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Identification and characterization of Singapore grouper iridovirus (SGIV) ORF162L, an immediate-early gene involved in cell growth control and viral replication

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ABSTRACT

Singapore grouper iridovirus (SGIV) is a major pathogen resulting in heavy economic losses to grouper aquaculture. In this study, SGIV ORF162L encoding a putative homolog of ICP46 was identified and characterized. Interestingly, ICP46 could be found in all sequenced iridoviruses and is considered as a core gene of the family *Iridoviridae*. SGIV ICP46 was classified as an immediate-early (IE) gene during *in vitro* infection using drug inhibition analysis, reverse transcription polymerase chain reaction and Western blot analysis. Subcellular localization revealed that SGIV ICP46 was distributed predominantly in the cytoplasm. Furthermore, SGIV ICP46 proved to be a structural protein of the nucleocapsid; its overexpression could promote the growth of grouper embryonic cells and contribute to SGIV replication. This is the first report of the characterization of a putative ICP46 homolog and these results should offer important insights into the pathogenesis of iridoviruses.

1. Introduction

Iridoviruses are large DNA viruses that have only been isolated from invertebrates and poikilothermic vertebrates, usually associated with aquatic environments including marine habitats. The family *Iridoviridae* comprises five genera: *Ranavirus*, *Lymphocystivirus*, *Megalocytivirus*, *Iridovirus* and *Chloriridovirus* (Chinchar et al., 2005). The iridoviruses, classified into genera of *Ranavirus* (type species, frog virus 3, FV3), *Lymphocystivirus* (type species, lymphocystis disease virus, LCDV) and *Megalocytivirus* (type species, infectious spleen and kidney necrosis virus, ISKNV), are mainly associated with severe systemic diseases and heavy economic losses in farmed food and ornamental fish, as well as in wild fish. The fish mortality caused by iridovirus infection ranged from 30% to 100%. Iridoviral pathogens have been identified from more than 30 fish species worldwide in the last decade (Ahne et al., 1989; Hyatt et al., 2000; Piaskoski and Plumb, 1999; Wang et al., 2007).

The mechanisms of iridovirus infection and pathogenesis are poorly understood. Similar to other large dsDNA viruses, the iridovirus genes are expressed sequentially in three temporal kinetic classes of immediate-early (IE), early (E) and late (L) during the viral infection (Williams, 1996; Williams et al., 2005; Lua et al., 2005; Teng et al., 2008). Generally, the transcripts of viral IE genes may function as regulatory trans-acting factors and manipulate important host cellular functions, including transcription, apoptosis, cell cycle control and immune responses (Hobbs and DeLuca, 1999; Moss and Shisler, 2001; Kinchington et al., 2001). The previous studies on the function of viral IE genes were mainly focused on human viruses, such as *herpes simplex virus* (HSV), *human cytomegalvirus* (HCMV), *hepatitis B virus* (HBV) and *varicella-zoster virus* (VZV) (Everett, 2000; Castillo and Kowalik, 2002; Stinski and Petrik, 2008). However, the studies on biological functions of iridovirus IE genes are limited.

Singapore grouper iridovirus (SGIV) was isolated from the brown-spotted grouper, *Epinephelus tauvina*, and characterized as a novel ranavirus (Qin et al., 2001, 2003). As a causative agent of serious systemic disease, SGIV resulted in significant economic losses in grouper aquaculture in China and Southeast Asia countries. The genomic and proteomic analysis revealed that the SGIV genome consisted of 140,131 bp, with 162 predicted open reading frames (ORFs) coding for proteins length varying from 41 to 1268 amino acids, and 77 ORFs showed homologs to known virus genes, 30 of

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which matched to functional iridovirus proteins involved in viral DNA replication, transcription, nucleotide metabolism, cell signaling, etc. (Song et al., 2004, 2006). The whole genome transcription profiles of SGIV has also been characterized using a DNA microarray and the temporal kinetic classes were classified as 15 immediate-early (IE), 89 early (E) and 53 late (L) viral transcripts (Teng et al., 2008).

SGIV ORF162L was revealed to be an IE gene encoding a putative homolog of ICP46 by genome and transcriptome analysis (Song et al., 2004; Teng et al., 2008). ICP46 was firstly found in FV3, an IE protein of approximately 46 kDa was encoded by FV3 and referred to be as the infected cell polypeptide (ICP) 46 (Willis et al., 1977; Beckman et al., 1988). By comparative genomic analysis, ICP46 was revealed as one of the core genes of the family *Iridoviridae* suggesting that ICP46 might play an essential role in the life cycle of iridoviruses (Eaton et al., 2007). So far, the knowledge about ICP46 family is very limited, only the sequence and promoter analysis of ICP46 were reported in FV3, Bohle iridovirus (BIV) and ISKNV (Willis and Granoff, 1978; Beckman et al., 1988; Deng et al., 2002; Pallister et al., 2005). The function of ICP46 family is still unknown. Characterization of the SGIV-encoded ICP46 will provide insights into virus–host interactions and contribute to understanding the pathogenesis of iridoviruses.

In present study, we cloned a putative ICP46 homolog from SGIV, and for the first time investigated its transcription characters, subcellular localization and its role during viral infection in cell cultures *in vitro*.

2. Materials and methods

2.1. Virus and cell cultures

Iridovirus (SGIV, strain A3/12/98) was originally isolated from diseased grouper, *E. tauvina*. Propagation of SGIV and isolation of the viral genomic DNA were performed as described (Qin et al., 2003). Grouper embryonic cells (GP) and fathead minnow cells (FHM) were grown in Eagle's minimum essential medium containing 10% fetal bovine serum at 25 °C.

2.2. Virus purification and detergent extraction

Purification of SGIV was performed as described previously by Qin et al. (2001, 2003) with minor modifications. Briefly, virus was inoculated onto confluent monolayers of the grouper cell line at a multiplicity of infection (MOI) of 0.1. When advanced cytopathic effect (CPE) was observed, the cell cultures including medium containing SGIV were harvested, followed by 3 cycles of rapid freezing/thawing and ultrasonication. The suspension containing the lysate, virus, and cellular debris was then centrifuged at 4000 × *g* for 20 min at 4 °C. The supernatant was centrifuged at 210,000 × *g* for 1 h at 4 °C. The pellet was resuspended in PBS, and overlaid onto 10–60% (w/v) sucrose gradients and centrifuged at 210,000 × *g* for 1 h at 4 °C. Virus band was collected and diluted with PBS. The virion pellets were finally collected after 1 h of centrifugation at 100,000 × *g* at 4 °C and suspended in PBS, then stored at −80 °C until used.

The viral envelope was removed from purified virions according to the methods of Heppell and Berthiaume (1992) with modification. In brief, the purified SGIV particles were treated with 1% Triton X-100 (150 mM NaCl, 50 mM Tris, pH 7.5) for 30 min at room temperature, and centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was collected as envelope fraction, and the pellet was treated with 1% Triton X-100 for 30 min and centrifuged again to further remove the viral envelope proteins, and then the resulting pellet was collected as the non-envelope fraction. Separation of the envelope and the non-envelope fractions was confirmed by Western blotting with antiserum against SGIV major capsid protein (MCP) (prepared in our laboratory; 1:1000).

Table 1
Primer sequences used in this study (enzyme cleavage site is underlined).

Primers	Primers sequence (5′–3′)
ICP46-P_1	GCGGGATCCGATTTAAAAATGGCTT (BamHI)
ICP46-P_2	GTTCTCGAGTCACTCTTCTTCGTCG (XhoI)
ICP46-P_3	GCCTCTCGAGTTGTGGAAGATTTAA (XhoI)
ICP46-P_4	CGTGGATCCTTCAGCTGACTCTTCT (BamHI)
ICP46-P_5	CAAACGGCAAGCATTACA
ICP46-P_6	CGTCGGGAAACTCCAACATA
Actin-F	CACTGTGCCCATCTACGAG
Actin-R	CCATCTCCTGCTCGAAGTC

2.3. Gene cloning and plasmid construction

Based on the genome sequences of SGIV, an open reading frame (ORF) 162L encoding ICP46 homolog was revealed by computer-assisted analysis. The full length of SGIV ICP46 (ORF162L) was cloned by PCR from SGIV genomic DNA using the primers of ICP46-P_1/ICP46-P_2 (Table 1). The PCR reaction was performed in a volume of 25 μl, containing 1 μl genomic DNA, 0.2 μM of each primer, 0.7 U of PrimeSTAR HS DNA Polymerase (TaKaRa), 2 μl of 2.5 mM dNTP, 5 μl of 5× PrimeSTAR buffer (Mg^{2+} plus). PCR was carried out under the following conditions: 4 min at 94 °C and then 30 s at 94 °C, 45 s at 52 °C, 1 min at 72 °C for 30 cycles, followed by 72 °C for 8 min. The fragment product was then cloned into prokaryotic vector pET-32a (+) (Novagen) to obtain plasmid pET-ICP46. The designed primers ICP46-P_1/ICP46-P_2 and ICP46-P_3/ICP46-P_4 (Table 1) were also used to amplify the SGIV ICP46 gene with the introduced restriction enzyme sites, then the fragments were cloned into eukaryotic vectors pcDNA3.1 (+) (Invitrogen) and pEGFP-N3 (Clontech), respectively, to obtain plasmids pcDNA-ICP46 and pEGFP-ICP46. The constructed plasmids of pcDNA-ICP46 and pEGFP-ICP46 were confirmed by restriction enzyme digestion and DNA sequencing, respectively.

2.4. Amino acid sequence comparisons and phylogenetic analysis

The peptide sequence homologs to the SGIV ICP46 were dug by computer-assisted analysis from the National Center for Biotechnology Information (NCBI) blast server. The multiple alignment of amino acid sequence of SGIV ICP46 with those from other 12 sequenced iridoviruses (Table 2) was performed using Clustal X 1.83 and edited by the GeneDoc program. The phylogenetic relationships were inferred with neighbor-joining (NJ) distance using MEGA (Version 3.1).

2.5. Prokaryotic expression, purification and antibody preparation

The recombinant plasmid pET-ICP46 was transformed into *Escherichia coli* BL21 cells and the fusion protein was expressed. The fusion protein was purified according to the protocol supplied with the HisBind purification kit (Novagen). To obtain antibody against SGIV ICP46, the purified recombinant ICP46 protein was mixed with equal volume of Freund's adjuvant (Sigma) to immunize mouse by hypodermal injection subcutaneously at 7-day intervals. The anti-ICP46 serum was collected at the seventh day after the fourth immunization and its specificity was tested by Western blot analysis. Simultaneously, the negative serum was produced as control by immunizing mouse with PBS and Freund's adjuvant (Sigma) using the same procedure.