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Identification and characterization of two homologues of interferon-stimulated gene ISG15 in crucian carp

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Abstract

ISG15 is one of the most strongly induced genes upon viral infection, interferon (IFN) stimulation, and lipopolysaccharide (LPS) stimulation, and only one copy has been found in mammals so far. Here two fish ISG15 genes, termed *CaISG15-1* and *CaISG15-2*, have been cloned and sequenced from UV-inactivated GCHV (grass carp haemorrhagic virus)-infected and IFN-produced CAB cells (crucian carp *Carassius auratus* blastulae embryonic cells) by suppression subtractive hybridization. The full-length cDNA sequences of two crucian carp *ISG15* encode a 155-amino-acid protein and a 161-amino-acid protein, both of which show 78.9% identity overall and possess the characteristic structures of mammalian ISG15 proteins including two tandem ubiquitin-like domains and the C-terminal canonical LRLRGG motif. In CAB cells treated with different stimuli including active virus, UV-inactivated GCHV and IFN containing supernatant (ICS), the expression of both *CaISG15-1* and *CaISG15-2* was upregulated but displayed different kinetics. Poly I:C and LPS were also able to induce an increase in mRNA for both genes. In CAB cells responsive to active GCHV, UV-inactivated GCHV, CAB ICS, Poly I:C and LPS, *CaISG15-1* was upregulated more significantly than *CaISG15-2*. These results suggest that there are two ISG15 homologues in crucian carp, both of which might play distinct roles in innate immunity against viral and bacterial infection.
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Keywords: *Carassius auratus*; *ISG15*; Differential expression; Interferon; Grass carp; Haemorrhagic virus; Antiviral-relevant genes; Innate immune response

1. Introduction

Type I interferon (IFNα/β) plays a pivotal role in innate immunity against viral and bacterial infection mainly through the JAK-STAT signalling pathway that culminates in the enhanced expression of a temporally coordinated subset of cell type-specific genes, named IFN stimulated genes (ISGs) [1]. *ISG15* is one of the most strongly induced ISGs in many cell types by IFNs, virus infection, double-stranded RNA (Poly I:C), lipopolysaccharides (LPS) [2]. Biochemical analysis reveals a cross-reactivity of ISG15 protein with anti-ubiquitin antibody, hence ISG15 is also called ubiquitin cross-reactive protein (UCRP) [3]. However, virus induction seems unique to ISG15 since the expression level of ubiquitin was not changed under the same condition [3].

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ISG15 was originally identified as an IFN-induced protein [4], and was the first example of a small class of Ubiquitin-like proteins (UBLs) that include SUMO, FAT10, Nedd8 and Ubl1 [5]. Most of these proteins show significant sequence homologous to ubiquitin and contain single ubiquitin-like (UBL) domains, while ISG15 is composed of two in tandem, each of which bears approximately 30% homology to ubiquitin [5]. A recent solved crystal structural analysis revealed that both ubiquitin-like folds of human ISG15 are joined by a six-residue extended linker sequence [6]. Similar to ubiquitin, ISG15 contains the canonical LRLRGG motif at its C terminus [2]. This motif is required for covalent conjugation of ISG15 via an isopeptide bond to cellular targets (protein ISGylation) as a primary response to IFNα/β induction and other markers of viral or bacterial infection [7]. Unlike ubiquitination, ISGylation does not appear to target proteins for proteasome degradation [8] but activates or suppresses the function of target proteins [9,10].

Through identification of ISG15 target proteins from IFN treated cells [7,8,11,12], ISG15 and ISG15 conjugation were suggested to relate to diverse cellular pathways including RNA splicing, chromatin remodelling/polymerase II transcription, cytoskeleton organization and regulation, stress responses and translation [12]. Several recent reports have provided indirect and direct evidence for involvement of ISG15 and protein ISGylation in innate antiviral response. Loss of mouse UBP43 (USP18), a protease specifically removed ISG15 from conjugated proteins [13], resulted in decreased life span, brain cell injury [14], hypersensitivity to type I IFN stimulation [15], and more resistant to both LCMV and VSV infection [16]. More recently, the antiviral role of ISG15 has been revealed in IFNα/β -receptor deficient mice in which ISG15 expression protected against Sindbis virus-induced lethality and decreased sindbis virus replication in multiple organs [17]. ISG15 is also the critical component in IFN-mediated inhibition of HIV-1 release [18]. Consistent with these findings, influenza A and B viruses have evolved different strategies to abolish the function of ISG15 in virus replication [19].

In order to study the molecular mechanism of fish innate response to virus infection, an ideal cell model system has been developed. When UV-inactivated GCHV (grass carp haemorrhagic virus) was used to infect CAB cells (crucian carp *Carassius auratus* L. blastulae embryonic cells), an innate IFN response was significantly induced in the infected cells [20]. As expected, many cellular genes homologous to mammalian ISGs were retrieved from this cell system by suppressive subtractive hybridization, including *TLR3*, *RIG-I*, *mda5*, *IFN*, *STAT1*, *IRF7*, *Mx1*, *Mx2*, *IFI56*, *IFI58*, *ISG15*, *PKR-like*, *Gig1 and Gig2* [20,21]. In addition, *ISG15* homologues have also been discovered in goldfish [22], Atlantic salmon [23], and more recently in Atlantic cod [24], but only one copy of *ISG15* gene was found in these species. In this study, we further determined the full-length nucleotide and amino acid sequences of two crucian carp *ISG15* genes, and their differential kinetics in CAB cells under distinct stimuli, including virus infection, IFN containing supernatant (ICS), Poly I:C and LPS treatment. These results indicate that there are two *ISG15* homologues in crucian carp, both of which might play distinct roles in host innate antiviral response.

2. Materials and methods

2.1. Cell, virus and interferon

Crucian carp (*Carassius auratus*) blastulae embryonic cells (CAB), and grass carp (*Ctenopharyngodon idellus*) kidney cells (CIK) were grown in a medium 199 supplemented with 10% foetal calf serum (FCS, purchased from Gibco BRL and heated before using, 56 °C for 30 min), 100 U ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin sulfate. According to previous reports [25], GCHV propagation and UV inactivation were performed, and the induction, ultracentrifugation and antiviral titer of CAB IFN-containing supernatant (ICS) were made. The purified CAB ICS was applied to study the expression of *CaISG15* genes.

2.2. RACE PCR

A SMART cDNA for rapid amplification of cDNA ends (RACE) was prepared with mRNA made from UV-inactivated GCHV-infected CAB cells by Clontech SMART PCR Synthesis Kit [20]. Specific pairs of primers (Table 1) were designed from two expressed sequence tags, CA53 and CA249, both of which were retrieved by suppressive subtractive hybridization and were homologous to mammalian ubiquitin-like genes [20]. Briefly, the universal primer Smart-F and specific reverse primers (CA53-R1 or CA249-R) were used for 5′-RACE PCRs, and the universal primer Smart-R and specific forward primers (CA53-F1 or CA249-F) used for 3′-RACE PCRs. The

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