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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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**Article info**

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 sequestre 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 over-expressing 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
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 *orthoreovirus* [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 (TCID50) 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 by using the primer pair (F-5′ cggCTCGA-GATGATGGACGACGACGCACG 3′ and R-5′ cccAAGCTTGTGTGTGTGTGG 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 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 (GACGCCUUAUC), 20 (CCACGCUUGUUCACGCUAC), or 30 (CAACCAAACGAAGCCAUUCGCUCAUUGCUCAUUGUC) 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 (Biotek, China) by staining the gels for 5 min.
2.6. GST pulldown assay and RNA immunoprecipitation

The S10 ORF of GCRV was amplified from the viral genome by RT-PCR using the primers F-5’ GAATTAACGACTCACTATAGGG AGAATTGCACTTCATGATTCG 3’ and R-5’ GAATTAACGACTCACTATAGGG AGAATTGCACTTCATGATTCG 3’. According to the procedure reported previously [30], the dsRNA corresponding to the viral S10 gene was transcribed using the Ribomax™ Large Scale RNA Production System-T7 kit (Promega, Shanghai) from a recombinant pMD19-T vector (Tiangen, China), where the GCRV S10 ORF was under the control of the T7 promoter. S10-dsRNA was purified with an RNA purification kit (Qiagen). The RNA was then incubated with GST-TIA1 or GST alone in a Glutathione S-Transferase purification mini spin column (Sangon, China) in nuclease-free water at an equimolar concentration. The clean lysate was treated with phenol-chloroform (Invitrogen) to clear the protein and then analyzed by Northern blot. The recombinant pMD19-T vector or negative control vector pEGFP-N1 were transfected into CIK cells seeded in 6-well plates. The cells in each well were then washed with PBS and lysed in 500 μl of RIPA lysis buffer. Cellular debris was cleared by centrifugation at 12,000 rpm for 10 min at 4 °C. The lysates were precleared by incubating it with 50 μl of Protein A/G Plus-Agarose (Santa Cruz) for 2 h at 4 °C. Then monoclonal antibodies against EGFP and 50 μl of protein A/G Plus-Agarose were added into the cleared lysates and incubated overnight. The beads were then pelleted (2500 rpm for 5 min at 4 °C), and the bound RNA was extracted using Trizol (Invitrogen) and analyzed by Northern blot or RT-PCR.

2.7. Northern blot assay

CIK cells were grown in 6-well plates and infected with GCRV at an MOI of 1. Then the cells were transfected with dsRNA-S10 with or without pEGFP-TIA1. At 48 h post transfection, cells were harvested and the RNA was extracted with Trizol. The RNA was separated on a 1.5% agarose formaldehyde denaturing gel and transferred to a nitrocellulose membrane using the upward cationic method. The membrane was pre-hybridized for 30 min using the Dig Easy Hyb Solution (Roche), and then hybridized for 6 h in Dig Easy Hyb Solution containing the synthesized digoxin-labeled dsRNA-S10 probe using Dig RNA Labeling Kit-T7 (Roche). The membrane was washed at room temperature in Wash Buffer I (2× SSC, 0.1% SDS) and Wash Buffer II (0.1× SSC, 0.1% SDS) using each buffer twice for 15 min. After hybridization and stringent washing, the membrane was rinsed briefly for 5 min in Wash Buffer III (0.1 M Maleic acid; 0.15 M NaCl; pH 7.5; 0.3% Tween 20), and incubated for 30 min in Blocking solution (Roche). Then secondary antibody, antidigoxigenin-AP, was diluted 1:10,000 in Blocking buffer and incubated with the membrane for 30 min. The membrane was then washed twice in Wash Buffer III and equilibrated for 5 min in Detection buffer (Roche). The signal was detected using NBT/BCIP (Roche).

3. Results

3.1. CITIA1 binds to both ssRNA and dsRNA in vitro

Mammalian TIA1 is an RNA chaperon that binds to mRNA in SG independent of the mRNA sequence; however, it was not clear whether grass carp CITIA1 bound to RNA in CIK cells. Compared with the GST control protein of about 22 kDa (Fig. 1A), purified recombinant CITIA1 protein (GST-TIA1) was expressed in Escherichia coli as a soluble GST-tagged fusion protein with a size of approximately 70 kDa (Fig. 1B). GST-TIA1 was also used to generate TIA1-specific polyclonal antibodies by immunizing New Zealand rabbits. The specificity of the induced rabbit serum in recognizing endogenous CITIA1 in CIK cells was validated by Western blot assay (Fig. 1C). Taking advantage of this antibody, we were able to monitor the steady-state level of CITIA1 during GCRV infection. In the cellular extracts that were harvested at various time points post viral infection, expression of CITIA1 was significantly upregulated at 12 h p.i. (Fig. 1D). As a control, the expression level of GAPDH remained constant during the viral infection course (Fig. 1D).

The ability of CITIA1 to bind RNA was tested by incubation with an equimolar concentration of synthesized single or double-stranded RNA oligonucleotides with 10, 20, or 30 residues in length. GST alone served as the negative control. The RNA oligonucleotides sequences were chosen arbitrarily from the GCRV-JX01 S10 gene. The protein-RNA mixture was analyzed by agarose gel analysis. A band shift on the agarose gel indicated that CITIA1 bound to both ssRNA and dsRNA (Fig. 2).

We next investigated the interaction between GST-TIA1 and S10-dsRNA. The S10-dsRNA and GST-TIA1 protein were mixed and incubated for protein-RNA interaction. The mixture was incubated with Glutathione S-transferase Sefinose Resin (Sangon), and then subjected for GST-protein purification. The purified protein-RNA complex was treated with phenol-chloroform to collect the RNAs bound to GST or GST-TIA1. A Northern blot analysis demonstrated that S10-dsRNA was pulled down by GST-TIA1, but not GST, indicating the specific interaction of viral dsRNA with GST-TIA1 (Fig. 3). Thus, CITIA1 had the potential to bind RNA with sequence specific for GCRV.

3.2. CITIA1 binds to viral RNA in vivo

RNA immunoprecipitation was performed on CIK cells transfected with both pEGFP-TIA1 (Fig. 4A) and S10-dsRNA. The TIA1 complex was immunoprecipitated using an anti-EGFP antibody. S10-specific RNA was detected from the immune-precipitated complex using RT-PCR (Fig. 4B). Northern blot analysis further confirmed that dsRNA specific for the GCRV-S10 gene was associated with the CITIA1 protein (Fig. 4C). These results were consistent with our in vitro experiments, indicating that CITIA1 potentially
binds viral dsRNA.

3.3. CiTIA1 protects dsRNA from degradation in vivo

Having shown that CiTIA1 was able to bind to GCRV RNA, we next assessed whether CiTIA1 protected the viral dsRNA from degradation in vivo. Both pEGFP-TIA1 and GCRV-S10-dsRNA were transfected into CIK cells. 48 h post transfection, the total RNA samples was extracted from the transfected cells (Fig. 5 line A); Northern blot analysis (Fig. 5 line A) indicated that the presence of overexpressed CiTIA1 reduced the degradation rate of dsRNA in comparison with control cells that were cotransfected with pEGFP and GCRV-S10-dsRNA. In consistence with previous report that GCRV infection resulted in the protection of dsRNA in CIK cells, viral genomic S10 in CIK cells infected with GCRV was protected from degradation into viral sequence-specific small molecular RNA (Fig. 5 line C). These results suggested that CiTIA1 might be responsible for protecting viral dsRNA from degradation during GCRV infection.

3.4. Overexpression of CiTIA1 results in enhanced viral replication level

It was not clear whether the interaction between CiTIA1 and GCRV dsRNA enhanced GCRV replication or inhibited viral infection. To assess the effect of TIA1 upregulation on GCRV replication, CiTIA1 was transiently overexpressed in CIK cells by transfection of CIK cells with pEGFP-TIA1. 24 h after transfection, the cells were infected with GCRV at an MOI of 1. The culture supernatant was collected at 4, 8, 12, and 24 h post infection (h pi) and subjected for a virus titration analysis reflected by the gene copy number of virus in the supernatant (Fig. 6). The increased replication levels suggested that CiTIA1 enhanced GCRV replication. As negative control, cells transfected with pEGFP-N1 demonstrated no impact on viral replication.

4. Discussion

GCRV is unique among the aquareoviruses because it is highly pathogenic for cultured grass carp. However, there is relatively little known about the molecular mechanisms underlying viral replication and pathogenesis. Here, we showed that CiTIA1 bound to both ssRNA and dsRNA with sequence specificity to GCRV genome in vitro. Furthermore, CiTIA1 binding to viral dsRNA in vivo was confirmed by GST-pull down and co-immunoprecipitation assays, which resulted in protecting viral dsRNA from degradation. In consistence with this, enhanced GCRV replication was achieved by overexpression of CiTIA1 in CIK cells. These data suggested that GCRV hijacked host TIA1 protein for efficient viral replication.

4.1. TIA1 accumulation and apoptosis

By controlling the duration of translational arrest, TIA-1 and
TIAR may play a role in determining whether stressed cells survive to repair the stress-induced damage or die by apoptosis [1]. During GCRV infection, the reduced expression of CiG3BP and increased level of CiTIA1 suggested less functional SGs formed during infection [30]. Fewer SGs might reduce the efficiency of both host translational arrest and sequestration of viral RNA, which would favor viral infection under conditions of normal cellular metabolism. Indeed, many genes were transcriptionally or translationally upregulated during GCRV infection [30]. Employing a polyclonal antibody against CiTIA1, we were able to show that expression level of CiTIA1 was significantly increased at 12 h p.i. (Fig. 1). Similar to the poliovirus, GCRV should unlink the accumulation of TIA1 from SG formation.

TIA1 accumulation may not always be beneficial for viral infection. TIA1 has been shown to regulate TNF-α mRNA and the alternative splicing of a variety of pre-mRNAs including the FAS receptor, suggesting TIA1 is an apoptosis-promoting protein [35]. While there are many factors that might contribute to the GCRV-induced apoptosis, expression of CiTIA1 in GCRV-infected CIK cells correlated with the development of apoptosis in GCRV-stressed cells. Here, we have shown that apoptosis-antagonizing protein CI1A1 induced by GCRV infection might be involved in protecting cells from apoptosis during the viral replication cycle. Interestingly, the Sendai virus trailer RNA binds to TIAR, and is involved in antagonizing TIAR-induced apoptosis [36]. This observation suggests that viral RNA might also be able to inhibit apoptosis by interacting with host pro-apoptotic factors. The role of CiTIA1 might be better understood by its more positive roles in infected cells beyond apoptosis. For example, in mammalian reoviruses, viral mRNAs escape translational repression when SGs are disrupted [16].

4.2. dsRNA binding of CiTIA1 and dsRNA-induced innate response

Translation in the host cell is suppressed during viral infection by the dsRNA kinase PKR, which homodimerizes and undergoes autophosphorylation after binding to dsRNA. In the case of Mammalian reovirus (MRV) infection, the cells attempt to inhibit viral replication by activating PKR and other stress kinases, phosphorylating eIF2, inhibiting the formation of ternary complexes, and forming SGs [16]. MRV-encodes the protein σ3, which binds dsRNA and prevents phosphorylation of PKR and eIF2; thus, no SGs form, and neither virus nor cellular translation is inhibited [16]. However, although GCRV and MRV face similar challenges with regard to dsRNA-induced PKR activation, GCRV does not encode a protein homologous to σ3. The finding that CiTIA1 associated with dsRNA might suggest that CiTIA1 potentially inhibits PKR activation by sequestering the dsRNA, which could be key for the virus to survive the dsRNA-activated innate immune response.

The mechanisms that allow reovirus genomic dsRNA to survive the RNAi pathway, which is triggered by dsRNA and initiated by Dicer, remain to be defined. A previous study suggested that during mammalian reovirus infection, dsRNA-binding proteins might
inhibit the RNAi pathway [33]. There is still little known about the escape mechanism used by GCRV. We have previously shown that the genomic dsRNA of GCRV was sensitive to the cellular RNAi pathway, and that unidentified RNAi suppressor protein(s) could contribute to the survival of the viral genome and efficient viral replication [31]. From the present study, an alternative hypothesis is that CiTIA1 accumulation may protect the dsRNA from Dicer cleavage and allow GCRV to escape the RNAi pathway. This hypothesis is supported by our finding that dsRNA bound by TIA1 was insensitive to degradation in vivo (Fig. 6).

4.3. GCRV benefits from CiTIA1 accumulation

SGs are dynamic, and the components are rapidly shuttled into and out of the complexes, so that changing stress (including virus stress) conditions can be dealt with rapidly. Many viruses appear to have mechanisms that disrupt the formation of SGs on viral mRNAs, suggesting that SGs are a host defense against infection. SGs have been shown to be induced by a number of viral infections and have been implicated in the cellular defense against infection [4,19]. For example, in Semliki Forest virus infection, SG formation is inhibited when eIF3B is bound and sequestered by the C-terminal domain of the viral nonstructural protein 3 [37]. Recent findings from studies of West Nile and Dengue viruses show that flaviviruses subvert TIA1 and TIA1 to interfere with SG formation, aid in viral gene amplification, and to prevent translation inhibition of both viral and cellular mRNAs [38]. While there is no experimental evidence that new foci formed by TIA1 contain other SG marker proteins, they may share certain functions. Our findings that GCRV unlinked TIA1 from SG formation and subverted TIA1 to enhance viral replication were consistent with the flavivirus studies.

4.4. Conclusions and unresolved questions for future research

Many viruses avoid and subvert subcellular defense structures to facilitate their amplification. Important questions about the role of CiTIA1 accumulation and SG formation remain to be answered. Some of the key questions going forward will be: (1) what factor(s) is blocking SG assembly during GCRV infection, (2) why viral replication is unaffected when CiTIA1 is sequestering viral and host RNA, and (3) what is the mechanism that induces the upregulation of CiTIA1 and downregulation of cG3BP1.

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