Virus Research 217 (2016) 133–142

Contents lists available at ScienceDirect

Virus Research

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

Tiger frog virus ORF080L protein interacts with LITAF and impairs EGF-induced EGFR degradation

Yong-Shun Chen[b,1], Nan-Nan Chen[a,b,1], Xiao-Wei Qin[b], Shu Mi[a], Jian He[a], Yi-Fan Lin[c], Ming-Shi Gao[b], Shao-Ping Weng[b], Chang-Jun Guo[a,b,*], Jian-Guo He[b]

[a] Guangdong Provincial Key Laboratory of Marine Resources and Coastal Engineering/South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, School of Marine, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, PR China
[b] MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, PR China
[c] Institute of Aquatic Economic Animals and Guangdong Province Key Laboratory for Aquatic Economic Animals, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, PR China

ARTICLE INFO

Article history:
Received 14 September 2015
Received in revised form 23 February 2016
Accepted 2 March 2016
Available online 5 March 2016

Keywords:
Endosome-lysosome pathway
Iridovirus
LITAF
Tiger frog virus
Viral replication

ABSTRACT

Tiger frog virus (TFV) belongs to the genus *Ranavirus*, family *Iridoviridae*, and causes severe mortality in commercial cultures in China. TFV ORF080L is a gene homolog of lipopolysaccharide-induced TNF-α factor (LITAF), which is a regulator in endosome-to-lysosome trafficking through its function in the endosomal sorting complex required for transport machinery. The characteristics and biological roles of TFV ORF080L were identified. TFV ORF080L was predicted to encode an 84-amino acid peptide (VP080L). It had high-sequence identity with mammalian LITAF, but lacked the N-terminus of LITAF, which contains two PPXY motifs. Transcription and protein level analyses showed that TFV ORF080L was a late viral gene. Localization in the virons also showed that TFV VP080L was a viral structural protein. Immunofluorescence staining showed that TFV ORF080L was predominantly colocalized with plasma membrane and partly distributed with the late endosome in infected HepG2 cells. SiRNA-mediated TFV ORF080L silencing decreased viral reproduction. Moreover, TFV ORF080L interacted with human/zebrafish LITAF and impaired EGF-induced EGFR degradation, thereby indicating that TFV ORF080L played a role in endosome-to-lysosome trafficking. These findings suggested that TFV ORF080L might negate the function of cellular LITAF to impair endosomal sorting and trafficking. Results provide a clue to the link between the dysregulated endosomal trafficking and iridovirus pathogenesis.

1. Introduction

Iridoviruses are large double-stranded DNA viruses exhibiting icosahedral symmetry, and they are commonly 120–200 nm in diameter (Knowles et al., 2012). They infect insects and cold-blooded vertebrates, including fish, amphibians, and reptiles (Williams, 1996). Virus genome is circularly permuted and terminally redundant, which is distinct for eukaryotic viruses (Tidona and Darai, 2000). *Iridoviridae* family has been subdivided into five genera, namely, *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus* (Jancovich et al., 2012). Ranaviruses grow in a wide variety of cultured fish, amphibian, and mammalian cells at temperatures up to 30 °C (Jancovich et al., 2012). Tiger frog virus (TFV) is isolated from diseased tadpoles (Weng et al., 2002), and it is the first to be sequenced among ranaviruses (He et al., 2002). Its genome comprises double-stranded DNA of 105,057 base pairs in length and is organized by 105 non-overlapping open reading frames (ORFs). TFV shows marked nucleotide sequence similarity to FV3, the type species of genus *Ranavirus*, and its proteins show marked homology. TFV enters via caveola-mediated endocytosis in a pH-dependent manner (Guo et al., 2011), but the pathogenesis of TFV infection remains unknown.

Lipopolysaccharide (LPS)-induced TNF-α factor (LITAF) has been isolated and characterized with activity in TNF-α transcriptional regulation (Myokai et al., 1999). LITAF binds to the promoter regions of TNF-α and other cytokines in response to bacterial LPS stimulation, thereby regulating the expression of TNF-α (Tang et al., 2005). LITAF is also known as a small integral membrane protein of lysosome/late endosome (SIMPLE), which is a 161-amino acid

* Corresponding author at: Guangdong Provincial Key Laboratory of Marine Resources and Coastal Engineering/South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, School of Marine, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, PR China.
E-mail address: relike2004@sina.com (C.-J. Guo).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.virusres.2016.03.001
0168-1702/

protein (Tang et al., 2005). In terms of other cellular processes, such as cell metabolism, hormone secretion, and innate immunity, endosomes are crucial in vesicle trafficking. The cell surface receptors bind with ligands, such as epidermal growth factor (EGF) and EGF receptor (EGFR); subsequently, they are targeted to the early endosome as cargo (Sorkin and Goh, 2009). The three destinations for the early endosome-sorted cargo are the lysosome, plasma membrane, and signaling pathways (Bonifacino and Traub, 2003; Piper and Katzmann, 2007). The cargo targeted to lysosomes is destined for receptor–ligand complex degradation or signal transduction. Otherwise, the cargo is recycled to the cell surface for reuse. The absorption of plasma membranes by endocytosis is counterbalanced by the return of membrane cargo to the cell surface through endosomal recycling. The recycling process maintains the homeostatic composition of the plasma membrane (Li and DiFiglia, 2012). Many protein complexes play roles in trafficking cargoes from endosomes to lysosomes, including the endosomal sorting complex required for transport-I (ESCRT-I) complex. EGFR is a type of cargo that is sorted into multivesicular bodies by ESCRT-I. LITAF is a functional partner of ESCRT in regulating endosome–lysosome trafficking and intracellular signaling (Chin et al., 2013). LITAF participates in the recruitment of ESCRT components, namely, STAM1, Hrs, and tumor susceptibility gene 101 (TSG101), to the early endosomal membrane and functions with the ESCRT complex to control endosomal sorting and lysosomal degradation of cargo proteins. This phenomenon is an evidence that links dysregulated endosomal trafficking to virus pathogenesis.

Based on a genome-wide bioinformatics analysis of TFV-encoded proteins, homology was predicted between the deduced amino acid sequences of TFV ORF080L, ORF136R of the Singapore grouper iridovirus (SGIV) (Huang et al., 2008), ORF075L of FV3 (Eaton et al., 2013), and LITAF. In the present study, we characterized TFV ORF080L and investigated its roles in viral infection and endosome-to-lysosome trafficking.

2. Materials and methods

2.1. Cells and antibodies

HepG2 (ATCC HB8065), HeLa (ATCC CCL-2), and HEK293T cells (ATCC CRL-1573) were cultured as a monolayer at 37 °C in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. TFV was originally isolated from diseased tadpoles in Naihai, Guangdong, China, and maintained in our laboratory. TFV was grown in fathead minnow cells at 27 °C.

Mouse polyclonal serum against TFV major capsid protein (MCP) antibody, mouse polyclonal serum against TFV ORF080L protein antibody, and mouse polyclonal serum against TFV ORF020R protein antibody were prepared as previously reported (Luo et al., 2009; Wang et al., 2008) by Xiao-Wei Qin. Other antibodies used in this study include the following: rabbit anti-EGFR (Cell Signaling Technology, USA), rabbit anti-EEA1 (Abcam, UK), rabbit anti-Rab9 (Abcam, UK), mouse anti-Myc antibody (Sigma-Aldrich, Germany), rabbit anti-Flag antibody (Sigma-Aldrich, Germany), and rabbit anti-GADPH antibody (Sigma-Aldrich, Germany). Alexa Fluor 488-conjugated goat anti-mouse IgG antibody, Alexa Fluor 555-conjugated goat anti-rabbit IgG antibody, EGF, MitoTracker Red CM-H2XRos and Hoechst 33342 were purchased from Life Technologies (USA).

2.2. Virus purification and Western blot

Confluent monolayers of HepG2 cells were infected with TFV (MOI = 10) and incubated at 27 °C. At 6 days post-infection (p.i.), the culture medium supernatant was harvested before cell detachment, and cell debris was removed by low-speed centrifugation. Virus purification through sedimentation in sucrose density gradients was conducted as described previously (Wang et al., 2008). Protein concentrations of the purified virus stocks were determined using the DC protein assay kit (Bio-Rad, USA). About 20 μg of the purified sample was boiled in SDS loading buffer and analyzed by SDS-PAGE on 12% or 15% polyacrylamide gel. Western blot was performed as described previously (Hill et al., 2008).

2.3. Plasmid construction and transient transfection

Recombinant DNA techniques were performed according to standard procedures. The TFV ORF080L DNA sequence (GenBank accession no. ABB92333) was cloned into pCMV-Myc (Clontech Laboratories, USA) and pFlag-CMV4 (Sigma-Aldrich, Germany) to generate Myc-ORF080L and Flag-ORF080L, respectively. The homo LITAF DNA sequence from HeLa cells (GenBank accession no. NP_004853) and zebrafish LITAF sequence (GenBank accession no. NP_001002184) from ZF4 cells were cloned into pCMV-myc to generate Myc-human LITAF and Myc-zebrafish LITAF, respectively. The transient transfection of recombinant DNA plasmids into HepG2 cells was performed using Lipofectamine™ 2000 (Invitrogen, USA), according to the manufacturer's instructions.

2.4. $TCID_{50}$ assay

Before infection, 96-well dishes were prepared by seeding with cells. Dilutions at 10^{-1}–10^{-10} of the original virus sample were performed in a culture medium. About 0.1 ml of virus dilution was added to 10 wells for such a dilution, and approximately 0.1 ml of culture medium was used as negative control. The dishes were placed at 27 °C, and the number of positive and negative wells was recorded. $TCID_{50}$ was calculated using Karber method (Miller and Ulrich, 2001).

2.5. Transcript analysis in the presence of inhibitors

To detect the transcription of TFV ORF080L, CHX (Cycloheximide from microbial; MP, USA) and cytosine arabinoside (AraC, MP, USA) were used for de novo protein synthesis and DNA synthesis inhibition, respectively. Briefly, HepG2 cells were either pretreated with CHX (200 μg/ml) or AraC (400 μg/ml) for 1 h prior to the TFV infection. Mock-treated cells were used as control. Total RNA was extracted and reverse-transcribed to cDNA, followed by PCR. TFV ORF097R, a previously characterized early gene during TFV infection (Xie et al., 2014), and TFV MCP, a known late gene, were used as an indicative control. TFV ORF097R forward primer: 5′- TGCCACATCCAGACATTC-3′, reverse primer 5′-CAATCTCTATCAGCCTCCTT-3′. TFV MCP forward primer: 5′-TCGCTGGTGGAGCCCTGGTA-3′, reverse primer: 5′-GGCGTTGGTCAGTCTGCCGTA-3′ GADPH forward primer: 5′-AAGGTGAAGGTCGGAGTC-3′, reverse primer: 5′-CTTGAGGCTGTTGTCATACT-3′. TFV ORF080L forward primer: 5′-GGAATTCAATGGACGACAAGTTTACTACC-3′, reverse primer: 5′-CGGGATCCTTATAACATTTTGTACACAAAC-3′.

2.6. Co-immunoprecipitation (Co-IP) assay

HepG2 cells were cultured in complete medium in 10 cm culture plates. Transfected cells were rinsed twice with cold PBS and lysed directly on the plate with 1 ml of cell lysis buffer (Beyotime, China) containing the phosphatase/protease inhibitor cocktail. Co-IP was performed according to the manufacturer's instructions (Dynabeads protein G immunoprecipitation kit; Life Technologies). Briefly, about 100 μl of cell lysate aliquots of were stored at −20 °C as a loading control, and approximately 800 μl of lysate was added