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# Molecular cloning and expression analysis of the *Ajuba* gene of grass carp (*Ctenopharyngodon idella*) involved in cellular response to viral infection

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ABSTRACT

Ajuba belongs to the LIM domain proteins, which are involved in the assembly of the extracellular matrix and, along with associated proteins, regulate target genes that connect the extracellular matrix and the cytoskeleton. In the present study, we characterized the entire cDNA sequence of the *Ajuba* gene from grass carp (*gcAjuba*). The *gcAjuba* cDNA contained an open reading frame (ORF) of 2121 bp encoding a polypeptide of 706 amino acids with an estimated molecular mass of 75.966 kDa and three LIM domains in the C-terminal. The transcriptional level of *gcAjuba* was significantly up-regulated following the stimulation of virus *in vitro*. Sub-cellular location of gcAjuba and GCRV-JX01 NS26 proteins did not overlap in the cytoplasm and no direct interaction between gcAjuba and the protein NS26 was detected by co-immunoprecipitation (CO-IP) test in grass carp kidney cells. Based on these results, the *gcAjuba* is determined to be an immediately inducible gene responding to viral infection and *in vivo* association of gcAjuba with NS26 could not be confirmed, which has been suggested by yeast two-hybrid assay in previous report.


## 1. Introduction

LIM domain proteins are involved with protein–protein interactions (Dawid et al., 1998) and the LIM domain is rich in conserved cysteine and histidine residues that enable a stable tertiary structure when combined with $Zn^{2+}$ (Ostendorff et al., 2002). Mammalian LIM domain proteins are categorized into four groups (Bach, 2000; Gill, 1995; Zheng and Zhao, 2007). The first group consists of two tandem N-terminal LIM domains and one transcription activation domain that are mainly involved in cell differentiation and cell fate; the second group consists of two or more LIM domains clustered at the N- or C-termini and associated with regulating cytoskeleton remodelling. The third group consists of three or four LIM domains at the C-termini and different N-terminal domains whose role is to bind with cytoskeletal protein in cell adhesion processes (Marie et al., 2003; Pratt et al., 2005). The fourth group, similar to group three in structure, participates in cell cycle regulation and actin polymerization (Zheng and Zhao, 2007).

The Zyxin/Ajuba family of cytosolic LIM domain-containing proteins (Ajuba, TRIP6, LPP, LIMD1, WTIP, Zyxin, Migfillin) is the third group (Petit et al., 2005). These proteins have the potential to shuttle from sites of cell adhesion into the nucleus and can be candidate transducers of environmental signals (Feng and Longmore, 2005). Among them, Ajuba consists of three LIM domains at the C-terminal and is located in the cytoplasm where it interacts with a variety of other proteins (Wang and Gilmore, 2003). Studies on the functions of Ajuba have to date been concentrated in mammals. Ajuba is a component of the IL-1 signalling pathway modulating IL-1-induced NF-κB activation by influencing the assembly and activity of the aPKC/p62/TRAF6 multiprotein signalling complex (Feng and Longmore, 2005). NF-κB is one of the best-characterized transcription factors. It is expressed ubiquitously and regulates the expression of many genes, most of which encode proteins that play an important and often determining role in the processes of immunity and inflammation (O'Neill and Kaltschmidt, 1997). So, Ajuba is believed to be involved in the immunity system by NF-κB signal transduction pathway.

Ajuba also contributes to the formation or strengthening of cell–cell adhesion and regulates cell growth and differentiation decisions (Feng and Longmore, 2005; Kanungo et al., 2000). Ajuba participates in the assembly of the extracellular matrix and regulates target genes involved in the connection process of the extracellular matrix and the cytoskeleton (Hou et al., 2008; Kisseleva et al., 2005). Ajuba also plays an important role in some diseases. For example, Ajuba can bind and activate the Aurora-A protein that is associated with tumour development (Hirota et al., 2003).

Grass carp (*Ctenopharyngodon idella*) is an important commercial fish that is widely grown in Eastern Asia (FAO, 2010). Grass carp

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haemorrhagic disease, caused by the Grass carp reovirus (GCRV), is a major problem for grass carp aquaculture (Chen et al., 2013). GCRV has been assigned to the genus Aquareovirus in the family reoviridae (Fauquet et al., 2005) and consists of 11 dsRNA genomic fragments, which encode seven structural proteins (VP1–VP7) and five non-structural proteins (NS16, 26, 31, 38 and 80) (Zhang et al., 2010). It remained a puzzle for the NS26 protein that had no homologue in Mammalian reovirus or any other protein in available database (Attoui et al., 2002). In a study to screen interacting cellular proteins with grass carp reovirus using yeast two-hybrid system, we identified that the GCRV-JX01 NS26 protein might interact with three proteins: gcAjuba, CiLITAF and LOC571286 (Wang et al., 2013a). Recent evidence indicated that NS26 could participate in cell–cell fusion through cooperation with NS16 in aquareovirus infection (Guo et al., 2013). Since the mammalian Ajuba family of LIM proteins are adaptor proteins within multi-protein complexes that connect cell–cell and cell matrix contact proteins to the cytoskeleton (Kim et al., 2012), we are interested to characterize the grass carp *Ajuba* gene and its possible involvement in cellular response to virus infection.

In this report, the coding sequence of the entire cDNA of *Ajuba* from grass carp (*gcAjuba*) was determined and analysed, *in vivo* association of gcAjuba with GCRV NS26 was investigated, and expression pattern of *gcAjuba* in response to GCRV infection was characterized.

## 2. Materials and methods

### 2.1. Molecular cloning of the entire cDNA of gcAjuba

Grass carp kidney (CIK) commercial cells used in this study were obtained from China Center for Type Culture Collection and grown in 25 cm culture flask in the medium M199 supplemented with 10% inactivated foetal calf serum (Gibco BRL) prior to being incubated at 28 °C. To avoid the loss of the essential genes, the passage times of original CIK cell lines were limited to 3–5 in our experiments. The total RNA was extracted from $5 \times 10^6$ CIK cells using 2 mL TRIzol Reagent (Invitrogen, USA) and reverse-transcribed into cDNA using the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's protocol. The primary PCR primers were designed according to sequences obtained from a previous study (Ajuba-F1 and Ajuba-R1, Supplementary Table S1). The primary PCR was set up using cDNA generated from the total RNA of CIK cells as the template. The PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA), then cloned into a pMD19-T Vector (TaKaRa, Japan) and transformed into DH5α competent cells.

To obtain the full-length cDNA sequence of gcAjuba, 3′ and 5′ rapid amplification of cDNA ends (RACE) was carried out using the SMARTer™ RACE cDNA Amplification Kit (Clontech). The 3′ and 5′ RACE were performed as previously described (Shen et al., 2014), but using total cellular RNA as the amplification template. Briefly, 3′RACE and 5′RACE were performed using gene-specific primers (gcAjuba-R2 and gcAjuba-F3, Supplementary Table S1) and adapter primers (UPM and NUP, Supplementary Table S1). The full sequence of gcAjuba was confirmed with the primers gcAjuba-F4/gcAjuba-R4 and gcAjuba-F5/gcAjuba-R5 (Supplementary Table S1). All amplification products were cloned and sequenced.

### 2.2. Bioinformatics analysis of gcAjuba gene

The BLAST program from the National Center for Biotechnology Information (NCBI) was used to search homologous sequences in GenBank (http://blast.ncbi.nlm.nih.gov). ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used to predict the open reading frame (ORF) and deduced protein sequence of gcAjuba. The ProtParam tool at ExPASy (http://web.expasy.org/protparam/) was used to analyse the deduced amino acid sequence. Multiple sequence alignments were generated with DNAMAN software. Phylogenetic analysis of the amino acid sequences was performed using the Neighbour-Joining (NJ) algorithm in MEGA version 5.0.

### 2.3. Virus preparation and infection

GCRV-JX01 were isolated from diseased grass carps with typical haemorrhagic symptoms for the first time and maintained in the laboratory (Wang et al., 2013b). The 90% confluent CIK monolayer cells in five bottles of 100 cm culture flasks were infected with GCRV-JX01. Culture supernatant was collected after the death of 90% of infected cells. In brief, the virus particles were purified from the supernatant of infected CIK cells for the observation of CPE by ultracentrifugation (Fang et al., 2008). Virus titration was performed via the standard $TCID_{50}$ (50% tissue culture infective dose) assay (He et al., 2011). CIK cells were maintained as stock cultures in DMEM and re-plated 2 days before infection with GCRV-JX01. Cells were infected for 1 h, and fed with 200 μL fresh medium. At 48 h post infection, 96-well plates were observed under light microscope for typical CPE. The $TCID_{50}$ value was calculated using the Reed–Muench method.

For virus infection, CIK cells cultured in six-well cell culture plates were infected with GCRV-JX01 at a multiplicity of infection (MOI) value of 1.0. Controls were left uninfected in the experiment. Three repeats (three wells) were performed in experimental groups and control groups. After allowing 2 h for absorption, all unattached viruses were removed and the infected cells were continuously cultured with the M199 medium supplemented with 10% FBS. Cells were collected at 0, 4, 8, 12, 24 h p.i.

### 2.4. RNA extraction and quantitative RT-PCR

Total RNA was isolated from both the infected cells and the control cells using TRIzol (Invitrogen) and then subjected to DNase I treatment (Promega) according to the manufacturer's protocol for real-time PCR analysis (RT-PCR). RNA was assayed by a Nanodrop 2000 spectrophotometer. Approximately 200 ng of RNA from each sample was reverse-transcribed using PrimerScript First Strand cDNA Synthesis Kit (Takara). The first-strand cDNA was subsequently used as the template for the RT-PCR. The grass carp *elongation factor* 1a (EF1a) gene was amplified as an internal standard reference gene (Su et al., 2011). The specific primers RT-gcAjuba-F6/R6 (Supplementary Table S1) used for quantitative RT-PCR (qRT-PCR) displayed a single peak in melting curve analysis with an amplification efficiency close to the theoretical 100%. RT-PCR was carried out in a 20 μL reaction volume containing 5 μL of 1:10 diluted original cDNA, 10 μL of 2 × SYBR Green Master Mix (Bio-Rad, USA), 2 μL of each primer (20 pmol/μL), and 3 μL of PCR grade water using the Bio-Rad CFX 96 Real-time PCR machine. The triplicate fluorescence intensities of each sample, as measured by crossing-point (Ct) values, were compared and converted to fold differences by the relative quantification method (Niu et al., 2014). The *gcAjuba* transcriptional level was calculated by $2^{-\Delta\Delta CT}$ method. The data were subjected to analysis of Student's t-test and the P values less than 0.05 ($P < 0.05$) were considered statistically significant.

### 2.5. Recombinant plasmid construction and sub-cellular localization

For the construction of recombinant plasmids pEGFP-gcAjuba and pcDNA3.1-NS26, primers pEGFP-gcAjuba-F/R, containing *Xho*I and *BamH*I sites (Supplementary Table S1), were used to amplify the ORF of *gcAjuba*. The PCR products were purified and cloned into a pEGFP-N1 expression vector to get pEGFP-gcAjuba. Similarly, primers