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Apolipoprotein mediates soft-shelled turtle systemic septicemia spherical virus (STSSSV) infection

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ABSTRACT

Soft-shelled turtle systemic septicemia spherical virus (STSSSV) is a devastating pathogen of farmed *Pelodiscus sinensis* and causes great economic losses. Little is known about the pathogenic mechanisms of this virus. In this study, STSSSV was found to bind three proteins with molecular masses of approximately 260, 212 and 134 kDa from the turtle *P. sinensis* via overlay assays. A matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis revealed that the 260 kDa protein shared the closest homology with apolipoprotein B (ApoB). A blotting assay showed that anti-mouse ApoB antibody cross-reacted with the *P. sinensis* apolipoprotein B (PApoB) protein. A high interaction affinity between PApoB and STSSSV was confirmed by co-immunoprecipitation. In an immunofluorescence assay, PApoB colocalized with STSSSV both on the surfaces of and within turtle cells. Moreover, the ApoB antibody was able to inhibit STSSSV infection. These data suggest that PApoB mediates the infection of *P. sinensis* by STSSSV.

1. Introduction

Pelodiscus sinensis, also known as *Trionyx sinensis* or Chinese soft shell turtle, is mainly distributed in China, Japan and Southeast Asia. It is an important freshwater aquaculture species in China. As with other aquacultured species, viral diseases have become an important factor limiting the sustainable development of aquaculture of this species (Hong et al., 2003). Soft-shelled turtle systemic septicemia (STSS) in *P. sinensis* is characterized by serious systemic sepsis with high morbidity and mortality and has caused farmers great economic losses (Li et al., 2013; Zhang et al., 2015). The soft-shelled turtle systemic septicemia spherical virus (STSSSV) is the pathogenic agent responsible for STSS (Li et al., 2012a; Li et al., 2012b). An indirect ELISA and a colloidal gold immunochromatographic strip have been developed to rapidly detect STSSSV (Li et al., 2013; Zhang et al., 2015). However, these previous studies focused on developing diagnostic techniques and little is known about the mechanism of STSSSV infection.

In this study, we attempted to identify *P. sinensis* proteins with affinities to STSSSV that facilitate infections by this virus.

2. Materials and methods

2.1. STSSSV and antibody

STSSSV was isolated and purified from the internal organs of infected *P. sinensis* via multiple differential centrifugation steps as previously described (Li et al., 2012a; Zhang et al., 2015). Virions were suspended in phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 dissolved in 1 L of distilled water at a pH of 7.4).

A chicken polyclonal anti-STSSSV specific antibody was prepared as previously described (Li et al., 2012a; Li et al., 2015; Zhang et al., 2015). Mouse anti-STSSSV serum was prepared according to Fang et al. (2015).

Abbreviations: STSSSV, soft-shelled turtle systemic septicemia spherical virus; PApoB, *Pelodiscus sinensis* apolipoprotein B; ApoB, apolipoprotein B; STSS, soft-shelled turtle systemic septicemia; Ab, antibody; VOPBA, viral overlay protein binding assay; PBS, phosphate buffered saline; ELISA, enzyme linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; NP-40, Nonidet P-40; NETN, Tris-EDTA-buffered saline with NP-40; MS, Mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; IgG, Immunoglobulin G; FITC, fluorescein isothiocyanate; TRITC, tetraethyl rhodamine isothiocyanate; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; VLDL, very low density lipoprotein; LDL, low density lipoprotein; LDL-R, low density lipoprotein receptor; ER, endoplasmic reticulum; VSV, vesicular stomatitis virus; HCV, hepatitis C virus; BVDV, Bovine viral diarrhea virus; HGV, GB virus C/hepatitis G virus.

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Briefly, hens and mice were immunized with purified STSSSV. Chicken polyclonal anti-STSSSV specific antibodies were purified from egg yolks.

2.2. Cell protein preparation

Cell proteins were prepared according to a previously described method (Fang et al., 2015). Cell proteins were extracted from *P. sinensis* liver tissue and were eventually resuspended in freshly made buffer M (100 mM Tris-HCl with a pH of 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.2% Triton X-100, and 1 mM PMSF) and stored at −80 °C for later use.

2.3. Virus overlay protein binding assay (VOPBA)

To identify *P. sinensis* cell proteins involved in virus binding, a virus overlay protein binding assay (VOPBA) was carried out as previously described (Fang et al., 2015). The chicken polyclonal anti-STSSSV antibody was used to locate the virus binding proteins in a PVDF membrane.

2.4. Mass spectrometry (MS) analysis

Protein bands that corresponded to the viral binding band of the VOPBA were excised and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

2.5. Western blotting assay and Co-immunoprecipitation (Co-IP) assays

Mouse antisera to STSSSV were produced as previously described (Fang et al., 2015) and rabbit anti-mouse apolipoprotein B (ApoB) IgG was purchased from Abcam (Cambridge, MA).

Western blot assays were used to verify that the 260 kDa protein identified by the VOPBA was indeed ApoB. Turtle cell proteins were separated by SDS-PAGE and a commercial anti-mouse ApoB IgG was used as a primary antibody.

Co-IP assays were carried out according to a previously described method (Fang et al., 2015) with some modifications. Briefly, cell protein supernatant from soft-shelled turtle liver tissue or PBS (as negative control) was incubated with purified STSSSV on ice for 2 h. IgG against ApoB was added and samples were incubated overnight at 4 °C. Protein A + G agarose beads (Santa Cruz) were added and incubated overnight, then were collected at 4 °C and washed three times with NETN buffer (20 mM Tris–HCl, 0.5% NP-40, 100 mM NaCl). Co-immunoprecipitates were subjected to immunoblotting using antisera to STSSSV. In a reciprocal experiment, 500 μL of cell protein supernatant from soft-shelled turtle hepatocytes was incubated with 100 μL of purified STSSSV or PBS (as negative control), immunoprecitated with antisera against STSSSV, and *P. sinensis* apolipoprotein B (PApoB) was detected using a commercial anti-mouse ApoB IgG in the immunoblotting stage.

2.6. Immunofluorescence staining

P. sinensis hemocytes and hepatocytes were fixed to glass slides with 10% formalin and were incubated for 15 min at room temperature. The fixed cells were incubated with STSSSV or PBS (as a negative control) on ice for 1 h. Next, the cells were washed three times with PBS incubated with 1:50 diluted rabbit anti-ApoB polyclonal antibodies and 1:50 diluted mouse anti-STSSSV serum for 1 h at 37 °C. After 3 washes with PBS, the cells were incubated with 1:50 diluted fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit and 1:50 diluted tetraethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-mouse antibodies for 1 h at 37 °C. Cell nuclei were stained by DAPI. Finally, the cells were washed 3 times with physiological saline and viewed under a fluorescence microscope (Nikon eclipse Ti-U).

2.7. Blocking assay

STSSV were labeled with FITC as previously described (Li et al., 2007). *P. sinensis* hemocyte suspensions were aliquoted into 96-well cell culture plates (Nest, China) and maintained at room temperature for 30–60 min, then were incubated with a rabbit polyclonal anti-ApoB antibody at room temperature for 1 h. After three washes with PBS, the hemocytes were mixed with FITC-labeled STSSSV for 1 h, then were washed 3 times with PBS to remove unbound FITC-labeled STSSSV. The cell nuclei were stained with propidium iodide (PI) (1 mg/mL) for 3–5 min.

3. Results

3.1. Screening for a virus binding protein in soft-shelled turtle liver cells by VOPBA

To identify the molecules of *P. sinensis* liver cells involved in STSSSV binding, the VOPBA technique was utilized. Cell proteins were separated by SDS-PAGE on two parallel 10% gels. One gel was stained with coomassie brilliant blue R-250 (Fig. 1, lane1), and the second gel was transferred onto a PVDF membrane for VOPBA. The VOPBA results identified three major virus binding bands with approximate sizes of 260, 212 and 134 kDa (Fig. 1, lane 2).

3.2. MALDI-TOF-MS analysis

In the MALDI-TOF and BLAST analysis, the 260 kDa virus binding protein exhibited homology to apolipoprotein B (Fig. 2).

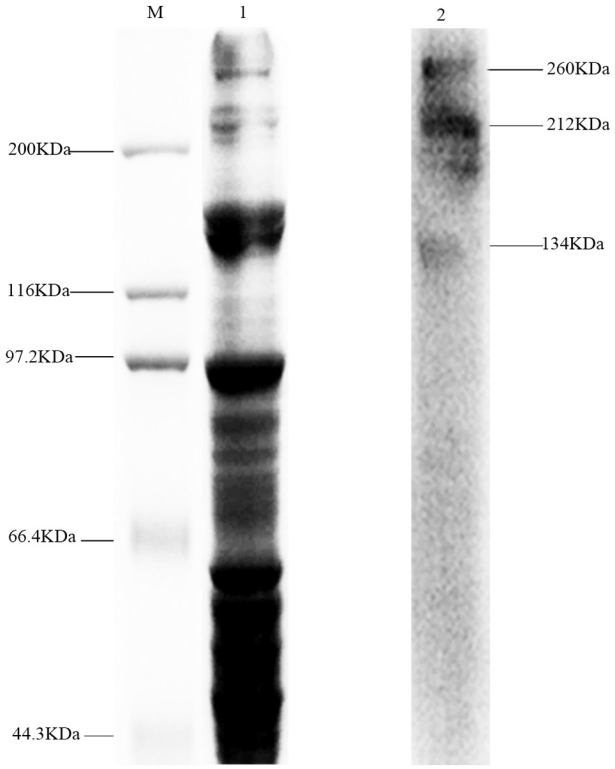

Fig. 1. VOPBA analysis of STSSSV binding to *P. sinensis* cell proteins. Lane M, protein maker; lane 1, cell proteins stained with coomassie brilliant blue; lane 2, VOPBA with an STSSSV overlay.