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Sequestration of RNA by grass carp *Ctenopharyngodon idella* TIA1 is associated with its positive role in facilitating grass carp reovirus infection

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ABSTRACT

Previous report demonstrated that grass carp reovirus (GCRV) infection resulted in unlinking cellular stress granule formation from aggregation of grass carp *Ctenopharyngodon idella* TIA1 (CiTIA1). Here, we provided evidence to show that CiTIA1 bound to synthesized ssRNA and dsRNA *in vitro*. Both GST-pull down assay and RNA immunoprecipitation analysis confirmed the association between GCRV-specific RNA and GST-tagged CiTIA1 in *C. idella* kidney (CIK) cells. Furthermore, CiTIA1 was shown to protect dsRNA of virus-origin from degradation in CIK cells through Northern blot analysis. Finally, transient overexpression of CiTIA1 enhanced the replication efficiency of GCRV in CIK cells. Taken together, our results suggested that cellular CiTIA1 might facilitate GCRV replication through sequestrating and protecting viral RNA from degradation.

1. Introduction

Stress granules (SGs) are discrete foci that contain cytoplasmic mRNAs. The SGs, in which the mRNAs are recruited by TIA1 (T cell internal antigen 1) and TIAR (T-cell restricted intracellular antigen related protein), are generally induced following exposure to a stressor. Mammalian TIA1 and TIAR are both ARE (adenine and uridine-rich element)–binding proteins that appear to selectively downregulate TNF-α production through post-transcriptional regulation, suggesting that they are translational silencers in unstressed cells [1]. Both TIA1 and TIAR are essential for mammalian embryo development, but their specific functions during development are not known [2]. As RNA-binding protein, TIA1 and TIAR appear to be functionally redundant, which allows these proteins to sequestrate untranslated mRNA, preventing the initiation of translation and leading to translational arrest [1]. Both TIA1 and TIAR are activated following the stress-induced phosphorylation of eIF-2α, a translation initiation factor [3]. Once the stressor is removed, the mRNAs are released from the SGs and translated by ribosomes or degraded in processing bodies (P-bodies) [3,4]. While there are over 500 RNA-binding proteins involved in the formation of mammalian SGs, the primary SG proteins are relatively limited, which include TIA1, TIAR, RasGAP-associated endoribonuclease (G3BP), elongation initiation factor 3 (eIF3), and poly-A binding protein (PABP) [5–7]. SG formation can be induced by overexpressing the component protein(s) including TIA1, TIAR, and G3BP. TIA1 and G3BP are thought to be the critical effectors of SG formation because truncated forms of the proteins inhibit SG formation [6,8,9]. Until now, very little has been known on SG biomarkers of aquatic animals and most knowledge on SG comes from the study on mammalian animals including human and mouse.

Viruses have evolved to interact with the SG pathway in very different ways. For example, respiratory syncytial virus and coronaviruses take advantage of the SGs as translational silencers and induce SGs to inhibit host translation [10–12]. In contrast, rotavirus and cardioviruses inhibit the formation of SGs during infection to prevent the adverse effect of SGs on the translation of viral genes [13,14]. Many viruses, including mammalian reovirus, poliovirus, semliki forest virus, and hepatitis C virus, could induce SGs during early infection to benefit from SG-mediated inhibition of host translation, but during late infection tend to inhibit SG formation to allow efficient viral replication [15–17]. Finally, TIA1 and TIAR appear to be critical for West Nile virus replication, possibly

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through their ability to bind specifically to the 3′ terminus of the viral minus-strand RNA template [18]. Interestingly, TIA1 can self-aggregate into novel foci that lack other SG-defining components in poliovirus-infected cells, suggesting a novel role for TIA1 independent of SGs [19].

Grass carp reovirus (GCRV) causes a hemorrhagic disease in cultivated grass carp (*C. idella*) leading to heavy economic losses [20]. GCRV is considered unique among the known aquareoviruses due to its high pathogenicity [21]. There are three known types of GCRV: type I (representative strain: GCRV-873) [22], type II (GCRV GD-108) identified in 2012 [23], and type III GCRV (GCRV-104) identified in 2013 [24]. All of the GCRV strains contain a segmented double stranded RNA (dsRNA) genome and share low homogeneity with each other. However, all of the GCRV strains belong to the genus *aquareovirus*, which is significantly different from the genus *othoreovirus* [25]. Type I GCRV is widely regarded as the archetype aquareovirus and has been the subject of research in developing diagnostic methods, elucidating virus-host cell interactions, and characterizing viral morphology [26–29]. Even with the relatively extensive knowledge pertaining to type I GCRV, little is known about the mechanisms on its replication and pathogenicity in grass carp cells. In this study, GCRV refers to the type 1 strain.

Previously, two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization tandem mass spectroscopy were performed to characterize altered protein expression profiles in *C. idella* kidney (CIK) cells following GCRV infection. The experiments revealed that grass carp *C. idella* TIA1 (CiTIA1) and G3BP1 (CiG3BP1) were significantly upregulated and downregulated, respectively [30], indicating GCRV might unlink CiTIA1 aggregation from SG formation that is similar to the effects of poliovirus in host cells [19]. TIA1 has not been functionally characterized in any fish species to date and potential differences might exist between fish and mammalian TIA1, which merit further characterization of this molecule as an RNA chaperon molecule in grass carp. Although GCRV can efficiently infect and replicate in CIK cells, the presence of GCRV dsRNA in host cells is a signal to induce RNA receptor-mediated antiviral innate immunity, including the RNAi pathway, which has been demonstrated to be effective in grass carp cells; recent studies also demonstrated that chemically synthesized siRNA targeting the viral dsRNA genome inhibited viral replication in CIK cells [31,32]. Given that dsRNA-binding proteins could potentially inhibit RNAi [33], the dsRNA binding activity of CiTIA1 was investigated here both *in vitro* and *in vivo*. Since whether CiTIA1 aggregation enhances or antagonizes GCRV replication is unknown, we also analyzed the impact of over-expressed CiTIA1 on viral replication in this study.

2. Materials and methods

2.1. Cell and virus

CIK cells were maintained in M199 medium (100 U/mL Penicillin and 100 μg/mL Streptomycin) supplemented with 10% fetal bovine serum (Gibco BRL). The cells were incubated and infected at 28 °C. Type I GCRV strain JX-01 was used in this study [34]. The virus was titrated using a standard 50% tissue culture infective dose ($TCID_{50}$) assay. Real-time RT-PCR was established to quantify the transcriptional expression profile of GCRV-S10 gene using CFX96™ Real-Time PCR Detection System (Bio-Rad) [34].

2.2. Plasmid construction

The *CiTIA1* ORF was amplified by RT-PCR from the total RNA extracted from CIK cells using the primer pair (F-5′ ccgCTCGAGATGATGGACGACGAGCAGC ′3 and R-5′ cccAAGCTTGTGTGTGTGG TATCCTGCAGT 3′). The amplified fragment was cloned into the eukaryotic expression vector pEGFP-N1 (Invitrogen) and prokaryotic expression vector pGEX-4T-3 (Invitrogen) to generate pEGFP-TIA1 and pGST-TIA1, respectively. Plasmid extraction and purification was performed using the PureYield Plasmid Midiprep System (Promega). The quantity and quality of the extracted DNA was determined by Nanodrop 2000 (Thermo). The plasmid DNA was stored at −20 °C.

2.3. Recombinant protein expression and polyclonal antibody generation

The prokaryotic expression plasmid pGST-TIA1 and the vector pGEX-4T-3 was transformed into BL21 competent cells (Tiangen, Shanghai), respectively. A single colony was picked and incubated in LB broth supplemented with 50 μg/mL ampicillin at 37 °C. The expression of GST-TIA1 or GST was induced with 1 mM IPTG at 20 °C overnight. 1 mL of the culture liquid was subjected for SDS-PAGE analysis. The bacteria pellet was then re-suspended in GST binding/wash Buffer (Sangon, Shanghai) containing a bacterial protease inhibitor cocktail. After ultrasonication and centrifugation, the cleaned lysates were used to purify recombinant GST-TIA1 using Glutathione™ Sepharose 4B beads (GE Healthcare) according to the manufacturer's protocol. Purified proteins were confirmed by Western blot analysis. The purified GST-TIA1 protein was used to elicit polyclonal antibodies in New Zealand rabbits. Briefly, groups of 4 animals were intraperitoneally immunized with a 50% emulsion of Freud's adjuvant (FCA, Sigma,USA) containing purified recombinant protein (100 μg per rabbit). The rabbits were boosted twice using the same dose at 3-week intervals. Immune sera were collected 7 days after the last immunization. The polyclonal antibodies were purified using GL biochem (Shanghai, China), and the serum specificity was confirmed by Western blot analysis.

2.4. Cell transfection

Approximately 24 h before transfection, CIK cells were grown in a 24-well plate at 4×10^5 cells per well in M199 supplemented with 10% FBS. When the CIK cells reached 70% confluence, they were washed with PBS and the culture supernatants were replaced with Opti-MEM (Invitrogen). The plasmid pEGFP-TIA1 (1 μg) was transfected into the CIK cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The vector pEGFP-N1 served as the negative control. EGFP-TIA1 protein expression was monitored 18 h post transfection by detecting the fluorescent signal under the fluorescence microscope (Olympus, Japan). Transfected CIK cells were also harvested for Western blot analysis to confirm the expression of EGFP-tagged protein.

2.5. RNA binding assay

Synthetic GCRV-S10-specific (JQ042807.1) ssRNA or dsRNA oligonucleotides in the length of 10 (GACGCCUUAC), 20 (CCACGCUUGUUCACGUCAAC), or 30 (CAACCAAACGAAGCCAUUCGCUCA UUAGUC) nucleotides were employed for the RNA binding assay. Briefly, purified GST-TIA1 or GST alone was diluted in 20 μL of binding buffer containing 0.1 μM RNA, 100 mM NaCl, and 50 mM Tris–HCl, pH 7.4. The reaction mixtures were incubated at 25 °C for 30 min to allow the protein to bind to the nucleic acid. The mixtures were then resolved by agarose gel electrophoresis at 4 V/cm using 2% gels buffered with TBE. Nucleic acids were visualized with SYBR Green II (Bioteke, China) by staining the gels for 5 min.