Fish & Shellfish Immunology 43 (2015) 449–459

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Proteomic identification and characterization of *Ctenopharyngodon idella* tumor necrosis factor receptor-associated protein 1 (*Ci*Trap1): An anti-apoptosis factor upregulated by grass carp reovirus infection

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ARTICLE INFO

Article history:
Received 10 October 2014
Received in revised form
20 January 2015
Accepted 26 January 2015
Available online 3 February 2015

Keywords:
Grass carp *Ctenopharyngodon idella*
Proteomic analysis
CiTrap1
Apoptosis
Grass carp reovirus

ABSTRACT

Human tumor necrosis factor receptor-associated protein 1 (Trap1) is a mitochondrial protein identical to heat shock protein 75 (HSP75) that plays an important role in protecting cells from oxidative stress and apoptosis. In this study, grass carp (*Ctenopharyngodon idella*) tumor necrosis factor receptor-associated protein 1 (designated as *Ci*Trap1) was identified through two-dimensional electrophoresis (2-DE) analysis and its pattern of expression was investigated in grass carp kidney (CIK) cells infected with grass carp reovirus (GCRV). The full length cDNA of *Ci*Trap1 contained an opening reading frame of 2157 bp that encoded a peptide of 718 amino acids. Phylogenetic analyses indicated that the *Ci*Trap1 shared 87% identity with its homologue from zebrafish (*Danio rerio*). The transcriptional level of *CiTrap1* in CIK cells was upregulated post virus infection as well as poly (I: C) stimulation. Following virus infection, grass carp *PTEN-induced putative kinase 1* (*PINK1*) and *Sorcin*, whose coding proteins interact with Trap1 in human, were simultaneously upregulated with *CiTrap1*. Typical characteristics of apoptosis were observed in CIK cells infected with GCRV by DAPI staining, DNA ladder electrophoresis, TUNEL assay and Annexin V labeling. RNAi-mediated silencing of *CiTrap1* in CIK cells resulted in the increased rate of virus-induced apoptotic cells. The results of this study suggest that *Ci*Trap1 is involved in the host's innate immune response to viral infection possibly through protecting infected cells from apoptosis.

1. Introduction

The grass carp *Ctenopharyngodon idella* is an economically important freshwater fish in China, with an annual output greater than any other freshwater species (~20% of the total production of the freshwater fishes). Grass carp reovirus (GCRV) is a pathogenic aquareoviruses first identified in 1986 that leads to grass carp hemorrhagic disease with high mortality [1,2]. GCRV consists of 11 double-strands of RNA (dsRNA) [3] encoding 12 proteins including seven structural proteins and five non-structural proteins [4]. GCRV causes a severe cytopathic effect in grass carp kidney cells (CIK) [5]. Therefore, GCRV and CIK cells provide a robust system with which to study the innate antiviral immune mechanisms of fish *in vitro*, including the identification of immune genes, mechanisms of infection, screening of antiviral drugs and the development of subunit vaccine [6–15]. Nevertheless, knowledge on the host's immune defense against viral infection is still not adequate for the development of effective management strategies for disease control.

The tumor necrosis factor receptor associated protein 1 (Trap1), first identified by the yeast-based two-hybrid method, interacts with tumor necrosis factor receptor 1 (TNFR1) and is homologous to the 90-kDa heat shock protein (HSP90) [16]. Studies have revealed that Trap1 is identical to the 75-kDa heat shock protein (HSP75) and is located in the mitochondria [17,18]. Trap1 differs from HSP90, however, in that it cannot bind stably to p23 and Hop (p60), but can bind retinoblastoma (Rb) with a unique LxCxE motif [17,19]. Trap1 may regulate genes involved in the cell cycle and protect cells from reactive oxygen species and related apoptosis [19,20]. Moreover, stable Trap1-expressing Saos-2 cells are more resistant to DNA damage induced by H_2O_2 and apoptosis [21]. Previous studies have also indicated that Trap1 interacts with the upstream protein, PTEN-induced putative kinase 1 (PINK1), and the downstream protein, Sorcin, to protect cells against apoptosis

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http://dx.doi.org/10.1016/j.fsi.2015.01.031
1050-4648/

[22–24]. Trap1 is also regarded as an inhibitor of apoptosis that protects cells from viral infection [25]. However, the mechanisms of Trap1 in the innate immune response to pathogen and in apoptosis are still poorly understood.

Apoptosis, a kind of programmed cell death, is a process of cell suicide that occurs under stimulation and it is different from regulated necrosis, autophagic cell death and mitotic catastrophe [26]. It is characterized by a series of morphological and biochemical changes, including cell shrinkage, cytoplasmic membrane blebbing, chromatin condensation, DNA fragmentation, translocation of phosphotidylserine and the formation of apoptotic bodies [27]. Apoptosis occurs via two fundamental pathways: extrinsic and intrinsic (caspase-dependent or − independent) [28]. The extrinsic pathway is processed by the combination of death ligands and receptors. Death receptors are transmembrane proteins (e.g. Fas and TNFR) that promote the formation of a death-inducing signaling complex (DISC) when they interact with corresponding ligands and activate downstream caspases to cause apoptosis. The intrinsic pathway is controlled by the mitochondria and triggered by stimuli-like reactive oxygen species (ROS), endoplasmic reticulum stress, lysosomal stress or calcium overloading. When the mitochondria are stimulated, cytochrome *c* is released into the cytoplasm activating caspases (e.g. caspase-9 and caspase-3) causing caspase-dependent intrinsic apoptosis [29,30]. On the other side, caspase-independent intrinsic apoptosis could be induced by apoptosis-inducing factor (AIF), endonuclease G (ENDOG) and high temperature requirement protein A2 (HTRA2) in a way of relocating to the nucleus, mediating large-scale DNA fragmentation and cleaving a wide array of cellular substrates [26]. A recent study suggested that GCRV induces oxidative stress and apoptosis in CIK [31].

Proteomics is a powerful technique to investigate the dynamics of host–virus interactions and has been applied to studies of spring viremia carp virus (SVC) [32], porcine circovirus type 2 (PCV2) [33], bombyx mori nuclear polyhedrosis virus (BmNPV) [34], white spot syndrome virus (WSSV) [35] and infectious spleen and kidney necrosis virus (ISKNV) [36]. In this study, we used two-dimensional electrophoresis (2-DE) to compare the protein expression profiles of GCRV-infected and mock-infected CIK cells. *Ci*Trap1, grass carp tumor necrosis factor receptor-associated protein 1, was identified as one of the significantly upregulated proteins at 24 h post-infection. The aim of this study was to characterize the *CiTrap1* gene and investigate its involvement in the host's innate immune response to virus infection by presenting data to demonstrate the correlation between increased GCRV-induced apoptosis and reduced expression of *CiTrap1*, the potential anti-apoptotic factor.

2. Materials and methods

2.1. Cell lines, virus, and plasmids

Grass carp reovirus was propagated using a strain of GCRV maintained in our laboratory (GCRV-JX01) [1]. CIK cells were grown in medium M199 supplemented with 10% inactive fetal calf serum (Gibco BRL) and incubated at 28 °C. GCRV particles were purified from the supernatant of infected CIK cells by ultracentrifugation [4]. Virus titration was performed by a standard 50% tissue culture infective dose ($TCID_{50}$) assay [37].

The open reading frame (ORF) sequence of *CiTrap1* was amplified by the Pyrobest® DNA polymerase (TAKARA, Japan) with gene specific primers, Trap1-GFP-F1 and Trap1-GFP-R1. The PCR product was cloned into eukaryotic expression vector pEGFP-N1 (Invitrogen) after digestion with a combination of XhoI and EcoRI (TAKARA, Japan). Plasmid DNA was stored at −20 °C.

2.2. 2-DE, image analysis and diversity protein spots analysis

CIK cells were infected with GCRV at a multiplicity of infection (MOI) of 1, and mock-infected cells served as negative control. At three time points post-infection (6 h, 12 h, and 24 h), cells were scraped and harvested by centrifugation at 8000 × g for 5 min. Total cellular proteins were extracted from the infected cells as described previously [32]. The protein concentration was determined by the Bradford method [38]. 2-DE was performed with 24-cm (linear, pH 3–10) IPG strips (Bio-Rad) [39]. The IPG strips were loaded with 250 μL of rehydration buffer (7M urea, 2 M thiourea, 2% CHAPS and 65 mM DTT) containing 80 μg of protein. All the experiments were performed in triplicate. The first dimensional IEF was performed at 17 °C with a voltage gradient of 500 V for 1 h, 1000 V for 1 h, 8000 V for 8 h, continued up to a total of 60 kVh. The isoelectric focused strips were incubated for 15 min in an equilibration buffer (6 M urea, 0.375 M Tris, 20% glycerol and 2% SDS) containing 1% DTT and then incubated for 15 min in an equilibration buffer containing 2.5% (w/v) iodoacetamide. Equilibrated strips were sealed onto the top of 12.5% SDS-PAGE gels for electrophoresis. The gels were visualized by silver stain [40] and a Bio-Rad GS-710 scanner (Bio-Rad). The protein spots were analyzed with ImageMaster 2D platinum software (version 7.0; GE Healthcare). A comparative analysis of the protein spots was performed by matching corresponding spots across different gels. Difference in the mean normalization percentage volumes of the protein spots between the treatment and control groups were assessed with a Student's *t*-test. The protein spots were then processed by in-gel digestion [41] and sequence analysis of the peptides was undertaken by mass spectrometry (MS) according to published methods [6]. Briefly, the protein spots were excised from the silver-stained gels and placed in centrifuge tubes. They were then re-dissolved in 0.8 μL of matrix solution (a-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid and 50% acetonitrile), and then spotted onto the 4800 Plus MALDI-TOF/TOF™ Analyzer (Applied Bio-systems, USA). All acquired sample spectra were processed using 4700 Explore™ software (Applied Bio-systems) with the default settings selected. Parent mass peaks with a mass of 800–4000 Da and a minimum signal to noise ratio of 50 were selected for tandem MS/MS analysis. MS data were analyzed using MASCOT software (Matrix Science, London, UK) and the National Center for Biotechnology Information non-redundant (NCBI nr) eukaryotic protein sequence database. Database searches were carried out using the following parameters: trypsin digest with one missing cleavage; variable modifications of methionine oxidation and cysteine carbamodomethylation; peptide and fragment mass tolerance of 100 ppm or 0.3 Da, respectively. Proteins with statistically significant search scores ($P < 0.05$) were considered to be identified reliably.

2.3. Cloning and sequencing analysis of the full-length CiTrap1 cDNA

Total RNA was isolated and extracted from CIK cells using TRLzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity of the extracted RNA was determined as the OD_{260nm}/OD_{280nm} ratio, with expected values between 1.8 and 2.0. The RNA was treated with RNase free-DNase I (Takara, Japan) to remove residual genomic DNA before being reverse-transcribed into cDNA using random hexamer primers and MMLV Reverse Transcriptase (Takara, Japan). The internal region of *CiTrap1* was amplified from grass carp cDNA by primers (Trap1-F1 and Trap1-R1) designed according to the sequence of zebrafish (*Danio rerio*) *Trap1* gene (GenBank accession no. NM_001113625.1). PCR products were purified with the Wizard® SV Gel and PCR Clean-up System kits (Promega), cloned into the pGEM-T easy vector