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Targeting Heat Shock Protein 70 as an antiviral strategy against grass carp reovirus infection

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ABSTRACT

Grass carp (*Ctenopharyngodon idella*) hemorrhagic disease, caused by grass carp reovirus (GCRV), has been a serious problem in grass carp aquaculture for several decades. Characterization of the primary host factors associated with host-virus interaction is critical for understanding how a virus infects its host cell and these host factors can be antiviral targets. This study aimed to screen host factors that interacted with GCRV in the *C. idella* kidney (CIK) cells and used them as antiviral targets. Twelve proteins were identified by virus overlay protein binding assay and LC-MS-MS. Among these twelve proteins, Heat Shock Protein 70 (HSP70) was outstanding. Results of flow cytometry and immunofluorescence assay indicated that HSP70 was on the cell membrane. HSP70 was expressed at low levels preceding GCRV infection, but its expression was induced upon GCRV infection. Inhibition of HSP70's function by inhibitors (VER155008 and pifithrin-μ) maintained HSP70 on the cell surface in infected cells, however GCRV quantity was decreased in the CIK cells (compared with the control group, the maximum inhibition rate of the treatment group was close to 85%), suggesting that fully functional HSP70 was required for GCRV infection. Moreover, GCRV showed a dose dependent reduction by inhibiting the entry stage of the viral life cycle following treated with VER155008 and pifithrin-μ. VER + PIF (1:1) were used at 15 μM and the expression of GCRV-VP6 downregulated nearly to 90%, which revealed that HSP70 played an important role in GCRV entering into CIK cells. This work speculated that HSP70 might be a host factor in the process of GCRV infecting CIK cells, therefore, it might be a potential antiviral target for GCRV infection.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is one of the most important aquaculture species in China; its output reached 5 million tons in 2016, accounting for 18% of freshwater aquaculture production in China. Farmers suffer severe economic losses annually due to mortalities resulting from grass carp hemorrhagic disease, caused by grass carp reovirus (GCRV). GCRV is a pathogen that is fatal to many aquatic animals including black carp (*Mylopharyngodon piceus*), topmouth gudeon (*Pseudorasbora parva*) and rare minnow (*Gobiocypris rarus*). It can cause severe hemorrhagic disease in fingerling and yearling populations of grass carp, one of the four major fish species that are crucial to freshwater aquaculture in China (Ding et al., 1991; Ke et al., 1990; Wang and Guo, 1994). GCRV is classified taxonomically in the genus *Aquareovirus*, family Reoviridae. Like many other reoviruses, GCRV is a multilayered spherically structured particle that contains a genome of 11 segments of dsRNA (named S1-S11) that encoded 12 proteins (two proteins encoded by segment 11) (Fan et al., 2013). GCRV produces a typical cytopathic effect (CPE) by causing the formation of large syncytia in *C. idellus* kidney (CIK) cell lines (Guo et al., 2013; Tu et al., 2013). Therefore, GCRV has served as a model to study the replication and pathogenesis of *Aquareoviruses*, both *in vivo* and in *vitro* (Wang et al., 2012). However, there is no effective therapeutic drug available against GCRV. The binding of viruses to host factors constitutes the first step in the viral cycle and is one of the main determinants for viral tropism. Accordingly, one of the most promising antiviral strategies is disturbing the interaction of viruses with their host factors. Hence, identification of host factors in the process of GCRV infection may contribute to the development of antiviral therapeutic agents screening for GCRV control and long term sustainability of grass carp farming.

Proteomics is a powerful technique to investigate the dynamics of host-virus interactions and has been applied to studies of spring viremia carp virus (SVCV) (Liu et al., 2013), bombyx mori nuclear polyhedrosis virus (BmNPV) (Qin et al., 2012) and white spot syndrome virus (WSSV) (Chai et al., 2010). Targeting host proteins for antiviral therapeutics provides an advantage over targeting viral proteins because host proteins are not susceptible (Noble et al., 2010). To further understand the pathogenesis of viral disease, it is crucial to find host

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factors of cells that interact with virus.

HSP70 is protein chaperone that has many cellular functions, which include the folding of nascent proteins, refolding of misfolded proteins, and protein transport between cellular compartments (Daugaard et al., 2007). HSP70 and its homologs comprise two functionally distinct domains, namely an N-terminal substrate-binding domain (SBD) and a C-terminal ATPase domain that allosterically-modulates the activity of the SBD (Saibil, 2013). Small molecule inhibitors pifithrin-μ (PIF) directly interacts with the SBD, thus interfering with its ability to bind to client proteins, whereas VER155008 (VER) targets the ATPase domain, preventing ATP from binding and consequently inhibiting HSP70 function by preventing allosteric regulation of the SBD. HSP70 has been implicated as a host factor in viral pathogenesis (Das, 2009; Valle et al., 2005). However, there are few reports about the role of HSP70 in GCRV invasion CIK cells.

In this study, the role of HSP70 in GCRV pathogenesis and the antiviral activity of small molecule inhibitors (VER and PIF) were evaluated. With the aim of identifying the CIK host factors that interacted with GCRV, we performed standard virus overlay protein binding assay (VOPBA) and LC-MS-MS. Using immune-fluorescent and flow cytometry determined the location of the HSP70. Afterwards, the function of HSP70 in GCRV invasion CIK cells was investigated with VER and PIF. Moreover, the antiviral activity of VER and PIF was also checked. This work highlighted HSP70 was a host factor in the process of GCRV infection, and it could be used as a potential antiviral target.

2. Materials and methods

2.1. Cell lines and viruses

CIK cells and $GCRV_{104}$ were kindly provided by Prof. Ling-bing Zeng in Yangtze River Fisheries Research Institute, Wuhan, Hubei, China. CIK cells were cultured at 28 °C under 5% CO_2 in Dulbecco's modified Eagle's minimum essential medium (DMEM; HyClone, USA) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin, and 100 μg/mL of streptomycin. The virus propagated in CIK cells and then was stocked at −80 °C.

2.2. Propagation and purification of virus

GCRV propagated in the CIK cell line in 25 cm^2 flasks (Corning). Confluent monolayers of CIK cells were exposed to the virus and cultured in DMEM medium supplemented with 5% FBS at 28 °C. When CPE was observed and continued until 3 d post-infection, the virus-infected cell culture supernatant was harvested. Afterwards, virus was purified by the method of sucrose density gradient centrifugation with the aid of supercentrifuge (Fang et al., 2005). The collection of suspension was centrifuged (8000 rpm) for 30 min to remove the redundant impurities. Then the GCRV particles were collected by ultracentrifugation from the supernatant in 33000 rpm for 2.5 h, and virus precipitation was resuspended in appropriate amount of TE buffer solution (0.01 M Tris, 0.001 M EDTA, pH = 8.0). Thereafter, the suspension was centrifuged (26000 rpm) for 2 h in 20%, 35% and 50% sucrose density gradient. Afterwards, the extractives which in the zones of 20%–35% and 35%–50% were collected and diluted with TE buffer to 12 mL. The diluents were centrifuged (26000 rpm) for 90 min to get rid of sucrose. All these steps were performed in 4 °C. Sediment was dissolved with TE buffer and stored at −80 °C until further use.

2.3. Preparation of polyclonal antibody

A rabbit was immunized with purified GCRV according to the following procedure. GCRV was emulsified with an equal volume of Freund's adjuvant. The mixture (600 μL) was intracutaneously injected into the rabbit (first immunization with freund's complete adjuvant, and the rest with freund's incomplete adjuvant) at 1, 7, 14, 21 and 28 d. After last immune, the rabbit was bled from an ear vein, and the antisera was collected by centrifugation (Chu and Ng, 2003). The antisera was tested by enzymelinked immunosorbent assay (ELISA) and a dilution of serum was considered positive when the ratio [OD (positive serum)]/[OD (negative serum)] was two or higher (Hermida et al., 2006). The specificity of polyclonal antibody was analyzed by western blot.

2.4. Virus overlay protein binding assay

Approximately 120 μg of CIK cells membrane proteins were loaded onto an Immobilized pH gradient (IPG) strip (24 cm, pH 4–7; BIORAD). Each strip was passively rehydrated overnight with 120 μg of total proteins that were premixed with a rehydration solution containing 1% DTT, 1% IPG buffer (pH 4–7, GE Healthcare), 1 × bromophenol blue solution. The first dimension was carried out in Ettan IPGphor3 system (GE Healthcare). The second dimension was conducted on miniVE Vertical Electrophoresis System (GE Healthcare). The equilibrated strips were placed onto the top of a 12.5% SDS-PAGE gel and then separated with a constant voltage of 120 V for 2.5 h. A 2-D gel was stained by coomassie brilliant blue. Another two pieces of 2-D gel were electrophoretically transferred onto 0.22 μm PVDF membranes. The membranes were blocked overnight with 5% skim milk. Afterwards, the experimental group was incubated with purified virus (1:500) in PBS at 4 °C for 8 h and the control group was incubated with PBS. After three washes with PBS, the membranes were incubated overnight at 4 °C with the specific anti-GCRV polyclonal antibody (1:1000) in 5% skim milk. Whereafter, membranes were washed three times with TBST, incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG (Beyotime Biotechnology, China). Washed membranes were developed with ECL (Advansta, USA) and then photographed. The specific spot was analysed by LC-MS-MS.

2.5. Immunofluorescence and flow cytometry analysis

To ascertain the location of HSP70 in CIK cells, an immune-fluorescent study was designed using monoclonal antibody against HSP70 (ENZO, USA). For this purpose, monolayers of CIK cells were seeded on cell climbing piece and fixed with 4% paraformaldehyde for 30 min at room temperature. The CIK cells were washed for three times using PBS and incubated with blocking solution (goat serum) to prevent non-specific binding of antibodies and conjugate. Subsequently, the experimental group was incubated with HSP70 monoclonal antibody (1:50) at 4 °C overnight and the control group was incubated with PBS. Prior to staining, cells were subjected to three washes using PBST and incubated with FITC conjugated goat anti-mouse antibody for 1 h at room temperature. Cells were stained with DAPI and observed using a fluorescence microscope (Leica, Germany). On the other hand, cells were seeded in 6-well plate. The experimental group was incubated with HSP70 monoclonal antibody (1:50) at 4 °C overnight. As a control, another group was incubated with PBS. After three washes with PBST, the two groups were incubated with FITC conjugated goat anti-mouse antibody for 1 h at room temperature. Subsequently, cells were washed with PBST for three times and the fluorescence intensitise of HSP70 was detected by flow cytometry (Beckman, USA).

2.6. HSP70 inhibition test

Small molecule inhibitors VER and PIF (APEXBIO) were both dissolved in DMSO (Sigma Aldrich) to make 10 mM stock, which were further diluted in DMEM supplemented with 5% FBS to the appropriate working concentrations. Firstly, cytotoxicity of VER and PIF was detected by MTT assay. Briefly, cells were seeded in each well of 96-well plate for about 24 h until cells reached approximately 90% confluence in each well. Inhibitors [VER, PIF, VER + PIF (1:1)] between 5 and 25 μM were tested with three replicates, which were incubated with