Fish & Shellfish Immunology 44 (2015) 515–524

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Proteomic analysis of cellular protein expression profiles in response to grass carp reovirus infection

Dan Xu [1], Lang Song [1], Hao Wang, Xiaoyan Xu, Tu Wang, Liqun Lu*

Key Laboratory of Aquatic Genetic Resources of the Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, PR China

ARTICLE INFO

Article history:
Received 19 November 2014
Received in revised form
4 March 2015
Accepted 6 March 2015
Available online 14 March 2015

Keywords:
Grass carp reovirus
Proteomics
Stress granule
*Ci*TIA1
*Ci*G3BP1

ABSTRACT

Grass carp (*Ctenopharyngodon idella*) hemorrhagic disease, caused by grass carp reovirus (GCRV), is emerging as a serious problem in grass carp aquaculture. To better understand the molecular responses to GCRV infection, two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization tandem mass spectroscopy were performed to investigate altered proteins in *C. idella* kidney (CIK) cells. Differentially expressed proteins in mock infected CIK cells and GCRV-infected CIK cells were compared. Twenty-three differentially expressed spots were identified (22 upregulated spots and 1 downregulated spot), which included cytoskeleton proteins, macromolecular biosynthesis-associated proteins, stress response proteins, signal transduction proteins, energy metabolism-associated proteins and ubiquitin proteasome pathway-associated proteins. Moreover, 10 of the corresponding genes of the differentially expressed proteins were quantified by real-time reverse transcription polymerase chain reaction to examine their transcriptional profiles. The T cell internal antigen 1 (TIA1) and Ras-GTPase-activating SH3-domain-binding protein1 (G3BP1) of the cellular stress granule pathway from grass carp *C. idella* (designated as *Ci*TIA1 and *Ci*G3BP1) were upregulated and downregulated during GCRV infection, respectively. The full-length cDNA of *Ci*TIA1 was 2753 bp, with an open reading frame (ORF) of 1155bp, which encodes a putative 385-amino acid protein. The 2271bp full-length cDNA of *Ci*G3BP1 comprised an ORF of 1455bp that encodes a putative 485-amino acid protein. Phylogenetic analysis revealed that the complete ORFs of *Ci*TIA1 and *Ci*G3BP1 were very similar to zebrafish and well-characterized mammalian homologs. The expressions of the cellular proteins *Ci*TIA1 and *Ci*G3BP1 in response to GCRV were validated by western blotting, which indicated that the GCRV should unlink TIA1 aggregation and stress granule formation. This study provides useful information on the proteomic and cellular stress granule pathway's responses to GCRV infection, which adds to our understanding of viral pathogenesis.

1. Introduction

Grass carp reovirus (GCRV), the type strain of *Aquareovirus C*, is one of the most virulent pathogens of the grass carp (*Ctenopharyngon idellus*) [1]. The grass carp is the most economically important fish species cultivated in China, with an annual production of more than 3.6 million metric tons. Frequent outbreaks of hemorrhagic disease resulting from GCRV infection have resulted in significant economic losses in the grass carp cultivation industry [2,3]. GCRV is a dsRNA virus with a double-layered protein capsid, while the GCRV genome comprises 11 dsRNA genomic fragments encoding 12 viral proteins [4]. GCRV produces a typical cytopathic effect (CPE) by causing the formation of large syncytia in *C. idellus* kidney (CIK) cell lines [5,6]. Therefore, GCRV has served as a model to study the replication and pathogenesis of *Aquareoviruses*, both *in vivo* and *in vitro* [7]. However, there is no effective therapeutic drug available against GCRV and only vaccines hold great promise as a strategy against this disease. A commercial vaccine protecting against GCRV infection was recently developed in China; however, the appearance of more genetically distant genotypes of GCRV limited the commercialization of the single viral strain-based vaccine product [6]. 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) [8], porcine circovirus type 2 (PCV2) [9], bombyx mori nuclear polyhedrosis virus (BmNPV) [10], white spot syndrome virus (WSSV) [11] and

* Corresponding author. College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, PR China. Tel.: +86 2161900453; fax: +86 2161900454.
E-mail address: lqlv@shou.edu.cn (L. Lu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.fsi.2015.03.010
1050-4648/

infectious spleen and kidney necrosis virus (ISKNV) [12]. To further understand the pathogenesis of GCRV disease, it is crucial to characterize the proteomic interactions between host cells and the virus; however, there are few reports on this aspect and the pathogenesis of this disease remains incompletely understood.

Stress granules (SGs) are large cytoplasmic mRNA-protein aggregates, formed following exposure of cells to various stresses [13] and following initiation of translation inhibition [14]. SGs contain various proteins, including cellular RNA-binding proteins, such as T cell internal antigen 1 (TIA-1) and Ras-GTPase-activating SH3-domain-binding protein1 (G3BP1), which bind to mRNPs (messenger ribonucleoprotein) [15,16]. After binding nucleotide sequence elements within mRNAs that are exposed upon polysome disassembly, G3BP1 and TIA-1 form SGs through self-aggregation [17–19]. TIA-1 is a typical multi-domain RNA binding protein, where individual RNA binding domains, connected by flexible linkers, cooperate in the recognition of target RNAs during the stress granules pathway [20]. The multiple protein phosphatase Mg^{2+} (PPM) domains of TIA-1 are independent structural modules in the absence of RNA: PPM2 and 3 bind cooperatively to pyrimidine-rich RNA ligands [20]. G3BP, a phosphorylation-dependent endoribonuclease, regulates RasGAP through an interaction in the SH3 domain of RasGAP [21]. G3BP is an RNA-binding protein involved in RNA metabolism and signal transduction. Much attention has been paid to its role in regulating SGs. G3BP has five conserved domains: nuclear transport factor2-like (NTF2), and acid-rich region, and RNA-recognition motif (RRM), an arginine-glycine-rich box (RGG) and, depending on splicing of its transcript, several PXXP motifs. G3BP determines the fate of mRNAs during cellular stress as an effector of SG assembly, and Ras signaling contributes to this process by regulating G3BP dephosphorylation [16]. The mechanism of SG formation is poorly understood, but involves mRNP remodeling that incorporates new proteins that may nucleate SGs and involves mRNP transport on microtubules [22]. However, the respective roles of these proteins in SG-associated functions have not yet been fully elucidated in fish, especially during GCRV infection.

Two-dimensional electrophoresis (2-DE) was used to analyze the protein expression profiles of GCRV-infected and mock-infected CIK cells in this study. Twenty-three differentially expressed spots were identified successfully, including two stress granules (SGs) related proteins: the T cell internal antigen 1 (TIA1) and Ras-GTPase-activating SH3-domain-binding protein1 (G3BP1). The objective of this study was to use proteomics to identify the proteins of the grass carp that respond to GCRV infection, especially *Ci*TIA1 and *Ci*G3bp1.

2. Materials and methods

2.1. Cell culture, virus infection and sample preparation

CIK cells were grown in M199 medium supplemented with 10% inactivated fetal calf serum (Gibco BRL). CIK cells were incubated and infected at 28 °C. The GCRV-JX01 particles were purified from the supernatant of infected CIK cells by a previously described ultracentrifugation method [23]. Virus titration was performed by a standard 50% tissue culture infective dose ($TCID_{50}$) assay [24]. CIK cells were infected with a multiplicity of infection (MOI) of 1. Total cellular proteins were extracted at different time points. Briefly, CIKs were inoculated with GCRV-JX01 or with medium (control). At 6 h, 12 h, and 24 h post-infection (hpi), the cells were scraped and harvested by centrifugation at 8000 × *g* for 5 min. The cells were then washed three times with ice-cold phosphate buffered saline (PBS) and lysed with lysis buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 1 mM PMSF) containing DNaseI (20 units/ml)/RNaseA (0.25 mg/ml) for 2 h on ice. The supernatant was collected by centrifugation at 20,000 × *g* for 60 min at 4 °C, and stored at −80 °C. The protein concentration was determined by the standard Bradford method [25].

2.2. Two-dimensional gel electrophoresis (2-DE), image analysis and differential protein spot analysis

According to the methods detailed by Cao Haipeng et al. [26], the 2-DE was performed with 13-cm (linear, pH 3–10) immobilized pH gradient (IPG) strips (Bio-Rad). The IPG strips were loaded with 250 μl of rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS and 65 mM DTT) containing 80 μg of protein. Three technical replicates were performed for each group. Isoelectric focusing (IEF) was performed at 17 °C with a voltage gradient of 500 V for 1 h, 1000 V for 1 h, and 8000 V for 8 h, and then continued up to a total of 60 kVh. The focused strip was equilibrated for 15 min with equilibration solution (6 M urea, 0.375 M Tris–HCl, 20% glycerol and 2% sodium dodecyl sulfate [SDS]) containing 2% DTT. The strips were then equilibrated for another 15 min with equilibration solution 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 with 0.1% Coomassie Brilliant Blue (CBB) R-250 stain and scanned using a Bio-Rad GS-710 scanner (Bio-Rad). The spots were analyzed using Image Master 2D platinum software (version 7.0; GE Healthcare). To evaluate the differentially expressed protein spots, Student's t-tests were applied, and a significance threshold of 95% was applied. Differentially expressed protein spots were excised and processed by in-gel digestion, according to Rosenfeld et al. [27], followed by sequence analysis of the peptides by mass spectrometry (MS), performed as described previously [28]. Briefly, the differentially expressed protein spots were manually excised from the silver-stained gel, placed in centrifuge tubes, re-dissolved in 0.8 μl of matrix solution (a-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic Acid (TFA) and 50% acetonitrile), and then spotted onto the 4800 Plus matrix-assisted laser desorption/ionization tandem mass spectroscopy (MALDI-TOF/TOF™) Analyzer (Applied Biosystems, USA). The Nd:YAG laser was operated at a wavelength of 355 nm, with an acceleration voltage of 2 kV. All acquired sample spectra were processed using 4700 Explore™ software (Applied Bio-systems) operating with default settings. Parent mass peaks with a mass of 800–4000 Da and a minimum signal-to-noise ratio of 50 were picked out for tandem MS/MS analysis. MS data were analyzed using the MASCOT software (Matrix Science, London, UK) and the NCBInr eukaryotic protein sequence database. The parameters were set as follows: trypsin digest with one missing cleavage; fixed modification of carbamidomethyl (C); oxidation of variable modification; peptide mass tolerance of ±100 ppm; and fragment mass tolerance of 0.3 Da. Proteins with a minimum ion score of 35 ($P < 0.05$) were considered to be identified reliably.

2.3. Quantitative real-time RT-PCR detection of mRNA encoding certain identifed proteins

According to the corresponding gene sequences of MALDI-TOF/TOF identified proteins, specific primers were designed using the Beacon Designer 7.9 analysis software (Table 1). Total RNA was extracted at designated times using Trizol Reagent (Invitrogen). Total cDNA was produced by reverse transcription (RT) of equal amounts of RNA (1 μg) using a CFX96 Real-time PCR (Bio-Rad) instrument, according to the instructions of the PrimeScript™ RT reagent Kit (Takara) in a total volume of 20 μl. Each reaction mixture consisted of 1 μL of DNA, 7 μl of nuclease-free water, 10 μl of 2 × SsoAdvancedTM SYBR Green Supermix (Bio-Rad), and 1 μL of