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Research Article

Interferon induced Mx protein from Indian snow trout *Schizothorax richardsonii* (Gray) lacks critical functional features unlike its mammalian homologues

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ABSTRACT

Viral attack within host cells triggers the production of type I interferons and leads to the induction of interferon stimulated genes (ISGs). One of the ISG Mx, encodes type I interferon inducible GTPase that is responsible for the establishment of an anti-viral state within cells. Intriguingly, several isoforms of Mx have been reported in fish, but the structural analysis of fish Mx proteins remains unexplored. For the first time, we have identified and unraveled the molecular structure of Mx protein from Indian snow trout, *Schizothorax richardsonii* (Gray) a Coldwater fish that inhabits the water bodies in the sub-Himalayan region. The snow trout Mx coding region consists of 2518 nucleotides with an open reading frame (ORF) of 1854 nucleotides. It codes for a polypeptide of 617 amino acids with a predicted molecular weight of 70 kDa. *In silico* analysis of snow trout Mx protein revealed signature of dynamin family (LPRGTGIVTR) along with a tripartite GTP-binding domain (GDQSSGKS, DLPG, and TKPD). Homology modelling established that the Mx protein is an elongated structure with a G domain, bundle signaling element (BSE) and a GTPase effector domain (GED). Moreover, the GED of Mx contains two highly conserved leucine zippers at the COOH-terminal of the protein suggesting its structural similarity with human homologues. However, snow trout Mx lacks the essential features of its mammalian homologues questioning its functional characteristics. Further, a ligand binding site in the said protein has also been predicted adjacent to the GTPase switch within the G domain.

1. Introduction

Type I interferons mediate signaling *via* JAK/STAT pathway and lead to the induction of variety of proteins due to stimulation of interferon stimulated genes (ISGs) (Schneider et al., 2014). Among ISGs, Mx is a well characterized type I interferon induced protein responsible for antiviral host defense (Haller et al., 2007). Mx proteins are usually present in all vertebrates and are known to appear in one to three isoforms (Verhelst et al., 2013). The ability of Mx in conferring selective resistance against influenza viruses has been well demonstrated besides blocking viral spread (Staeheli et al., 1986a,b; Arnheiter et al., 1990). It is established that Mx can induce antiviral state against a number of viruses across different animal species (Haller et al., 2007; Verhelst et al., 2013). Mx proteins possess distinguishing antiviral activities and can be localized either in the nucleus or cytoplasm. Nuclear forms for example, mouse Mx1, protects against influenza (Krug et al., 1985; Staeheli et al., 1986a,b; Grimm et al., 2007; Zimmermann et al., 2011) and Thogo virus (Haller et al., 1995) that are known to replicate in the nucleus while the cytoplasmic form, like mouse Mx2, are known to inhibit the replication of viruses in the cytoplasm like the vesicular stomatitis virus and hantavirus (Jin et al., 1999, 2001; Zurcher et al., 1992). The nuclear Mx possess a nuclear localization signal important to shuttle the Mx protein into the nucleus besides binding to the viral capsid protein (Zurcher et al., 1992; Schulte et al., 2015).

Mx proteins belong to the family of GTPase that include dynamins (Praefcke et al., 1999; Haller and Kochs, 2002). They possess a tripartite GTP binding domain, a middle domain and a C terminal GTPase effector domain (Praefcke et al., 1999; Haller and Kochs, 2002) with a characteristic leucine zipper (Verhelst et al., 2013; Haller and Kochs, 2011). Sequence variations have been observed among different Mx proteins with C-terminal GTP-

Abbreviations: ISGs, interferon stimulated genes; GED, GTPase effector domain; ORF, open reading frame; BSE, bundle signaling element; Poly (I:C), polyinosinic: polycytidylic acid; UTRs, untranslated regions.

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binding motif being more conserved which may account for functional differences between the Mx isoforms (Lee and Vidal, 2002).

Antiviral activity of Mx proteins has also been reported from fish against a number of fish viruses (Verhelst et al., 2013; Lee and Vidal, 2002; Caipang et al., 2003; Larsen et al., 2004; Kibenge et al., 2005; Wu and Chi, 2007). Usually one to three Mx isoforms have been reported in fish, except European eel, *Anguilla anguilla*, which has four (Staeheli et al., 1989; Trobridge et al., 1997; Huang et al., 2013; Nygaard et al., 2000; Robertsen et al., 1997; Plant and Thune, 2004; Chen et al., 2006). As there is a great variation in the Mx isoforms within different fish species, it was intriguing to unravel the characteristics of Mx gene from snow trout, *Schizothorax richardsonii* (Gray), an important food and game fish inhabiting one of the most difficult high altitudinal regions in the foothills of Indian Himalayas. This investigation is a first report on identification and molecular characterization of interferon induced Mx protein from any Indian Coldwater fish.

2. Material and methods

2.1. Fish collection and stimulation

Schizothorax richardsonii, Indian snow trout were collected at an attitude of 1184 m above sea level from *Kalsa* stream Chaafi. The coordinates of the site located in the Kumaon hills of Uttarakhand, India were 29.365 N, 79.586 E. Fish were acclimatized in laboratory aquariums for a week. Fish ranging in weight from 8 to 10 g were injected intraperitoneally with poly (I:C) at a dose of 200 μg per fish. Liver spleen and kidney were collected from the poly (I:C) treated fish 72 h post treatment. Prior to dissection, the fish were anesthetized by dipping them in 0.05 ml/l clove oil (Velisek et al., 2005).

2.2. cDNA synthesis and cloning

For RNA extraction the collected tissues were homogenized. Total RNA was extracted using Ribozol (Amresco) following the manufacturer's recommendations. In a reaction volume of 20 μl, 5 μg total isolated RNA was reverse transcribed along with 0.5 μg random hexamers, 20 pmoles dNTP mix, 20 U RNase inhibitor and 200 U of RevertAid Reverse Transcriptase (Thermo Scientific). The reaction was incubated at 25 °C for 10 min followed by 42 °C for an hour. Finally the reaction was stopped by heating the contents at 70 °C for 10 min.

After aligning the available Mx coding sequences of *Cyprinids*, two sets of primers were designed (Table 1) from the conserved region for amplification of two overlapping fragments approximately 900 base pair each. Amplifications were carried out in a reaction volume of 25 μl using 2.5 μl of standard *Taq* Buffer, 10pmoles of forward and reverse primers, 10 pmoles of dNTP mix, 2.5 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (NEB) and 2.5 μl of c-DNA template. PCR reaction were amplified by initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 2 min and a final extension of 10 min at 72 °C. Two PCR products of 900 base pair as expected, were gel purified and cloned using InsTAclone PCR cloning kit (Thermo scientific) to carry out nucleotide sequencing.

2.3. Rapid amplification of cDNA ends – polymerase chain reaction (RACE-PCR)

RACE was employed to carry out amplification of 5′ and 3′ ends of Mx coding region along with the UTRs. Gene specific primers for 5′ and 3′ RACE were designed (Table 1) using the partial nucleotide sequence of snow trout Mx coding region (Accession No KU529282). 5′ and 3′ RACE reactions were carried out with their respective gene specific primers using SMARTer® RACE 5′/3′ Kit (Clontech) following the manufacturer's recommendations. Amplification was carried out using cycling condition consisting of 30 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 1 min and extension at 72 °C for 3 min. The fragments obtained were cloned using InsTAclone PCR cloning kit (Thermo scientific) to obtain the nucleotide sequence.

2.4. In silico analysis of Mx protein

To analyze protein domains, bioinformatics analysis of Mx coding region was carried out. The amino acid sequence was deduced using protein translation tool-Expert Protein Analysis System (EXPASY) (Gasteiger et al., 2003). Functional analysis of the translated protein sequence into families, presence of domains and repeats was carried out using PROSITE (De Castro et al., 2006). Different characteristics of the predicted protein, physicochemical properties like molecular weight, theoretical pI (isoelectric point) and grand average of hydropathy (Gravy) were computed by protparam tool (Gasteiger et al., 2005). The prediction of protein localization at cellular level was carried out using WoLF PSORT II (Horton et al., 2007) for predicting nuclear or cytoplasmic activity. To compare the various fish and human Mx protein multiple sequence alignment was carried out using ClustalW (http://www.genome.jp/tools/clustalw/).

2.5. Phylogenetic analysis

Amino acid sequences of Mx protein from different fish species were retrieved from GenBank (Table 2). The deduced amino acid sequence of snow trout Mx was compared with the available retrieved amino acid sequences of Mx protein using multiple sequence alignment tool ClustalW (Larkin et al., 2007) in MEGA 7 (Kumar et al., 2016) to obtain a phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987).

Table 1
List of primers used for snow trout Mx amplification.

Primer ID	Primer sequence 5′–3′	Amplified sequence	Product length
Mx gene S2F	CACGCTGTCCTCTGGTATTG	Mx mid fragment	980 bp
Mx gene S2R	GAGGGTTTCCTCCGTCTTAATG	Mx mid fragment	
Mx gene S3F	GATACAACTGAAGAAGGGCTACA	Mx mid fragment	970 bp
Mx gene S3R	CAGCATGTGGTACTGGACTATC	Mx mid fragment	
3′RACE Frw	GATTACGCCAAGCTTGTGCAGATGTACGTGAGATGGCGCAGC	3′ RACE Mx	–
5′RACE Rev	GATTACGCCAAGCTTGCCTCGGCATTTAACAATCATGTAGCCCT	5′ RACE Mx	–
MxNcoIF	CATGCCATGGCTGAAAGTAGTAGTCTGAGCCAG	Full length Mx	1851 bp
MxXhoIR	CCGCTCGAGTGCAGAATGCACAAATTTGGACA	Full length Mx	