LQMLQKENL	EFLKEDFDI	GHKRAALQSR	LNRLMKARAY	L	---	---	VEF	---	---	---	---	---	---	---	---	---	---
AS_Mx1	580	...	T.E.D.I	Q.....	S.....	NK	K.....	S.....	---	---	---	---	---	---	---	---	---	---	---
AS_Mx2	580	...	T.E.D.I	Q.....	S.....	NK	K.....	S.....	---	---	---	---	---	---	---	---	---	---	---
AS_Mx3	580	...	T.E.D.I	Q.....	S.....	S...K	K.....	S.....	---	---	---	---	---	---	---	---	---	---	---
RBT_Mx1	587	...	T.E.D.I	Q.....	S.....	S...K	K.....	S.....	---	---	---	---	---	---	---	---	---	---	---
RBT_Mx2	592	...	T.E.D.I	Q.....	S.....	S...K	K.....	HN	---	---	---	---	---	---	---	---	---	---	---
RBT_Mx3	580	...	T.E.D.I	Q.....	S.....	NK	K.....	S.....	---	---	---	---	---	---	---	---	---	---	---
Jp_Flounder	577	...	---	EL...	C.....	HK.....	T.....	---	---	---	---	---	---	---	---	---	---	---	---
At_Halibut	579	...	---	I...	F.....	R...S.....	HK.....	T.....	---	---	---	---	---	---	---	---	---	---	---
Fugu	583	...	L...R.T	LP...	L.V	T.....	---	---	---	---	---	---	---	---	---	---	---	---	---
Grouper	583	...	---	M	D...C...	S...G...	---	---	---	---	---	---	---	---	---	---	---	---	---
Sea_Bream	575	...	V...S	---	S.....	N.....	K.TQ	T...	A.L	---	---	---	---	---	---	---	---	---	---
Catfish	592	LM...NAI	DH...H	N.NV	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Zebrafish	589	...	L...DGV	DN...C	Q.EN	L	QT	IEGTQP	GHLLE	T.I	DYCNILMQ	---	---	---	---	---	---	---	---

Fig. 1. Amino acid alignment of the barramundi Mx with other fish Mx sequences using Clustal W alignment program. Sequence identity is represented by dots and gaps are indicated as dashes. The empty boxes indicate the GTP-binding motif and the gray box indicates the dynamin family signature. The putative leucine zipper is depicted in the region by underlining leucine and isoleucine residues.

Table 1

Comparison of sequence identity between barramundi Mx protein (Genebank accession number [AY821518](#)) and the Mx protein of other species

Species	NCBI accession no.	Protein region compared	% identity
Atlantic Salmon Mx1	U66475	1–624	83
Atlantic Salmon Mx2	U66476	1–624	83
Atlantic Salmon Mx3	U66477	1–624	84
Rainbow Trout Mx1	U30253	1–624	83
Rainbow Trout Mx2	U47945	1–624	80
Rainbow Trout Mx3	U47946	1–624	83
Japanese flounder	AB110446	1–624	85
Atlantic halibut	AF245513	1–624	81
Fugu	AF525215	1–624	78
Grouper	AY574372	1–624	87
Sea bream	AF491302	1–623	81
Catfish	AY095349	1–624	73
Zebrafish	AF533769	6–621	70
Human MxA	M30817	2–624	56
Human MxB	M30818	2–624	52
Mouse Mx1	M21117	8–624	51
Rat Mx1	X52711	4–624	51

3.4. Induction of Mx expression by virus infection

The cBB cells were infected with one of the following viruses: fish nodavirus (B00GD), birnavirus (IPNV-SP), or iridovirus (TGIV). After viral infection, time course expression of Mx in cBB cells was detected by real-time PCR. In nodavirus infection, Mx expression was elevated at 24 h post-infection (hpi) and remained at a high level until 72 hpi. In birnavirus and iridovirus infections, Mx expression could only be detected for 6 to 24 hpi when CPE had not appeared, and the levels of Mx expression were much lower than that induced by nodavirus infection. The level of Mx gene expression in nodavirus infection was 20- and 50-fold higher than those in birnavirus and iridovirus 24 hpi respectively (Fig. 4).

Fig. 2. Mx protein phylogenetic tree. The amino acid alignment of Fig. 1 was used to construct a neighbor-joining tree of Mx protein. Bootstrap support values are given under the branches.

Fig. 3. Detection of Mx expression in cBB cells transfected with poly I:C at different incubation temperature by real-time PCR. The cBB cells were transfected with poly I:C ($1 \mu\text{g ml}^{-1}$) and incubated at (A) 28°C , and (B) 20°C . Column bars with different letters are significantly different at $P < 0.05$ ($N = 3$).

3.5. Antiviral activity of poly I:C-transfected cBB cells

The IFN response including Mx expression was first induced in cBB cells by poly I:C transfection, and then the antiviral activity against three kinds of fish viruses was measured (Fig. 5). The optimal MOI for inducing complete CPE in cBB cells was different for each virus, 100 for nodavirus, 0.1 for birnavirus and 10 for iridovirus. Without poly I:C transfection, the survival rate of cBB cells infected by fish nodavirus, birnavirus, and iridovirus infections were 2%, 0.4%, and 18% respectively. With poly I:C transfection, the survival rate of cBB cells infected by nodavirus and birnavirus infections significantly rose to 98.4% and 114.8% respectively, but there was no significant difference ($P > 0.05$) in the iridovirus-infected cBB cells which showed only a slight increase in the survival rate of cells (29.9%). Therefore, poly I:C-transfected cBB cells showed antiviral activity against nodavirus and birnavirus, but not against iridovirus.

3.6. The influence of Mx gene expression on the replication of fish virus

The cBB cells were first transfected with siRNA to inhibit Mx gene expression, and then transfected with poly I:C to induce IFN response and the expression of antiviral genes except Mx gene in cBB cells. The result revealed that Mx-specific siRNA was able to inhibit about 75% of Mx gene expression, compared with non-silencing siRNA (Fig. 6A). When non-silencing siRNA-transfected cBB cells were subsequently infected with three kinds of fish viruses, the viral replication level of nodavirus and birnavirus was decreased to 21% and 12.5% respectively. However, Mx-specific siRNA transfection of cBB cells caused a significant increase in the replication levels of nodavirus (68%) and birnavirus (32%), which were approximately 3-fold higher than those in non-silencing siRNA-transfected cBB cells. On the other hand, the replication levels of iridovirus in cBB cells showed no significant differences ($P > 0.05$) between Mx-specific and non-silencing siRNA-transfected cBB cells (Fig. 6B,C,D).

Fig. 4. Detection of Mx expression by real-time PCR in (A) Nodavirus-infected cBB cells, (B) Birnavirus-infected cBB cells, and (C) Iridovirus-infected cBB cells. Column bars with different letters are significantly different at $P < 0.05$ ($N = 3$).

Fig. 5. Antiviral activity in poly I:C-transfected cBB cells against 3 kinds of fish viruses. The cBB cells were transfected with poly I:C (black bar) or not (white bar). After 24 h treatment, cells were washed with PBS and then infected with one of the following: nodavirus, birnavirus, or iridovirus. The OD₅₉₅ values of all wells were measured by crystal violet staining when complete CPE appeared in the cells without poly I:C transfection. Results were presented as the percentage of surviving cells, calculated as the OD₅₉₅ of infected cells ($n = 4$) divided by the OD₅₉₅ of cells with neither treatment nor viral infection. Bars represent standard deviations. ** $P < 0.01$.

Fig. 6. The influence of Mx gene expression on viral replication. (A) Mx gene expression in cBB cells was inhibited by specific siRNA. The cBB cells were pre-transfected with Mx-specific siRNA (Mx-siRNA) to inhibit Mx gene expression; non-silencing siRNA (N-siRNA) was used as negative control. After 4 h incubation, cells were transfected with poly I:C, and the level of Mx gene expression was detected by real-time PCR 18 h later. Thereafter, the treated cells were infected with (B) Nodavirus, (C) Birnavirus, (D) Iridovirus. The cells were harvested 24 h post-infection, and the progeny virus was titrated. Viral titer was presented as the percentage of viral replication level (viral titer from any treated cells/viral titer from non-poly I:C-transfected cells). The mean viral titers given are the average of three independent experiments. Error bars indicate standard deviations. Repeated experiments which have the same results will lack the standard deviations. Column bars with different letters are significantly different at $P < 0.05$ ($N = 3$).

4. Discussion

In this study, a cDNA clone of barramundi Mx gene was constructed from the extracted mRNA of cBB cells after NNV infection. The motif characteristics of Mx proteins, including the GTP-binding site, the dynamin family signature, and the leucine zipper are all present in barramundi Mx. GTP-binding motif is important for antiviral activity because GTP binding induces a conformational change of the Mx protein that allows specific recognition of viral targets [34]. Mutations in the GTP-binding domain resulted in loss of antiviral activity [35]. The dynamin family signature is found in all members of the dynamin family that is essential for receptor-mediated endocytosis and synaptic vesicle recycling [36]. The leucine zipper is a region for protein-protein interaction and is thought to play a role in the oligomerisation for antiviral activity [37].

Poly I:C, an artificial double-stranded RNA, can induce Mx expression *in vivo* and *in vitro*. Many kinds of fish species had been injected with poly I:C for induction of Mx gene expression [16–18,21–23,38]. *In vitro*, Mx gene expression could also be induced by poly I:C in some fish cell lines, such as RTG-2 cells [16], CHSE-214 [39], SAF-1 [21], and CCO cells [22]. In this study, cBB cells expressed Mx gene by poly I:C transfection either at 28 °C or 20 °C, but the expression level of Mx at 20 °C was lower and delayed compared to that at 28 °C, which was an optimal culture temperature for barramundi. It might be that lower temperature resulted in slower growth and gene expression. Although Mx gene expression abated at low temperature, it could still be turned on by poly I:C.

Mx induction by fish viruses has been reported in many cases, including channel catfish virus (CCV) in catfish [22], nodavirus and iridovirus in grouper [23], infectious hematopoietic necrosis virus (IHNV) in rainbow trout [16], IPNV in Atlantic halibut [18], hirame rhabdovirus (HIRRV) or aquabirnavirus in Japanese flounder [19,40] *in vivo*, and

nodavirus in SAF-1 cells [21] *in vitro*. In the present study, we found that cBB cells were sensitive to nodavirus, birnavirus and iridovirus infection. High MOI (10) was used for synchronous virus infection in the time-course experiment. The Mx expression in response to fish nodavirus in cBB cells was elevated to high levels from 24 to 72 hpi (the last sampling time point). However, the level of Mx gene expression in response to fish birnavirus and iridovirus in cBB cells was much lower than that in nodavirus-infected cBB cells, and could only be detected from 6 to 24 hpi. It was also observed that complete CPE appeared in birnavirus- and iridovirus-infected cBB cells within 2 days, but in nodavirus-infected cBB cells, it was delayed until 14 days post-infection. Although the beginning of nodavirus replication is earlier than the expression of Mx gene in nodavirus-infected cBB cells, the increased expression of Mx gene after 24 hpi was enough to interfere with NNV replication and delay CPE.

Although low MOI (0.01) was applied for birnavirus infection of cBB cells, half of the cells exhibited CPE within 48 hpi, and the level of Mx expression were still as low as that with high MOI (10) (data not shown). Two hypotheses were suggested to explain the rapid appearance of CPE in birnavirus- or iridovirus-infected cBB cells. One is that birnavirus or iridovirus infection might suppress cellular gene expression, and the second possibility is the lack of specific Toll-like receptors (TLR) for recognizing birna- or iridovirus nucleic acids. It had been reported that IPNV could suppress type I interferon signaling [41], and FV3, frog iridovirus, could down-regulate cellular macromolecular synthesis [42]. The low level expression of Mx gene in birnavirus- and iridovirus-infected cBB cells might be due to the inhibition of cellular gene expression, including Mx expression or IFN-related gene expression, or macromolecular synthesis during viral infection. Therefore, in the absence of cellular defense, these viruses can proliferate more successfully, and cause CPE within 48 h in cBB cells. Nodavirus is single-stranded sense RNA virus, birnavirus is double-stranded RNA virus, and iridovirus is double-stranded DNA virus. It has been reported that viral dsRNA and poly I:C can be recognized by TLR3; while, viral ssRNA is recognized by TLR7 and TLR8, and viral or bacterial unmethylated CpG DNA motifs are recognized by TLR9 [43]. Therefore, the low level expression of Mx gene in birnavirus- and iridovirus-infected cBB cells might be due to the lack of TLR3 and TLR9. In our previous study, we observed that Mx gene expression in cBB cells is only induced by poly I:C-transfection but not by poly I:C-incubation, suggesting that cBB cells might lack TLR3 [30]. Additionally, some viral glycoproteins are reported to be able to elicit an IFN response [44–48]. Thus, the glycosylated capsid protein of nodavirus might also be potent in inducing an IFN response in cBB cells.

Antiviral activity had been shown in human MxA [49], rat Mx1, Mx2 [50], mouse Mx1 [13,51], Mx2 [14,52], porcine Mx1 [53], chicken Mx [54], Japanese flounder Mx [24], and Atlantic salmon Mx1 [25], but not in human MxB, duck Mx and rat Mx3 [55]. In addition, the antiviral activity of Mx protein has been shown to be virus specific. For example, mouse Mx2 could inhibit vesicular stomatitis virus (VSV), but not influenza virus [14]. Moreover, we found that poly I:C could induce high level Mx expression in cBB cells 24 h post-transfection, and the transfected cBB cells showed very high antiviral activity against fish nodavirus and birnavirus, but not against fish iridovirus. Therefore, it is suggested that antiviral activity of barramundi Mx is virus-specific, and barramundi Mx has a role in protecting the cells from nodavirus- and birnavirus-induced cell lysis.

Here, we have further demonstrated that Mx-specific siRNAs could down-regulate Mx expression in the treated cBB cells. However, the levels of nodavirus and birnavirus replication in the treated cBB cells were only restored to 68% and 32% respectively. One possibility is that other IFN-induced antiviral proteins were also involved in the inhibition of nodavirus and birnavirus replication. Another possibility is that a low level of Mx gene was still expressed, and inhibited the nodavirus and birnavirus replication. In the present study, six clones of barramundi Mx cDNA had been sequenced and compared, but all the sequences were the same (data not shown). Whether there are more than one form of barramundi Mx genes needs further study. In the future, we will find an NNV-permissive cell line without Mx gene, and transfect it with barramundi Mx cDNA to determine whether a certain form of barramundi Mx is responsible for the anti-nodavirus or anti-birnavirus activities.

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