Fish & Shellfish Immunology 35 (2013) 1048–1054

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

Characterization of four Mx isoforms in the European eel, *Anguilla anguilla*

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ARTICLE INFO

Article history:
Received 23 January 2013
Received in revised form
23 June 2013
Accepted 9 July 2013
Available online 17 July 2013

Keywords:
Mx
Isoform
European eel
Anguilla anguilla
Real-time PCR

ABSTRACT

Mx protein is known to play an important role in vertebrate immune response to viral infection. In this study, cDNA sequences of four Mx isoforms, designated as MxA, B, C and D were characterized in the European eel, *Anguilla anguilla*. These sequences contained an open reading frame of 1899, 1896, 1866, 1779 bp, flanked by 95, 53, 138, 69 bp of 5′ untranslated region and 389, 241, 136, 124 bp of 3′ untranslated region, respectively. A phylogenetic tree constructed with Mx peptide sequences from vertebrates revealed that MxA, C and D in the European eel formed into a clade containing zebrafish MxA and MxB and Mx proteins in other teleosts, whereas MxB in the eel was clustered together with zebrafish MxD, MxG and MxF. The transcription level of all Mx isoforms increased in a poly I:C dose-dependent manner in peripheral blood leukocytes of eels, as revealed by real-time PCR. A further experiment was conducted to reveal the temporal change in expression of these isoforms in various organs/tissues following poly I:C stimulation, and significant increase in expression was observed at various degrees in different organs or in different sampling occasions within the 12 h experimental period. In particular, MxA had the highest level of increase, while MxB had the lowest; and three isoforms, MxA, MxB and MxD had the highest increase in intestine, while the highest increase of MxC expression was observed in liver. These four isoforms of eel Mx are thus expressed differentially, and further work is certainly required to clarify the activity of promoter elements and antiviral activity of these Mx isoforms.

1. Introduction

Myxovirus (Mx) resistance proteins along with guanylate-binding proteins (GBPs), immune-related GTPase proteins (IRGs), and large inducible GTPase proteins (VLIGs) belong to the superfamily of interferon-inducible GTPases with characteristics of high molecular weight, a propensity to self-assembly, a relative low affinity for GTP, and a high intrinsic rate of GTP hydrolysis [1]. This GTPase superfamily members could all be induced by IFNs and orchestrate host defense in response to viral or microbial infection [2–4]. Mx proteins are characterized with a conserved tripartite GTP-binding domain, a middle domain, and a C-terminal GTPase effector domain (GED) which contains an Mx specific leucine zipper required for protein interaction [5,6]. Mx proteins have a molecular weight of about 70–80 kDa and exhibit only modest overall protein sequence similarity (30–40%) to non-Mx members of the dynamic IFN-inducible GTPase superfamily [1].

Mx proteins were originally identified in an inbred mouse strain with extraordinary high level of resistance towards infection with orthomyxovirus influenza A [7]. Subsequently, Mx genes have been found in other vertebrates, including mammals and birds with direct antiviral activity against a broad range of viruses [8–12]. Li et al. [13] conducted a systematic survey of Mx genes in bilaterians using bioinformatic approaches and found that Mx genes were originated before the radiation of chordate subphyla and might have evolved in a low rate in respect to gene copy number. On the other hand, the antiviral activities of Mx are highly conserved in vertebrates [13]. In human, two genes, coding for the separate Mx isoforms, termed MxA and MxB, are adjacent to each other on chromosome 21. But only MxA has detectable antiviral activity against several RNA viruses including influenza virus and vesicular stomatitis virus [14–19]. A sole Mx was identified in chicken with high polymorphism. A non-synonymous mutation (G to A) at position 2032 in nucleotide sequence which causes a specific amino acid substitution (Ser to Asn) has been demonstrated to be related to resistance to antiviral activity [20]. However, subsequent studies have produced conflicting findings that neither the Asn631 nor Ser631 Mx alleles showed any inhibition on the expression of

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http://dx.doi.org/10.1016/j.fsi.2013.07.012

virus-directed genes when cells were infected with Newcastle disease virus [21]. In teleost fish, Mx genes have been identified in several species of fish, such as in perch (*Perca fluviatilis*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), Atlantic halibut (*Hippoglossus hippoglossus*), Japanese flounder *(Paralichthys olivaceus*), fugu (*Takifugu rubripes*), channel catfish (*Ictalurus punctatus*), rock bream (*Oplegnathus fasciatus*) and zebrafish (*Danio rerio*) [13,22–31]. It is well established that different forms of Mx may exist in some species of fish. Three different isoforms of Mx genes have been found in rainbow trout, Atlantic salmon and rock bream, while only one reported in fugu and perch [13,22,28]. In zebrafish, seven functional Mx genes have been identified with the presence of a pseudogene, the number of which is far more than that in other vertebrates including other species of fish, frog and amniotes [13,27]. In this research, four Mx isoforms were cloned and characterized in the European eel, *Anguilla anguilla*, an important commercial fish species for aquaculture and fisheries. The phylogenetic relationship of these isoforms was analyzed with those in other species of fish and in other classes of vertebrates. The expression of these isoforms was examined in response to the stimulation of poly I:C, and to the infection of a pathogenic bacterium, *Edwardsiella tarda* of eels.

2. Materials and methods

2.1. Fish and stimulation with poly I:C and pathogenic bacterium Edwardsiella tarda

European eels, weighing about 100 g each, were maintained in aquarium with aerated water for 2 weeks before being used in the experiment. To examine the temporal changes of transcripts in different organs/tissues, one group of nine eels were each injected intraperitoneally with 0.2 mg poly I:C (Sigma) dissolved in phosphate buffer saline (PBS), and another group of nine fish were injected with PBS as control. At 3, 6, and 12 h post injection (hpi), fish were anaesthetized in 0.05% 2-phenoxyethanol, and thymus, pronephros, kidney, spleen, liver, intestine, gill, skin and muscle were separately dissected out for total RNA isolation. These observation time points were chosen since a previous study showed that Mx transcripts reached a peak at 12 hpi in poly I:C treated fish [32].

Edwardsiella tarda was cultured at 25 °C with tryptic soy broth (TSB, BD Bioscienes) prepared with distilled water. The bacterial cells were harvested by centrifugation at 3500 × *g* for 10 min and suspended in PBS for obtaining an appropriate concentration. Two groups of three fish were each injected intramuscularly with 200 μl PBS or live microbial PBS suspension (1×10^6 cfu/ml), respectively. 24 hpi, fish were then sacrificed and tissue/organs as described above were collected for real time PCR analysis.

2.2. Isolation of peripheral blood leukocytes (PBLs) and poly I:C stimulation in vitro

Leukocytes were isolated from peripheral blood of European eel by Percoll gradients (GE Healthcare, UK). Blood was diluted at 1:10 with L-15 medium (Invitrogen Life Technologies) and supplemented with 10 U/ml heparin, 10 mM HEPES, 60 mM NaCl, 5% FBS, 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco). The cell suspension was placed on 34/51% Percoll gradient and centrifuged at 400 × *g* for 40 min at 4 °C. The cells suspended from 34/51% Percoll interface were collected and washed three times with pre-cold L-15 medium. Leukocytes were resuspended in the pre-cold L-15 medium with cell numbers counted.

For stimulation experiment, 2 ml suspension of peripheral blood leucocytes (PBLs) was seeded in a 24-well plate at a density of 1×10^6 cells per well, and incubated with 100 μl poly I:C at a final concentration of 0.5, 5, 50 or 500 μg/ml, respectively. The cell cultures were incubated for 12 h at 28 °C, with each treatment prepared in triplicate. The harvested leukocytes were centrifuged at 400 × *g* for 5 min at 4 °C, and resolved in Trizol for RNA extraction.

2.3. Cloning and analysis of cDNA sequences

Total RNA from head kidney and spleen was extracted using Trizol (Invitrogen Corp) according to the manufacturer's instruction and reverse transcribed into cDNA by Powerscript II reverse transcriptase with gene specific primer of eel Mx (using SMART rapid amplification of cDNA end (RACE) cDNA Amplification kit, Clontech). The Mx sequences from other teleost (listed in Table 1) were used to search against the eel transcriptome database (http://compgen.bio.unipd.it/eeelbase/) with the tblastn tool. After obtaining contigs, primers were designed according to respective contig sequences, with a local version of the Primer Premier 5.0 design software tool (http://www.premierbiosoft.com/primerdesign/index.html) and PCR was performed to amplify partial cDNA sequences of eel Mx genes. PCR products were cloned into pMD18-T (Takara) and sequenced. To recover the full-length cDNA sequences, primer sets specific for 5′ untranslated region and 3′ untranslated region of each Mx cDNA sequences were designed using the primer design software tool as described above. RACE-PCR was performed by using the gene specific primers and adaptor primers. All PCR products were purified using an EZNAO Gel Extraction Kit (Omega, Bio-Tek) and ligated into pMD18-T by T/A cloning following the manufacturer's instructions. Several clones were sequenced using the dideoxy chain-termination method on ABI 3730 automatic sequencer (Applied Biosystems, Foster City).

Blast analyses were performed at the National Center for Biotechnology information website (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein domain features were identified by PROSITE (http://us.expasy.org/tools/scanprosite). Sequence alignments were calculated by using the CLUSTALW 1.8 program with manual adjustments. Homology between sequences was calculated using the Megalign program with the DNASTAR package. The phylogenetic tree based on the multiple protein alignment was

Table 1
Mx protein sequences used for searching against eel transcriptome database and phylogenetic tree construction.

Gene	Species	Abbreviation	Accession
MxA	*Homo sapiens*	*H. sapiens*	AAA36337.1
MxB	*Homo sapiens*		AAA36338.1
Mx1	*Mus musculus*	*M. musculus*	AAA39777.1
Mx2	*Mus musculus*		ENSMUSG00000023341[a]
Mx1	*Anolis carolinensis*	*A. carolinensis*	ENSACAG00000005450[a]
Mx2	*Anolis carolinensis*		ENSACAG00000023592[a]
Mx	*Gallus gallus*	*G. gallus*	ENSGALG00000016142[a]
Mx1	*Xenopus laevis*	*X. laevis*	NP_001243698.1
MxA	*Danio rerio*	*D. rerio*	CAD67755.1
MxB	*Danio rerio*		CAD67756.2
MxC	*Danio rerio*		CAD67757.1
MxD	*Danio rerio*		CAD67758.1
MxE	*Danio rerio*		CAD67759.1
MxF	*Danio rerio*		CAD67760.1
MxG	*Danio rerio*		CAD67761.2
Mx	*Hippoglossus hippoglossus*	*H. hippoglossus*	AAF66055.1
Mx1	*Oncorhynchus mykiss*	*O. mykiss*	AAA87839.1
RBTMx2	*Oncorhynchus mykiss*		AAC60214.1
RBTMx3	*Oncorhynchus mykiss*		AAC60215.1
Mx1	*Salmo salar*	*S. salar*	AAB40994.1
Mx2	*Salmo salar*		AAB40995.1
Mx3	*Salmo salar*		AAB40996.1
Mx	*Takifugu rubripes*	*T. rubripes*	AAO37934.1

[a] Sequences were retrieved from Ensembl database.