Fish and Shellfish Immunology 86 (2019) 355–360

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

Characterization of the interaction between outer-fiber protein VP55 of genotype III grass carp reovirus and Fibulin-4 of grass carp

Longlong Wang[a,b,1], Fei Yu[a,b,1], Hao Sun[a,b], Liqun Lu[a,b,c,*]

[a] National Pathogen Collection Center for Aquatic Animals, Shanghai Ocean University, Shanghai, PR China
[b] Key Laboratory of Agriculture Ministry for Freshwater Aquatic Genetic Resources, Shanghai Ocean University, Shanghai, PR China
[c] National Experimental Teaching Demonstration Center for Fishery Sciences, Shanghai Ocean University, Shanghai, PR China

ARTICLE INFO

Keywords:
Grass carp reovirus
Fibulin-4
Outer-fiber protein
Virus-host interaction

ABSTRACT

Genotype III grass carp reovirus (GCRV; representative strain, GCRV-104) belongs to the subfamily Spinareovirinae and encodes an outer-fiber protein, VP55, responsible for mediating the infection of target tissues by the virus and assisting the virus into cells. Fibulin-4/EFEMP2 protein was previously identified as a putative binding partner for VP55 in a yeast two-hybrid screening. Here, we have further characterized the association between Fibulin-4 and VP55 by using protein interaction assays. An intracellular co-localization assay showed that RFP-Fibulin-4 co-localized with GFP-VP55 in grass carp ovary (GCO) cells. Bacterially expressed GST-tagged Fibulin-4 was shown to associate with baculovirus-expressed His-tagged VP55 in a dot-blot overlay assay; moreover, baculovirus-expressed His-tagged VP55 was able to pull down GFP-Fibulin-4 expressed in the GCO cells. We performed real-time PCR and immunoblotting analysis and showed that endogenous Fibulin-4, although suppressed to a lower level in the late infection phase, is present throughout the infection course of GCRV-104 in CIK cells. In conclusion, our results indicate that grass carp Fibulin-4 interacts with VP55. The presence of Fibulin-4, a well-known secreted protein, during the infection course of GCRV-104 in grass carp cells implies its potential role during viral egression through interaction with VP55.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) reovirus (GCRV) is the causative agent of a hemorrhagic disease that results in high mortality and huge economic losses in the largest aquaculture industry in China, which yields 4 million tons of grass carp per year [1–3]. Unlike its mammalian counterparts and most other aquareoviruses, GCRV is believed to be highly host-specific [4,5]. However, there is much debate on this specificity because novel genetically distant viral strains have caused pandemics [3,6–8]. Three different GCRV types have been identified on the basis of genomic differences: type I, identified in the 1980s, is represented by the type strain GCRV-873; type II, identified in 2012, is represented by the strain GCRV-HZ08; and type III, identified in 2013, is represented by the strain GCRV-104 [9]. In 2013, We detected the co-infection of two grass carp reoviruses for the first time [10]. The genome of the well-characterized type I GCRV contains 11 double-stranded RNA segments, which encode seven structural proteins (VP1–VP7) and five non-structural proteins (NS16, NS26, NS31, NS38, and NS80) [11], and shows very low homogeneity with type III GCRV, which belongs to Spinareovirinae and has large spikes, or turrets, on the outer capsid [9].

Viruses encode envelope proteins or outer capsid proteins for cell attachment, which is the first step of the infection. The outer capsid proteins of GCRV are responsible for mediating the infection of target tissues and assisting the virus into cells. VP7 and VP5 are the outer capsid proteins of genotype-I GCRV. Our previous study demonstrated that they interact with each other *in vivo* and *in vitro* [12]. The GCRV virion contains 200 trimers of VP5-VP7 heterodimers [13]. Complete removal of VP7 alone from the outer shell by limited protease treatment resulted in the significant enhancement of viral infectivity [14], which suggests that VP5, not VP7, may be necessary for cell attachment or receptor binding by GCRV. In our previous study, we had performed both virus attachment and infection inhibition assays and observed that grass carp LamR is involved in GCRV infection [15]. Analysis of diseased grass carp samples collected from different regions of China showed that the type II reovirus of grass carp may be a representative prevalent strain in southern China [16]. The outer-fiber protein VP56, the outermost protein on the virion of the type II reovirus, has been

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

https://doi.org/10.1016/j.fsi.2018.11.067
Received 14 October 2018; Received in revised form 22 November 2018; Accepted 27 November 2018
Available online 28 November 2018
1050-4648/

suggested to play a key role during viral entry and is involved in cell attachment [17]. VP55, the outer-fiber protein of type III GCRV, is considered to be the homologue of VP56 [9]. However, there is limited information on the role of VP55 during the viral infection.

We had used a yeast two-hybrid system to identify putative binding partner(s) of GCRV-104 VP55 in a grass carp cDNA library and detected EGF (Epidermal Growth Factor)-containing fibulin-like extracellular matrix protein 2 (EFEMP2, also names as Fibulin-4) as a potential interacting protein for VP55 [18]. However, further biochemical and molecular characterization of the interaction is necessary. The open reading frame (ORF) of the *fibulin*-4 gene in grass carp encodes 440 amino acids. The protein consists of (in order) an N-terminal signal peptide, six calcium-binding epidermal growth factor modules in a tandem arrangement, and one C-terminal fibulin-like domain [19]. Transcriptional distribution analysis of Fibulin-4 in various tissues of healthy grass carp showed that it was highly expressed in the muscle, moderately expressed in the intestine and brain, and slightly expressed in the other examined tissues. Interestingly, the expression pattern was consistent with the tissue tropism of GCRV [18].

In humans, tissue fibrosis, a life-threatening autoimmune disease, is related to the fibulin family of proteins [20]. Fibulin-4 in particular is associated with elastic fiber formation and connective tissue development, and it is an essential component of the extracellular matrix [21,22]. Katsanis et al. performed northern blot analysis and observed the expression of a 2-kb EFEMP2 (Fibulin-4) transcript in all adult human tissues, with the highest levels in the heart [23]. Likewise, EFEMP1 is expressed in a wide range of adult and fetal tissues. In contrast to EFEMP1, EFEMP2 (Fibulin-4) was not significantly overexpressed in senescent or quiescent human fibroblasts, suggesting this EGF-like domain subfamily has diverse functions. EFEMP2 (Fibulin-4) interacts with the tumor protein p53 through these domains, which mediate protein–protein interactions [24]. Solid-phase binding assays have shown that these proteins bind differentially to extracellular proteins. Mouse Fibulin-4 was observed to bind to collagen XIV, nidogen-2, tropoelastin, and collagen XV-derived endostatin [25]. In this study, we performed protein interaction assays to characterize the association between Fibulin-4 and VP55. Molecular characterization of the putative interaction between Fibulin-4 and VP55 will pave the way for further insights into the consequences of GCRV/Fibulin-4 interactions during hemorrhagic disease in grass carp.

2. Materials and methods

2.1. Cells and virus

Genotype III GCRV-104 strain was obtained from the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences. Grass carp ovary (GCO) and kidney (CIK) cells were grown at 28 °C in M199 medium with 10% fetal bovine serum (FBS; Gibco, USA). Insect cells (SF9 cells) were cultured at 28 °C in Sf-900™ II SFM medium (Gibco, USA).

2.2. Plasmid construction

pEGFP-N1-Fibulin-4 and pGEX-4T-3-Fibulin-4 were constructed in previous study [26]. For Bac-to-Bac baculovirus expression of His-tagged recombinant VP55 protein, the *vp*55 ORF was amplified from GCRV-104-infected CIK cells by using RT-PCR with the primer pair CCGGAATTCATGGACGATCAAGCGCTCGCAAAC and CCCAAGCTTGG AGTGACAGGCCGCCACCATGAC, inserted into the pFastbacHTA vector to construct pFastbacHTA-VP55. The *vp*55 ORF was also amplified with the primer pair CCGGAATTCCGATGGACGATCAAGCGCTCG and CGC GGATCCCGGCAAGTGACAGGCCGCCAC and inserted into the pEGFP-N1 vector to construct pEGFP-N1-VP55. The coding region of *fibulin*-4 was amplified from CIK cells by using RT-PCR with the primer pair CCGCTCGAGATGCGGCCCGGGTGTGTTTTGGTCT and CCGGAATTCG GTAAAAAGCATAAGGCCCAACGTAG and inserted into the pDsRed-N1 vector to construct pDsRed-N1-Fibulin-4. All enzymes were purchased from TaKaRa, Japan, and each plasmid was confirmed by sequencing at Sangon, China.

2.3. Bac-to-Bac baculovirus expression of the His-VP55 protein

Recombinant baculoviruses were generated according to the protocol of the Bac-to-Bac baculovirus expression system (Thermo Fisher Scientific, USA). The His-VP55 protein was expressed from SF9 cells infected with the recombinant baculovirus stocks, according to the provided manual. Recombinant proteins were purified using Hislink™ Protein Purification Resin (Promega, USA), according to the manufacturer's protocol. The expressed protein was further analyzed by immunoblotting analysis with an anti-His monoclonal antibody (1:3000; Abmart, China).

2.4. Dot-blot overlay assay

The dot-blot overlay analysis was performed according to a previously described method [27,28]. The GST and GST-Fibulin-4 proteins were expressed and purified according to a previous study [29] and confirmed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, different concentrations (20 μL) of GST-Fibulin-4 or GST were blotted onto a 0.45-μm polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Germany) in a dot-blot apparatus (Bio-Rad, USA), and the blots were blocked with purified His-VP55 overnight at 4 °C. The samples were then blocked with blotting buffer (140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Tween 20, and 5% non-fat milk) for 2 h at room temperature (RT). The membranes were rinsed in phosphate-buffered saline (PBS) and then incubated with anti-His monoclonal antibody (1:3000; Abmart, China) for 2 h at RT. The blots were rinsed three times with PBST and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:3000; Abmart, USA) for 1 h at 37 °C. The blots were developed using Tanon™ High-sig ECL Western Blotting Substrate (Tanon, China).

2.5. His-pulldown assay

For the pulldown assay, GCO cells were grown to approximately 80–90% confluence in T25 flasks and then transfected with 5 μg of pEGFP-N1-Fibulin-4 by using Lipofectamine 3000 (Invitrogen, USA). About 36 h post-transfection, the cells were washed twice with PBS and collected in NP-40 lysis buffer (Beyotime, China); then, according to the Pierce™ His Protein Interaction Pull-Down Kit (Thermo Fisher Scientific, USA) protocol, prey protein from the GCO cell lysate was incubated with immobilized bait protein from the baculovirus-infected insect cell lysate at 4 °C for at least 3 h. The bait-prey elution was analyzed using 10% SDS-PAGE, and western blotting was performed using monoclonal antibodies specific for the GFP tag. The presence of GFP, GFP-Fibulin-4, and His-VP55 were confirmed in the input cell lysates by immunoblotting with the anti-Fibulin-4 polyclonal antibody (1:3000) and anti-His monoclonal antibody (1:3000; Abmart, China). The final eluate was also analyzed by immunoblotting with anti-His monoclonal antibody (Abmart, China).

2.6. Intracellular co-localization

To determine the intracellular location of Fibulin-4 and VP55, GCO cells were seeded into six-well cell culture plates for about 4–8 h (with an 80–90% confluent monolayer) by using M199 medium containing 10% FBS for transfection. About 5×10^6 GCO cells were co-transfected with 2.5 μg of pEGFP-N1-VP55 and 2.5 μg of pDsRed-N1-Fibulin-4 by using Lipofectamine 3000 (Invitrogen, USA), according to the manufacturer's protocol. After 36 h, the expressed positions of Fibulin-4 and VP55 protein were detected. The GCO cells which co-transfected with

plasmids pEGFP-N1 and pDsRed-N1-Fibulin-4 served as negative control. The expressed VP55 and Fibulin-4 proteins were further analyzed using specific monoclonal antibodies against RFP tag or GRP tag, as described above (Abmart, China). Fluorescence microscopy images were acquired using an OLYMPUS IX71 upright microscope (Olympus, Japan). The nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole).

2.7. Virus infection and immunoblotting assay

To determine the transcription and translation levels of Fibulin-4 during the GCRV infection, CIK cells were seeded into 12-well cell culture plates overnight (with an > 90% confluent monolayer) by using M199 medium containing 10% FBS. The cells were infected with GCRV-104 at MOI = 1. Samples were collected at 0, 12, 24, 36, 48, 60, 72, and 84 h post-infection with 50 μL of 2 × SDS-PAGE Sample Loading Buffer (Beyotime, China) or 1 mL of TRIzol™ Reagent (Invitrogen, USA).

The immunoblotting assay was performed as described previously [6]. Briefly, the protein samples were resolved with 10% SDS-PAGE and then transferred onto 0.45-μm PVDF membranes (Merck Millipore, Germany); the membranes were blocked for 2 h at RT in 5% non-fat milk dissolved in PBST. Primary (anti-VP3, 1:3000; anti-Fibulin-4, 1:5000; produced in the lab) and secondary (HRP-conjugated anti-mouse IgG, 1:5000; Abmart, China) antibodies were used to probe the target protein. Expression of GAPDH (anti-GAPDH, 1:4000; Abclonal, China) was used as the internal control. VP3 (anti-VP3, 1:4000; produced in the lab), the core shell protein of GCRV-104, served as the infection indicator. Images were visualized using Tanon™ High-sig ECL Western Blotting Substrate (Tanon, China).

2.8. Real-time RT-PCR

The total RNA was extracted using TRIzol Reagent (Invitrogen, USA). Using the PrimeScript™ RT Master Mix (TaKaRa, Japan), 200 ng of total RNA was reverse-transcribed into cDNA in a 20-μL reaction volume, according to the manufacturer's protocol. The expression levels of Fibulin-4 in the CIK cells were measured in triplicate by using real-time RT-PCR and normalized to the 18S rRNA level [30]. Quantitative PCR was performed in triplicate by using the CFX96 real-time PCR system (Bio-Rad, USA) and SYBR®Premix Ex Taq II (TaKaRa, Japan). The reaction conditions were as follows: 95 °C for 10 min, followed by 41 cycles at 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 20 s [18]. The amplification efficiencies of the reference and target genes were similar, reaching 100%. The relative expression levels of the target gene were calculated by using the $2^{-\triangle\triangle CT}$ method. Data analysis was performed using Microsoft Excel software and significant differences (T-test, double tails, paired samples) were considered at $p < 0.05$ and highly significant at $p < 0.01$.

3. Results

3.1. Grass carp Fibulin-4 binds to the outer-fiber protein VP55 in vitro

Previously, Fibulin-4 was identified as a potential interacting protein for VP55 by using a yeast two-hybrid screening system. To characterize the interaction between Fibulin-4 and VP55, GST and GST-Fibulin-4 were purified from *E. coli* (Fig. 1A), and the soluble His-tagged VP55 was expressed and purified from SF9 cells infected with the recombinant baculovirus (Fig. 1B). The dot-blot overlay assay indicated that the affinity-purified His-tagged VP55 proteins were bound to purified GST-tagged Fibulin-4, with a trend suggesting that the binding was dose-dependent (Fig. 1C). In contrast, GST, which served as the negative control, did not bind to VP55.

Taking advantage of eukaryotically expressed recombinant proteins, the association between rFibulin-4 and rVP55 was determined with the His-pulldown assay. Expression of GFP and GFP-Fibulin-4 by GCO cells was confirmed with fluorescence microscopy (Fig. 2A) and immunoblotting (Fig. 2B and C). The presence of GFP, GFP-Fibulin-4, and His-VP55 were confirmed in the input cell lysates by using immunoblotting with anti-Fibulin-4 polyclonal and anti-His monoclonal antibodies (Fig. 2D). The anti-GFP antibody was used to detect bait-prey elution. The association of His-VP55 with GFP-Fibulin-4, but not GFP, was revealed using western blot analysis of the bait-prey elution with anti-GFP monoclonal antibody (Fig. 2D). The His-pulldown assay provided confirmation of a physical interaction between Fibulin-4 and VP55.

3.2. Co-localization of Fibulin-4 with VP55

To investigate the sub-cellular location of Fibulin-4 with GCRV-104 VP55, we co-transfected GCO cells with the recombinant plasmids pEGFP-VP55 and pDsRed-N1-Fibulin-4. After 36 h, immunofluorescence was used to observe the expressed position of Fibulin-4 with VP55. Then, the GCO cells were stained with DAPI and observed with a fluorescence microscope. The RPF-fused Fibulin-4 protein (red) was expressed ubiquitously, and the GFP-fused VP55 protein (green) was patchily distributed in the cytoplasm (Fig. 3A). Western blot analysis was conducted using the cell samples transfected with pEGFP-VP55 and pDsRed-N1-Fibulin-4. The results showed that Fibulin-4 and VP55 were indeed expressed in the GCO cells (Fig. 3B), indicating that both Fibulin-4 and VP55 were expressed in the cytoplasm and RFP-Fibulin-4 co-localized with GFP-VP55 in the GCO cells.

3.3. GCRV induces the expressional suppression of Fibulin-4 in host cells

We used a polyclonal antiserum (produced in the lab) against grass carp Fibulin-4 to monitor its translational expression pattern in response to GCRV infection. Samples were collected at different time points (Fig. 4A), and the primary (anti-Fibulin-4) and secondary antibodies were used to probe the target protein. The translational expression levels of Fibulin-4 were significantly inhibited after the onset of the virus infection until reaching the minimum level at 60 h post-infection, which was consistent with its transcriptional expression pattern after the viral challenge (Fig. 4A and B).

4. Discussion

In this study, we provided molecular and biochemical evidences that the outer-fiber protein VP55 of type III GCRV interacted with the grass carp Fibulin-4 protein. Using prokaryotically expressed GST-Fibulin-4 and baculovirus-expressed His-VP55, we demonstrated the dose-dependent binding of VP55 to Fibulin-4 with the dot-blot overlay assay. Baculovirus-expressed His-VP55 also pulled down GFP-Fibulin-4 from the cell lysate. Ubc9, a putative GCRV-104 VP55 binding partner identified using yeast two-hybrid screening, interacted with VP55, and the N-terminal coiled-coil domain of VP55, containing a single lysine residue, was responsible for the interaction between VP55 and Ubc9 in yeast [18,31]. Furthermore, Ubc9 was shown to bind to outer-fiber proteins from type II GCRV, avian reovirus, and mammalian reovirus in yeast [31]. Thus, multiple interacting partners exist for VP55. In this study, VP55 did co-localize with Fibulin-4; however, Fibulin-4 was distributed in a wider region than VP55 in the cytoplasm and should have the capacity for interactions with more partners.

The name fibulin originates from the Latin word *fibula*, which means clasp or buckle, and fibulins have a diverse array of protein ligands [32]. The importance of fibulins in development and disease has been highlighted by gene-targeting experiments in animal models and identification of spontaneous mutations in humans. In mice, Fibulin-1 deficiency causes extensive hemorrhaging and perinatal death [33]. In humans, Fibulin-4 mutations result in cutis laxa characterized by inelastic and saggy skin, vascular tortuosity, increased risk of ascending aortic aneurysms, emphysema, and other clinical symptoms [34].

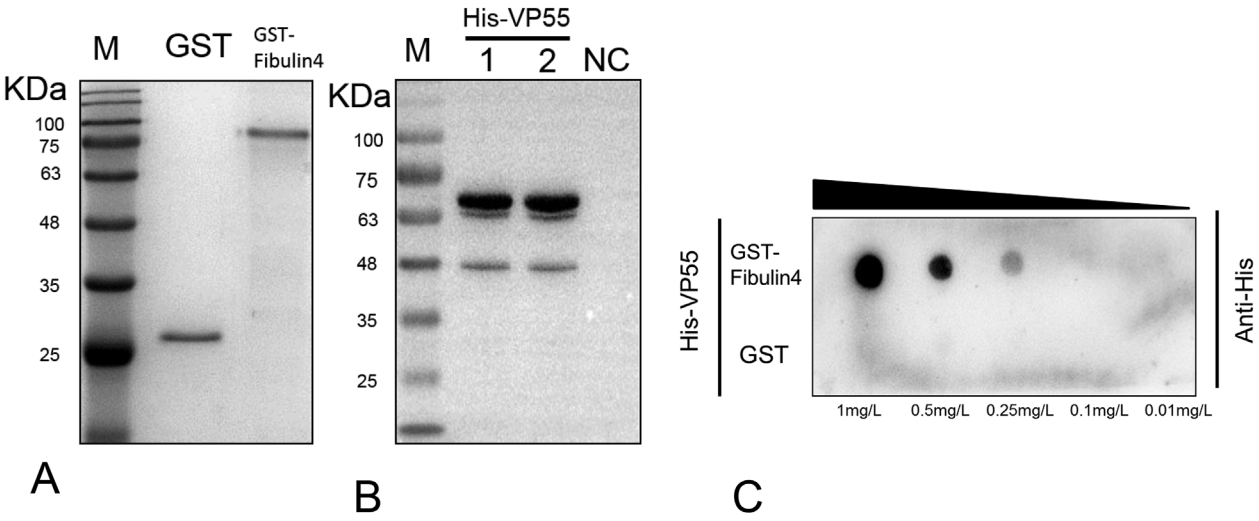

Fig. 1. Dot-blot overlay assay shows that VP55 binds to Fibulin-4. A: Purified GST and GST-Fibulin-4 recombinant proteins were analyzed using SDS-PAGE. B: Baculovirus-expressed His-tagged VP55 was analyzed using immunoblotting with anti-His monoclonal antibody. His-VP55 1 & 2 were the same samples; non-infected SF9 cells were used as the negative control (NC). C: Dot-blot overlay analysis of Fibulin-4 and VP55 interactions. Various amounts of purified GST-Fibulin-4 or GST were immobilized on a PVDF membrane and incubated with baculovirus-expressed VP55. Positive signals were detected with anti-His monoclonal antibody.

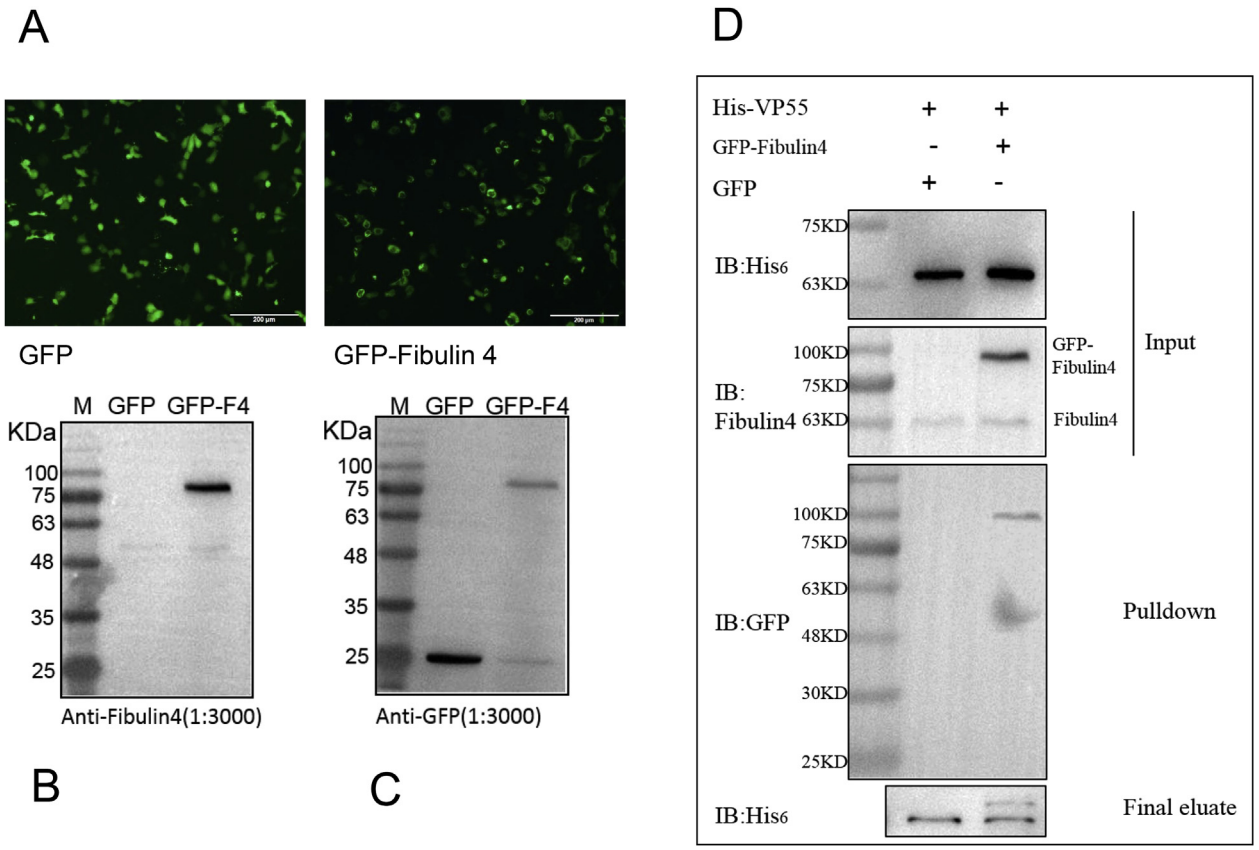

Fig. 2. Interaction of Fibulin-4 with VP55 in His-pulldown assays. A: Overexpression of GFP-Fibulin-4 (right) and GFP (left) was confirmed using fluorescence microscopy and immunoblotting analysis with anti-Fibulin-4 antibody (B) and anti-GFP monoclonal antibody (C). D: His-VP55 efficiently pulled down GFP-Fibulin-4 *in vitro*. The input cell lysates were confirmed using immunoblotting with an anti-Fibulin-4 polyclonal antibody and anti-His monoclonal antibody. The bait-prey elution was analyzed using immunoblotting with anti-GFP monoclonal antibody. The final eluted samples were also analyzed using immunoblotting with anti-His monoclonal antibody.

Overexpression of Fibulin-4 in macrophages promotes DNA synthesis; Fibulin-4 expression is augmented in macrophages by lipopolysaccharide treatment, which suggests a role in the response to sepsis [34]. Solid-phase binding assays have detected strong calcium-dependent binding of short fibulins to immobilized heparin, suggesting that these fibulins (Fibulin-3, Fibulin-4, and Fibulin-5) may bind to heparan sulfate located on the cell surface [35]. Our study confirmed the interaction between grass carp Fibulin-4 and GCRV outer-fiber protein VP55, and the significance of this interaction could be reasonably speculated to be helpful in facilitating GCRV to attach to target tissue

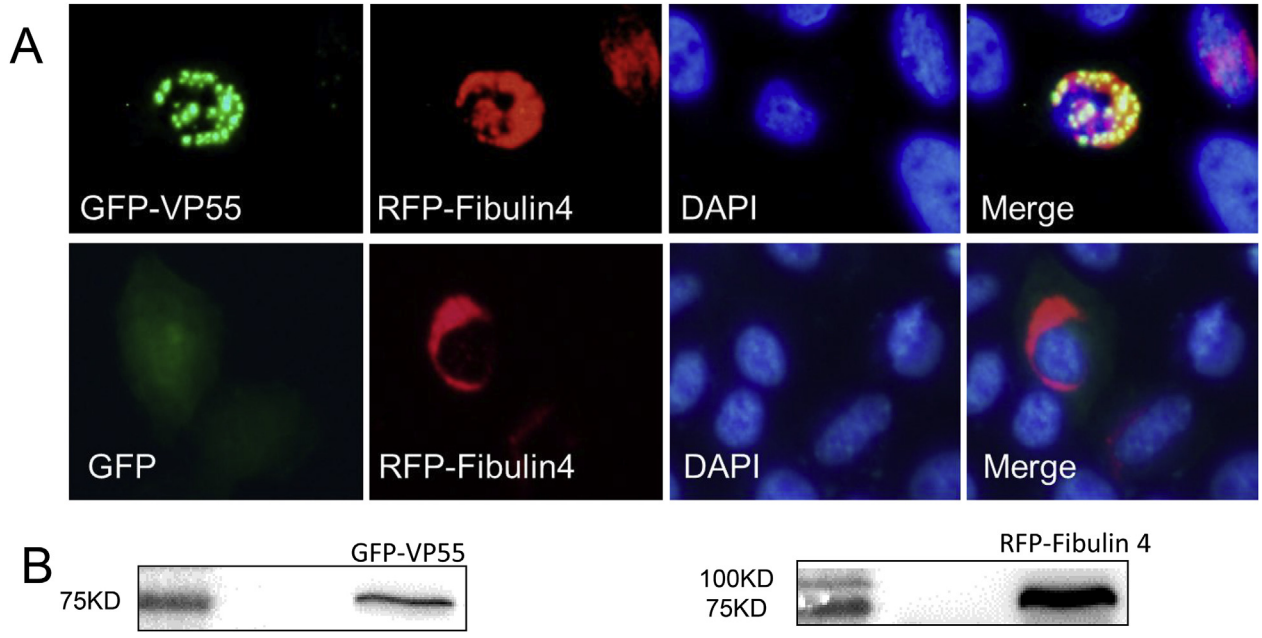

Fig. 3. Co-localization of RFP-Fibulin-4 and GFP-VP55 in GCO cells. A: Sub-cellular localization of recombinant VP55 (green) and Fibulin-4 (red). Cells were co-transfected with the plasmids pEGFP-N1-VP55 and pDsRed-N1-Fibulin-4. The cellular nucleus was stained with DAPI, and the images were merged together. The GCO cells which co-transfected with plasmids pEGFP-N1 and pDsRed-N1-Fibulin-4 served as negative control. B: The sample of plasmid-transfected cells was analyzed using immunoblotting with anti-GFP and anti-RFP antibodies. GCO cells served as the negative control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

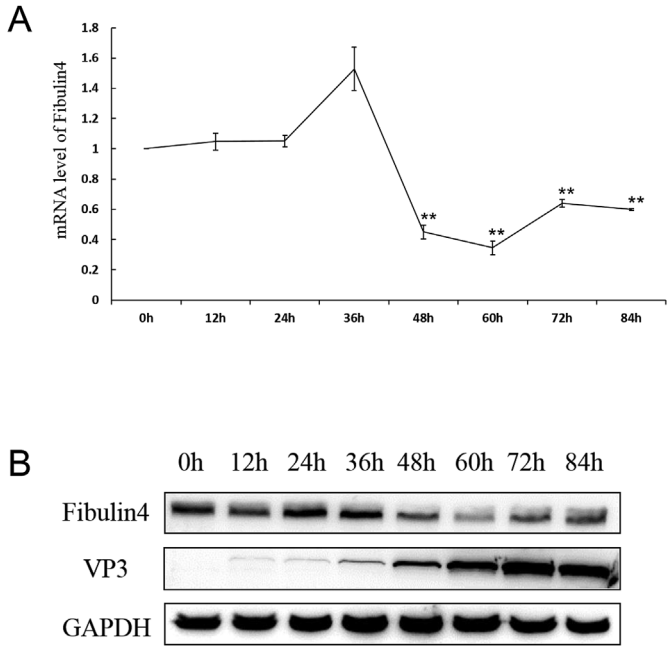

Fig. 4. Kinetics of Fibulin-4 expression in infected CIK cells. A: The CIK cells were infected by GCRV-104 at MOI of 1, and the relative mRNA levels of Fibulin-4 were determined using real-time RT-PCR and normalized to 18S rRNA levels and calculated against the expression of the 0-h group with the $2^{-\triangle\triangle Ct}$ method. Significant differences (compare to 0-h group) were considered at * $p < 0.05$ and highly significant at **$p < 0.01$. The RNA samples were collected at the indicated time. B: The CIK cells were infected by GCRV-104 at MOI of 1, and the protein samples were analyzed using immunoblotting (Fibulin-4, VP3, and GAPDH antibodies). The core shell protein VP3 served as the GCRV-104 infection indicator, and GAPDH acted as the internal reference protein. The protein samples were collected at the indicated time.

and cell membranes at the initial phase of infection. During GCRV-JX01 infection, the expression of grass carp Fibulin-4 in CIK cells was slightly reduced, but present, throughout the infection course [26]. Because of the secreting nature of synthesized Fibulin-4 in grass carp cells, interaction with GCRV virion through VP55 might also enable more efficient egression of GCRV particles from infected cells during late infection. It is unclear whether the Fibulin-4/VP55 interaction interferes with the host innate immune response.

The findings of this study would improve our understanding of both the GCRV replication strategy and Fibulin-4-mediated cellular response to viral stress. Fibulin-4 may be an ideal target for developing broad-spectrum drug targets for different genotypes of GCRV.

Acknowledgements

The funding for this study was provided by the National Natural Science Foundation of China (No. 31672690) to L. Lu.

References

[1] Q. Fang, L.H. Ke, Growth characteristics and high titer culture of grass carp hemorrhage virus (GCHV)-873 in vitro, Virol. Sin. 04 (3) (1989) 315–319 http://virosin.org/Paper/1989/04/03/VS19890403.0315/.
[2] Y. Jiang, W. Ahne, Some properties of the etiological agent of the hemorrhagic disease of grass carp and black carp, in: W. Ahne, E. Kurstak (Eds.), Viruses of Lower Vertebrates, Springer, Berlin, Heidelberg, 1989, https://doi.org/10.1007/978-3-642-83727-2_20.
[3] Y. Fan, S. Rao, L. Zeng, et al., Identification and genomic characterization of a novel fish reovirus, Hubei grass carp disease reovirus, isolated in 2009 in China, J. Gen. Virol. 94 (10) (2013) 2266–2277 https://doi.org/10.1099/vir.0.054767-0.
[4] L. Yan, G. Hong, X. Sun, et al., Characterization of grass carp reovirus minor core protein VP4, Virol. J. 9 (1) (2012) 1–7 https://doi.org/10.1186/1743-422X-9-89.
[5] A.A. Rangel, D.D. Rockemann, F.M. Hetrick, et al., Identification of grass carp haemorrhage virus as a new genogroup of aquareovirus, J. Gen. Virol. 80 (1) (1999) 2399 Pt 9 https://doi.org/10.1099/0022-1317-80-9-2399.
[6] F. Yu, L. Wang, H. Wang, et al., Repression of SUMOylation pathway by grass carp reovirus contributes to the upregulation of PKR in an IFN-independent manner, Oncotarget 8 (42) (2017) 71500–71511 https://doi.org/10.18632/oncotarget.20309.
[7] C. Pei, F. Ke, Z.Y. Chen, et al., Complete genome sequence and comparative analysis of grass carp reovirus strain 109 (GCReV-109) with other grass carp reovirus strains

reveals no significant correlation with regional distribution, Arch. Virol. 159 (9) (2014) 2435–2440 https://doi.org/10.1007/s00705-014-2007-5.
[8] X. Ye, Y.Y. Tian, G.C. Deng, et al., Complete genomic sequence of a reovirus isolated from grass carp in China, Virus Res. 163 (1) (2012) 275–283 https://doi.org/10.1016/j.virusres.2011.10.014.
[9] M.L. Nibert, R. Duncan, Bioinformatics of recent aqua- and orthoreovirus isolates from fish: evolutionary gain or loss of FAST and fiber proteins and taxonomic implications, Plos One 8 (7) (2013) e68607https://doi.org/10.1371/journal.pone.0068607.
[10] T. Wang, J. Li, L. Lu, Quantitative in vivo and in vitro characterization of co-infection by two genetically distant grass carp reoviruses, J. Gen. Virol. 94 (Pt 6) (2013) 1301 https://doi.org/10.1099/vir.0.049965-0.
[11] J.F. Mohd, A.E. Goodwin, M. Belhouchet, et al., Complete characterisation of the American grass carp reovirus genome (genus Aquareovirus: family Reoviridae) reveals an evolutionary link between aquareoviruses and coltiviruses, J. Virol. 373 (2) (2008) 310 https://doi.org/10.1016/j.virol.2007.12.006.
[12] W. Liu, H. Wang, F. Yu, et al., Grass carp reovirus outer capsid proteins VP5 and VP7 interact in vitro, Archives of Virology 162 (8) (2017) 2375–2380 https://doi.org/10.1007/s00705-017-3354-9.
[13] L. Cheng, Q. Fang, S. Shah, et al., Subnanometer-resolution structures of the grass carp reovirus core and virion, J. Mol. Biol. 382 (1) (2008) 213 https://doi.org/10.1016/j.jmb.2008.06.075.
[14] Q. Fang, E.K. Seng, Q.Q. Ding, et al., Characterization of infectious particles of grass carp reovirus by treatment with proteases, Archives of Virology 153 (4) (2008) 675–682 https://doi.org/10.1007/s00705-008-0048-3.
[15] H. Wang, F. Yu, J. Li, et al., Laminin receptor is an interacting partner for viral outer capsid protein VP5 in grass carp reovirus infection, Virology 490 (2016) 59 https://doi.org/10.1016/j.virol.2016.01.011.
[16] W. Zeng, Q. Wang, Y. Wang, et al., Establishment of multiplex PCR for detection of grass carp reovirus and its application, J. Fish. Sci. China 20 (2) (2013) 419–426 https://doi.org/10.3724/SP.J.1118.2013.00419.
[17] Y. Tian, Z. Jiao, J. Dong, et al., Grass carp reovirus-GD108 fiber protein is involved in cell attachment, Virus Gene. 53 (4) (2017) 1–10 https://doi.org/10.1007/s11262-017-1467-6.
[18] F. Yu, H. Wang, W. Liu, et al., Grass carp Ctenopharyngodon idella Fibulin-4 as a potential interacting partner for grass carp reovirus outer capsid proteins, Fish Shellfish Immunol. 48 (2016) 169 https://doi.org/10.1016/j.fsi.2015.11.029.
[19] S.D. Vega, T. Iwamoto, Y. Yamada, Fibulins: multiple roles in matrix structures and tissue functions, Cell. Mol. Life Sci. 66 (11–12) (2009) 1890 https://doi.org/10.1007/s00018-009-8632-6.
[20] T. Krieg, D. Abraham, R. Lafyatis, Fibrosis in connective tissue disease: the role of the myofibroblast and fibroblast-epithelial cell interactions, Arthritis Res. Ther. 9 (S2) (2007) S4 https://doi.org/10.1186/ar2188.
[21] C.L. Papke, H. Yanagisawa, Fibulin-4 and Fibulin-5 in elastogenesis and beyond: insights from mouse and human studies, Matrix Biology Journal of the International Society for Matrix Biology 37 (2014) 142 https://doi.org/10.1016/j.matbio.2014.02.004.
[22] M. Horiguchi, T. Inoue, T. Ohbayashi, et al., Fibulin-4 conducts proper elastogenesis via interaction with cross-linking enzyme lysyl oxidase, Proc. Natl. Acad. Sci. U. S. A. 106 (45) (2009) 19029–19034 https://doi.org/10.1073/pnas.0908268106.
[23] N. Katsanis, S. Venable, J.R. Smith, et al., Isolation of a paralog of the Doyne honeycomb retinal dystrophy gene from the multiple retinopathy critical region on 11q13, J. Hum. Genet. 106 (1) (2000) 66–72 https://doi.org/10.1007/s004390051011.
[24] M. Argentini, MBP1: a novel mutant p53-specific protein partner with oncogenic properties, Oncogene 18 (24) (1999) 3608–3616 https://doi.org/10.1038/sj.onc.1202937.
[25] N. Kobayashi, G. Kostka, J.H. Garbe, et al., A comparative analysis of the fibulin protein family. Biochemical characterization, binding interactions, and tissue localization, J. Biol. Chem. 282 (16) (2007) 11805–11816 https://doi.org/10.1074/jbc.M611029200.
[26] J. Sheng, F. Yu, D. Chen, et al., Infection of grass carp reovirus induced the expressional suppression of pro-viral Fibulin-4 in host cells, Fish Shellfish Immunol. (2018) 294–297 https://doi.org/10.1016/j.fsi.2018.04.014.
[27] Y. He, H. Xu, Q. Yang, et al., The use of an in vitro microneutralization assay to evaluate the potential of recombinant VP5 protein as an antigen for vaccinating against Grass carp reovirus, Virol. J. 8 (1) (2011) 1–6 https://doi.org/10.1186/1743-422X-8-132.
[28] J. Wojtera-Kwiczor, F. Groß, H.M. Leffers, et al., Transfer of a redox-signal through the cytosol by redox-dependent microcompartmentation of glycolytic enzymes at mitochondria and actin cytoskeleton, Front. Plant Sci. 3 (1) (2012) 284 https://doi.org/10.3389/fpls.2012.00284.
[29] M. Zhang, J. Sheng, F. Yu, et al., Studies on the interaction of grass carp fibulin-4 protein with grass carp reovious outer capsid proteins, Chin. J. Virol. 34 (4) (2011) 557 https://doi.org/10.13242/j.cnki.bingduxuebao.003423.
[30] J. Su, R. Zhang, J. Dong, et al., Evaluation of internal control genes for qRT-PCR normalization in tissues and cell culture for antiviral studies of grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immunol. 30 (3) (2011) 830–835 https://doi.org/10.1016/j.fsi.2011.01.006.
[31] F. Yu, H. Wang, L. Wang, et al., Orthoreovirus outer-fiber proteins are substrates for SUMO-conjugating enzyme Ubc9, Oncotarget 7 (48) (2016) 79814 https://doi.org/10.18632/oncotarget.12973.
[32] S.D. Vega, T. Iwamoto, T. Nakamura, et al., TM14 is a new member of the fibulin family (Fibulin-7) that interacts with extracellular matrix molecules and is active for cell binding, J. Biol. Chem. 282 (42) (2007) 30878–30888 https://doi.org/10.1074/jbc.M705847200.
[33] G. Kostka, R. Giltay, W. Bloch, et al., Perinatal lethality and endothelial cell abnormalities in several vessel compartments of fibulin-1-deficient mice, Mol. Cell Biol. 21 (20) (2001) 7025 https://doi.org/10.1128/MCB.21.20.7025-7034.2001.
[34] J. Djokic, C. Fagottokaufmann, R. Bartels, et al., Fibulin-3, -4, and -5 are highly susceptible to proteolysis, interact with cells and heparin, and form multimers, J. Biol. Chem. 288 (31) (2013) 22821 https://doi.org/10.1074/jbc.M112.439158.
[35] H. Heine, R.L. Delude, B.G. Monks, et al., Bacterial lipopolysaccharide induces expression of the stress response genes hop and H411, J. Biol. Chem. 274 (30) (1999) 21049–21055 https://www.jbc.org/content/274/30/21049.long.