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Identification and characterization of a tumor necrosis factor receptor like protein encoded by Singapore grouper iridovirus

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ABSTRACT

Virus encoded tumor necrosis factor receptors (TNFRs) have been demonstrated to facilitate virus to escape from apoptosis or other host immune response for viral replication. Singapore grouper iridovirus (SGIV), a large DNA virus which belongs to genus *Ranavirus*, is a major pathogen resulting in heavy economic losses to grouper aquaculture. Here, SGIV ORF096 (VP96) encoding a putative homolog of TNFR was identified and characterized. Multiple sequence alignment indicated that SGIV-VP96 contained two extracellular cysteine-rich domains (CRDs) with conserved four or six cysteine residues, but lacked the transmembrane domain at the C-terminus. SGIV-VP96 was identified as an early (E) gene and localized in the cytoplasm in transfected or infected cells. Overexpression of SGIV-VP96 *in vitro* enhanced cell proliferation, and improved cell survival against SGIV infection. Furthermore, virus infection induced apoptosis and caspase-3 activity were inhibited in SGIV-VP96 expressing FHM cells compared to the control cells. Taken together, our results suggested that SGIV might utilize virus encoded TNFR like genes to modulate the host apoptotic response for effective virus replication.

1. Introduction

Apoptosis is an important event in the normal development and homeostasis of multicellular organisms, and considered to be a host defense mechanism against viral replication. Apoptosis was also utilized for viruses to evade the host immune system to ensure viral replication, propagation, and persistent infection (Rahman and McFadden, 2006). Increasing evidences revealed that viruses could encode TNFR homologs and hijack the cellular TNF/TNFR pathway to favor viral infection by regulating the host apoptotic response (Benedict et al., 2003). Several viral TNFR homologs have been identified in the genomes of large DNA viruses, including poxvirus, herpesvirus, iridovirus and African swine fever virus (ASFV) (Arav-Boger et al., 2006; Poole et al., 2006; Saraiva et al., 2002; Sedger et al., 2006). To escape from the host immune response, these vTNFR homologs mimicked function by sequestering host cytokines in distinct ways and at different stages of the immune response. The Myxoma virus T2 protein (M-T2) could inhibit TNFα-mediated cytotoxicity and virus-induced lymphocyte apoptosis (Sedger and McFadden, 1996). CrmE protected cells only from the cytolytic activity of human TNF (Reading et al., 2002). To our knowledge, information concerning the function of vTNFR homologs from lower vertebrate virus remains largely unknown.

Iridoviruses are large DNA viruses, and the family *iridoviridae* are currently divided into five genera: *Ranavirus*, *Lymphocystivirus*, *Iridovirus*, *Chloriridovirus* and *Megalocytivirus* (Jancovich et al., 2011). It has been reported that typical apoptosis was evoked by iridovirus infection, including Chilo iridescent virus (CIV), red sea bream iridovirus (RSIV), lymphocystis disease virus (LCDV), Rana grylio virus (RGV), soft-shelled turtle iridovirus (STIV), frog virus 3 (FV3) and grouper iridovirus (GIV) (Chitnis et al., 2008; Huang et al., 2011a,b, 2007a; Pham et al., 2012; Imajoh et al., 2004; Hu et al., 2004; Chinchar et al., 2003). Singapore grouper iridovirus (SGIV), a novel ranavirus, was isolated from diseased grouper (Qin et al., 2001). Previous studies demonstrated that SGIV infection induced typical apoptosis in Fathead minnow (FHM) cells (Huang et al., 2011a,b). Based on the elucidation of SGIV genome, some potential viral gene products were predicted to be associated with apoptosis, including a lipopolysaccharide-induced TNF-a factor (LITAF) homolog and three TNFR homologs (Huang et al., 2008; Song et al., 2004). Whether SGIV TNFRs could regulate apoptosis during SGIV infection remains unknown.

In the present study, we firstly described a TNFR like gene encoded by SGIV ORF096 (VP96). SGIV-VP96 was able to increase the cell proliferation, and suppressed virus infection induced apoptosis in FHM cells. These results not only provided new insight

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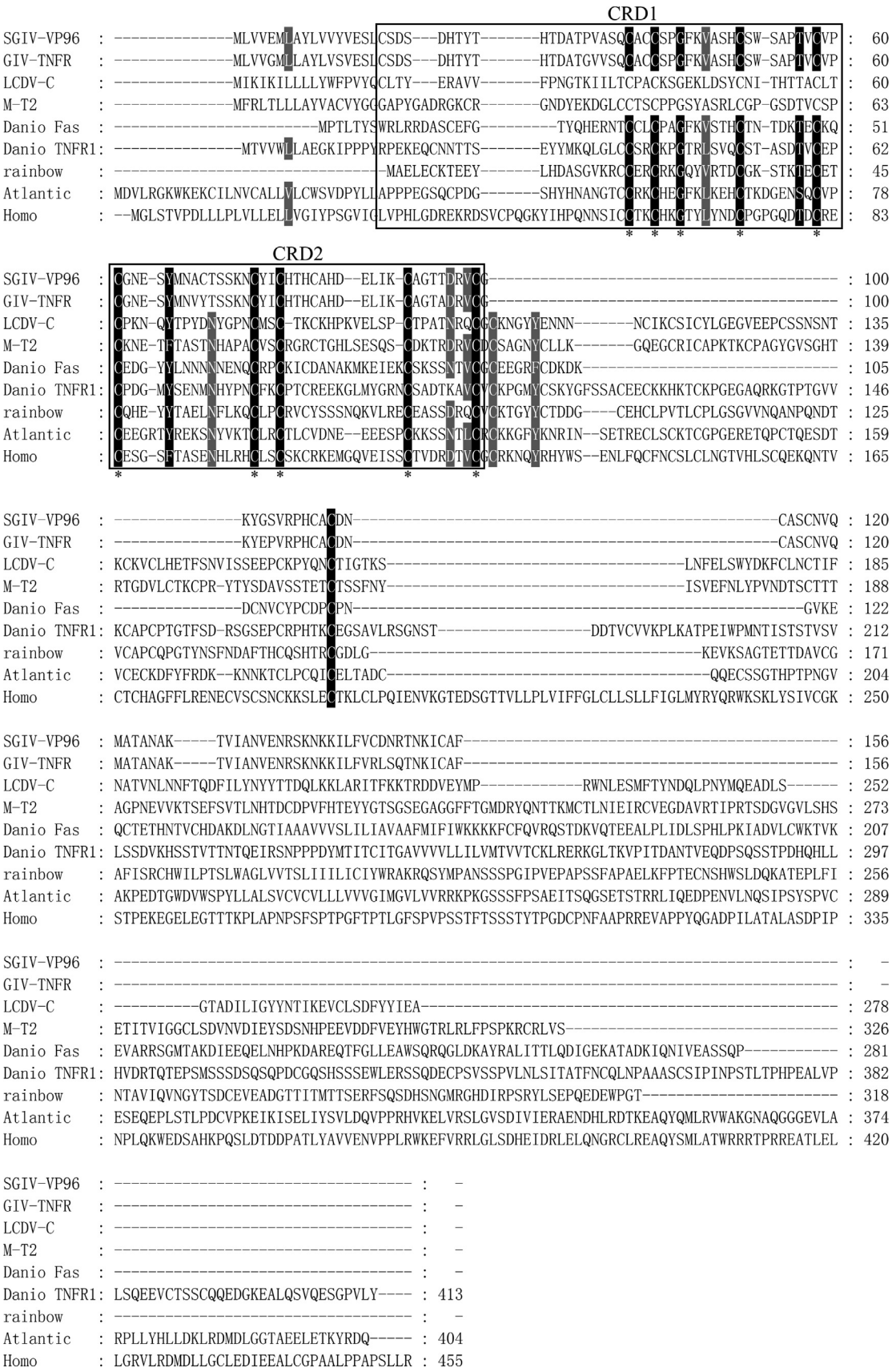

Fig. 1. Amino acid sequence alignment of SGIV-VP96 with other TNFR homologs from human, fish and viruses. The conserved cysteines (C) were indicated by asterisks under the alignment. Boxes above the sequences were the putative cysteine-rich domains (CRDs). Accession numbers of the sequences used for the above analysis were listed as follows: SGIV VP96, YP_164191; Grouper iridovirus (GIV) TNFR, AAV91081; Lymphocystis disease virus-isolate from China (LCDVC) TNFR, YP_073525; Myxoma virus T2 (M-T2), NP_051879; Atlantic Salmon TNFR, ACI68577; Rainbow trout TNFR, NP_001165342; Zebrafish TNFR, ABG91567; Human HVEM, CAX30822.

into exploring the function of TNFR homolog from iridovirus, but also contributed to better understanding of SGIV pathogenesis.

2. Results

2.1. SGIV-VP96 encoded a TNFR-like protein

Sequence analysis of SGIV ORF096R (SGIV-VP96) (YP_164191) revealed that it encoded a 156 amino acid protein with 16.8 kDa. Blastp search indicated that SGIV-VP96 shared 34%, 31% and 31% identity to TNFR members from Atlantic salmon, zebrafish and human, respectively. Amino acid alignment showed that SGIV-VP96 contained two extracellular cysteine-rich domains (CRDs) with conserved four or six cysteine residues (Fig. 1). TMHMM prediction indicated that the vTNFRs, including VP96, GIV TNFR, LCDV-C TNFR and M-T2 lacked the membrane anchor and cytoplasmic domains, while the later two contained a short C-terminal extension. Extracellular conserved CRDs at N-terminal were typical hallmarks of the TNFR superfamily (Locksley et al., 2001). Thus, SGIV-VP96 was proposed to be a novel virus encoded TNFR-like gene.

2.2. SGIV-VP96 was an early (E) viral gene

The temporal expression pattern of SGIV-VP96 was characterized by detecting its transcript and protein synthesis at different infection time points. As shown in Fig. 2, the SGIV-VP96 gene-specific fragment was detected at 4 h p.i., and high-level transcription continued until 48 h p.i. (Fig. 2A). The drug inhibition assays using CHX and AraC have been well developed to classify the transcript of iridovirus gene as immediate early (IE), early (E) and late (L) gene (Huang et al., 2008). The transcript of SGIV-VP96 gene could be detected in the present of AraC for 48 h, but not in the presence of CHX for 6 h (Fig. 2C), indicating that SGIV-VP96 transcription was an E viral gene. Differently, the transcription of IE gene (SGIV ORF086) was not affected by the addition of CHX or AraC, while the transcription of L gene (SGIV ORF072, MCP) was significantly inhibited in the presence of CHX or AraC.

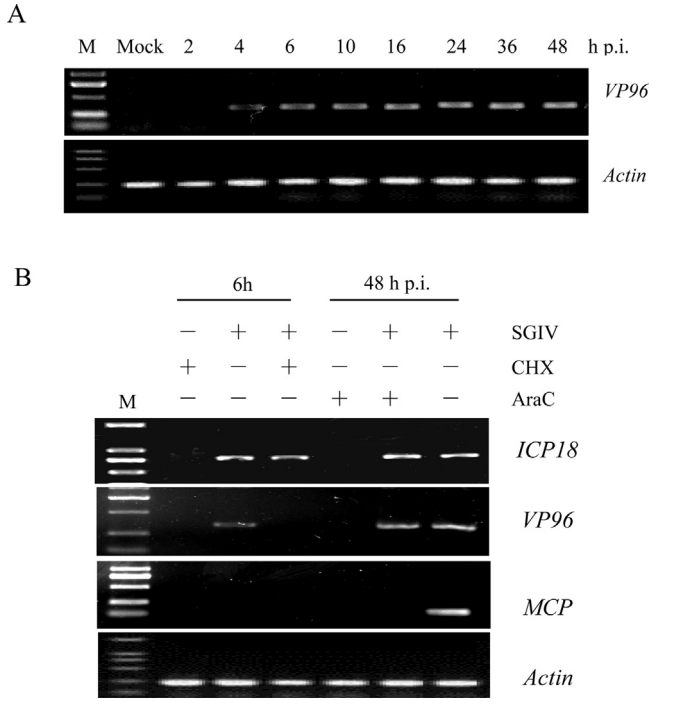

Fig. 2. VP96 was an early (E) gene during SGIV infection. (A) RT-PCR examination of SGIV *VP96* transcript at different time points. (C) RT-PCR detection of SGIV *VP96* transcripts under drug treatments.

2.3. SGIV-VP96 localized in cytoplasm

Subcellular localization of SGIV-VP96 was determined by pEGFP-VP96 fusion protein expression and immunofluorescence assay. In pEGFP-VP96 transfected cells, green fluorescence was observed in the cytoplasm. Some aggregated spots were also observed in a small minority of cells. As a control, the pEGFP-N_3 vector transfected cells emitted green fluorescence in both the cytoplasm and the nucleus (Fig. 3A). To examine the intracellular expression of SGIV-VP96 during SGIV infection, immunofluorescence assay was performed using anti-VP96 antiserum. As shown in Fig. 3B, the green fluorescence was distributed in the cytoplasm in the majority of SGIV infected cells (Fig. 3B, middle row). Some bright spots were observed in a small minority of infected cells (below row). No fluorescence signal was detected in mock-infected cells (Fig. 3B, upper row).

2.4. Overexpression of SGIV-VP96 improved cell viability and affected infectious virus production

To detect the function of SGIV-VP96 *in vitro*, we established stable FHM cell lines expressing full length VP96 and its mutants (Fig. 4A), including FHM-VP96 (VP96), FHM-VP96-ΔCRD1 (VP96-ΔCRD1), FHM-VP96-ΔCRD2 (VP96-ΔCRD2) and FHM-pcDNA (vector/control). The expression of VP96, VP96-ΔCRD1 and VP96-ΔCRD2 in stable cells was identified by RT-PCR (Fig. 4B). The effect of SGIV-VP96 on cell proliferation in FHM cells was examined. As shown in Fig. 4C, the number of VP96 overexpressing cells was about 1.4 folds than that of VP96-ΔCRD1, VP96-ΔCRD2 overexpressing or vector alone cells at day 6, indicating that SGIV-VP96 could promote fish cell proliferation, and the two CRDs were essential for this function.

To evaluate the effect of VP96 on the cell viability after SGIV infection, trypan blue exclusion assay was performed and the percentage of viable cells was calculated. As shown in Fig. 4D, the viability of vector cells was $48.5 \pm 2.00\%$, $27.3 \pm 1.08\%$, $10.36 \pm 3.09\%$ at 24 h, 36 h and 48 h p.i., respectively. In contrast, the viability of SGIV-VP96 expressing cells was $66.5 \pm 3.05\%$, $48.62 \pm 4.18\%$, $27.45 \pm 2.63\%$, respectively. Virus titer assay revealed that overexpression of VP96 significantly inhibited the virus production compared with vector cells (Fig. 4E). Together, our results indicated that overexpression of SGIV-VP96 could enhance the cell viability and affected virus production after SGIV infection.

2.5. Overexpression of SGIV-VP96 decreased virus infection induced apoptosis

Our previous studies showed that SGIV could induce apoptosis in FHM cells (Huang et al., 2011a,b). To determine the roles of SGIV-VP96 on virus-induced apoptosis in FHM cells, the nuclear morphology was examined under fluorescence microscopy. As shown in Fig. 5A , although the apoptotic bodies could be observed in both infected cells, the number of the apoptotic bodies in vector cells was greater than that in SGIV-VP96 expressing cells. In detail, the apoptotic percentage was decreased from $33.05 \pm 1.29\%$ (vector cells) to $18.75 \pm 0.64\%$ (VP96 overexpressing cells) at 18 h p.i. (Fig. 5B). Given that induction of apoptosis results in the activation of caspases-3, we also detected the effect of VP96 overexpression on caspase-3 activity induced by SGIV infection. The results showed that SGIV infection induced caspase-3 activity was significantly reduced in VP96 overexpressing cells compared with vector cells. Detailedly, the caspase-3 activity in SGIV-infected VP96 expressing

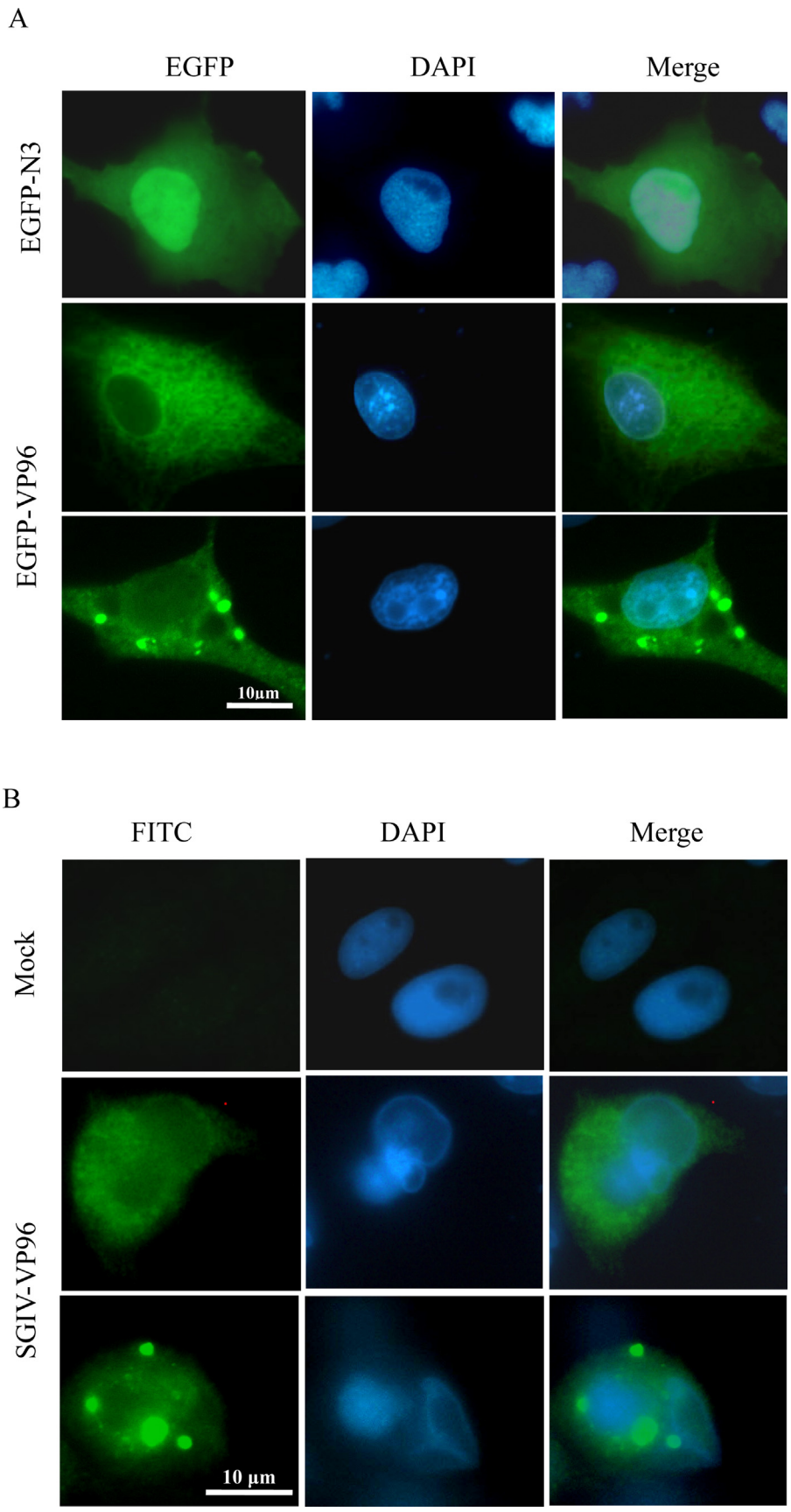

Fig. 3. SGIV-VP96 localized in cytoplasm. (A) The subcellular localization of SGIV-VP96 in transfected cells. The plasmid pEGFP-VP96 or pEGFP-N_3 was transfected into GS cells, and then stained with DAPI. The fluorescence was observed under fluorescence microscopy. (B) Intracellular localization of SGIV-VP96 in virus-infected GS cells. Cells were infected with SGIV and immuno-fluorescence assay was performed using anti-VP96 serum.

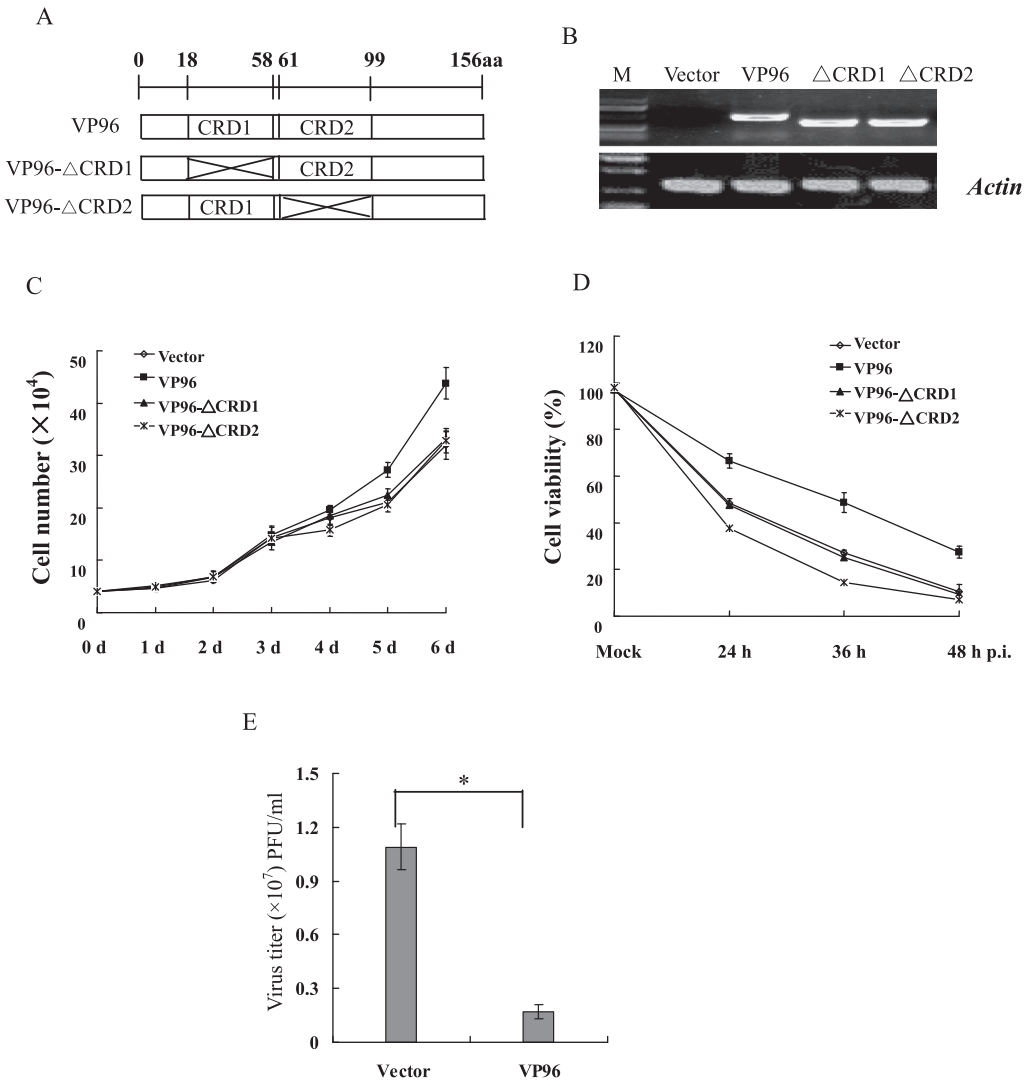

Fig. 4. VP96 overexpression enhanced cell proliferation and increased cell viability after SGIV infection. (A) The diagram of VP96 mutants VP96-ΔCRD1 and VP96-ΔCRD2. (B) VP96, VP96-ΔCRD1 or VP96-ΔCRD2 expression was confirmed by RT-PCR in stable cell line. (C) Overexpression of VP96 enhanced cell proliferation *in vitro*. (D) Overexpression of VP96 increased the cell viability after SGIV infection. The viability evaluation was performed using trypan blue exclusion assay. (E) Overexpression of VP96 decreased virus production.

cells was decreased by 1.5 fold compared with that in vector cells at 18 h p.i. (Fig. 5C). While, no significant increase was observed in SGIV infected control cells after treatment with caspase-3 inhibitor Z-DEVD-FMK. Evidently, SGIV-VP96 overexpression had a preventive effect on apoptosis induced by SGIV infection in FHM cells.

3. Discussion

Apoptosis is an important tactic for host to limit viral replication by killing virus infected cells (Roulston et al., 1999). Virus could also exploit certain viral products that effectively suppress or delay apoptosis, thereby either prolonging the survival of infected cells for the production of abundant progeny virus or facilitating the establishment of virus persistent infection (Cuconati and White, 2002; Benedict et al., 1999; Meseda et al., 2000). Orthopoxviruses encoded several functional TNFRs, and these vTNFRs were capable of binding with high affinity to different TNF or LTα (Benedict et al., 1999; Smith et al., 1996). It has been reported that the binding of TNF or LTα to the cellular TNF receptors (TNFRs) triggered intracellular signaling for regulating the immune and inflammatory responses and programmed cell death (Locksley et al., 2001; Benedict et al., 2003). Therefore, vTNFRs could prevent the biological activity of TNF or LTα and contribute to virus replication by binding to cellular TNF or LTα (Smith et al., 1996). For cowpox virus, cytokine response modifier E (CrmE) was found to bind human TNF and block the binding of TNF to high-affinity TNFRs on the cell surface (Saraiva and Alcami, 2001). Exploring the roles of virus encoded TNFR like proteins might provide insight into the development of novel immune-modulating therapies (Loparev et al., 1998). Like other large DNA viruses from mammals, iridoviruses also encoded one or more TNFR homologs (Song et al., 2004; Tidona and Darai, 1997; Tsai et al., 2005; Zhang et al., 2004). LCDV-1 TNFR-like protein has been proposed to be a "viroceptor" and expected to bind to the fish cytokine TNF and neutralized its effects (Essbauer et al., 2004). To our knowledge, the function of iridovirus vTNFRs remained largely unknown.

The complete genome annotation indicated that SGIV genome contained three TNFR like genes, including SGIV-VP50 (ORF50L), SGIV-VP51 (ORF51L) and SGIV-VP96 (ORF96R) (Song et al., 2004). Based on the amino acid alignment analysis, we found that SGIV-VP96 contained two CRD domains which are typical characteristics

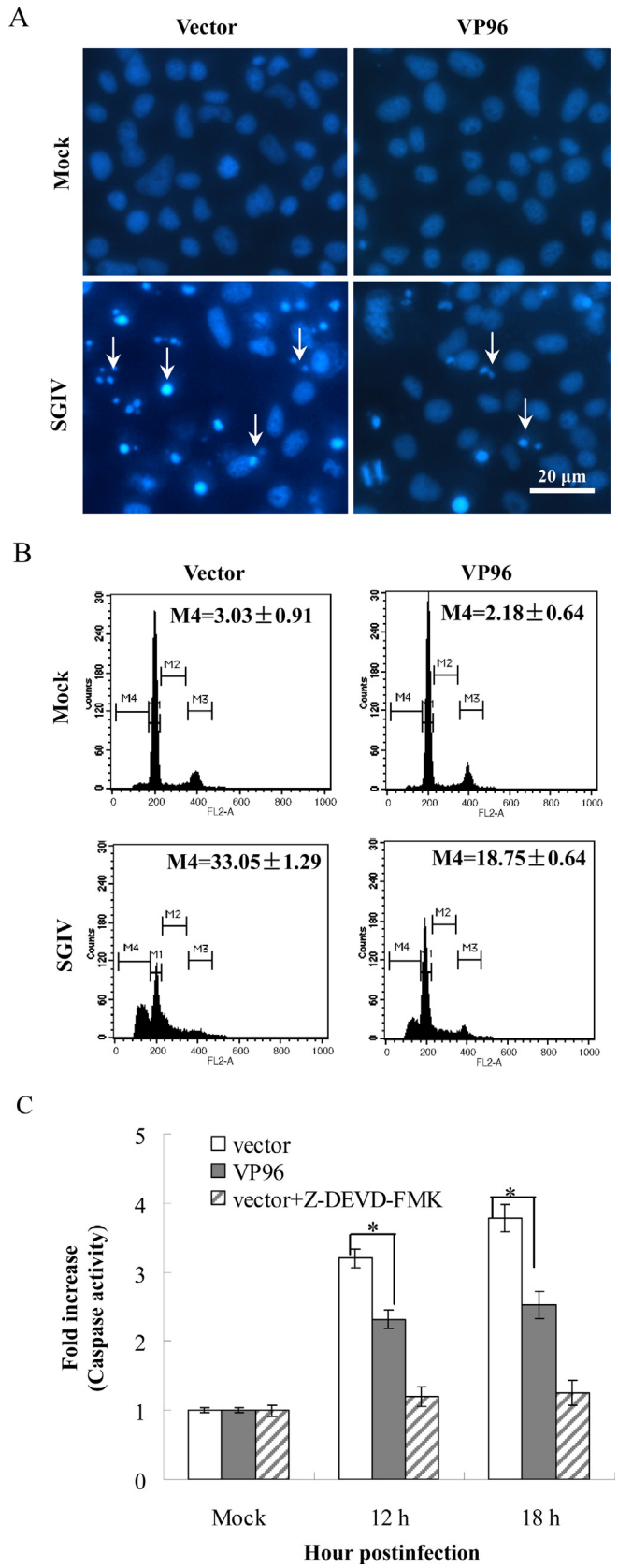

Fig. 5. Overexpression of VP96 inhibited SGIV infection induced apoptosis in FHM cells. (A) Nuclear morphology of SGIV infection in VP96 expressing cells and vector cells. Cells were infected with SGIV at 2 MOI, and stained with Hoechst 33342 at 18 h p.i. The fluorescence was examined under fluorescence microscopy. (B) Flow cytometry analysis of apoptotic cells in VP96 expressing cells and vector cells at 18 h p.i. The percentage of sub-G0/G1 phase was calculated and indicated on the histogram. (C) Overexpression of VP96 inhibited the caspase-3 activation induced by SGIV in FHM cells. Cells or Z-DEVD-FMK treated cells were infected with SGIV, and the cell lysates were collected at indicated time points (0, 12, 18 h p.i.). The caspase-3 activity was examined as described in Section 4.

of the TNF receptor superfamily members (Hehlgans and Pfeffer, 2005). Similar to other vTNFRs, SGIV-VP96 shared sequence similarity to regions of the extracellular domain of cellular TNFRs, and lacked both the membrane anchor and cytoplasmic domains for signaling (Saraiva et al., 2002; Seet et al., 2003). The structural variation between vTNFRs and their cellular counterparts led to the different function of them during virus infection. Once binding to vTNFRs, TNF or LTα could not bind to cellular TNFRs and their biological activity were prevented, including the initiation of intracellular signaling for regulating the immune and inflammatory responses and programmed cell death (Saraiva et al., 2001). Consistent with the data from viral DNA microarray (Chen et al., 2006), the drug inhibition assay revealed that SGIV-VP96 was an early gene and different form the expression profile of the other two TNFR homologs, SGIV-VP50 and VP51 (data not shown). We speculated that SGIV encoded three TNFR like genes might play different roles at different stages of virus infection. For cowpox virus, the CrmB gene was expressed at early stage of infection, whereas CrmC and CrmD were expressed at late stage of cowpox virus infection (Saraiva et al., 2002).

It has been demonstrated that overexpression of viral inhibitor of apoptosis genes could enhance virus infection induced cell viability. These functions of viral genes were considered as a notable strategy for virus to regulate the host apoptosis-off system for enhancing progeny production (Hong et al., 2002). As a large DNA virus, iridovirus from different genus have been proved to induce apoptosis in fish cells. Several iridovirus protein products were capable of inhibiting apoptosis induced by different stimuli, suggested that these viral genes might contribute to virus replication by regulation of apoptosis during virus infection (Lin et al., 2008; Huang et al., 2007a,b). In our study, overexpression of SGIV-VP96 *in vitro* increased cell proliferation significantly. Moreover, SGIV infection evoked apoptotic cell death and virus titer were markedly inhibited in SGIV-VP96 expressing cells. Notably, the CRD1 and CRD2 domains were essential for SGIV-VP96 to perform these functions. We speculated that SGIV might utilize VP96 to regulate the host apoptosis-off system for enhancing progeny production like infectious pancreatic necrosis virus (IPNV) VP5 protein (Hong et al., 2002). As a TNFR homolog encoded by Myxoma virus, M-T2 not only could inhibit TNF-alpha-mediated cytotoxicity, but also prevented virus-induced lymphocyte apoptosis by an as yet undefined mechanism (Schreiber et al., 1997). In addition, overexpression of poxvirus CrmE protein was able to inhibit TNF-alpha or TNFR1-induced cell death (Sedger et al., 2006). Whether SGIV-VP96 regulated cell apoptosis by binding to cellular TNF or LTα, and prevented TNF/TNFR moelcules modulated cell death still remained unknown. Accompanied by the increase of gene information of grouper TNF/TNFR molecules, we will investigate the interaction between VP96 and cellular TNFRs in the further study.

In conclusion, our data described a novel vTNFR-like gene encoded by SGIV. SGIV-VP96 was identified as a viral early gene which localized in cytoplasm. Moreover, the overexpression of SGIV-VP96 could increase the cell proliferation *in vitro*, and inhibit virus induced apoptosis. These results provided the vital clue to study the role of vTNFR homologs from iridovirus, and contributed to understanding the iridovirus pathogenesis.

Table 1
Sequences of primers used in this study.

Name	Sequence (5′–3′)
VP96-P1	GAAGGATCCATGTTAGTAGTAGAGATGTTAG
VP96-P2	AGTTCCTCGAGCTAGGTAGAAAATTCTTAA
VP96-P3	GAAAAGCTTCCATGTTAGTAGTAGAGATGTTA
VP96-P4	AAAGGATCCAAACGCGCAAATTTTGT
VP96-P5	AATGGATCCAGATGTTAGTAGTAGAGATGTTAGC
VP96-P6	GATCTCGAGGCTAGGTAGAAAATTCTTAA
VP96-P7	GTGGAATCGCTTGTCCCGTGCGGAAACGAATCGTAT
VP96-P8	TCCGCACGGGACAAGCGATTCCACATAGACTAC
VP96-P9	GTCTGCGTCCCGGGCAAATACGGATCGGTGCGC
VP96-P10	TCCGTATTTGCCCGGGACGCAGACGGTGGGCGCAGA
VP96-P11	AGTAGTCTATGTGGAATCGCT
VP96-P12	AGTCGCCATCTGCACATTA
Actin-PF	TACGAGCTGCCTGACGGACA
Actin-PR	GGCTGTGATCTCCTTTTGCA

4. Materials and methods

4.1. Cells and virus

Grouper embryonic (GE) cells from the brown-spotted grouper E. tauvina were cultured in Eagle's minimum essential medium containing 10% fetal bovine serum (Chew-Lim et al., 1994), and Grouper spleen (GS) cells from red-spotted grouper Epinephelus akaara were grown in Leibovitz's L-15 medium containing 10% fetal bovine serum (FBS) at 25 °C (Huang et al., 2009). Fathead minnow (FHM) cells were grown in M199 containing 10% fetal bovine serum at 25 °C. Cells were infected with SGIV at a multiplicity of infection (MOI) of 2. SGIV were propagated in GE cells and virus genomic DNA isolation was carried out as described previously (Qin et al., 2003).

4.2. Computer-assisted analysis, plasmid construction and antiserum preparation

Homology analyses were performed using the BLAST network server of the National Center for Biotechnology Information. Conserved domain and motif analyses were carried out using SMART search program. The multiple alignment of amino acid sequence of SGIV ORF096R with TNFRs from other species was performed by using ClustalX 1.83, followed by manual adjustments with GeneDoc program.

The full length of SGIV *ORF096R* (VP96) was cloned by PCR from SGIV genomic DNA using a pair of primers: P1/P2 (Table 1). The fragment was cloned into prokaryotic expression vector pET32-a (+) to obtain recombinant plasmid pET-VP96. Using another pair of primers (P3/P4), we cloned *VP96* gene into eukaryotic expression vectors pEGFP-N_3, and obtained recombinant plasmid pEGFP-VP96. To identify the function of VP96 and the roles of its CRDs domain *in vitro*, VP96, VP96-ΔCRD1 and VP96-ΔCRD2 were cloned into pcDNA3.1-HA vector using different sets of primers P5/P6, P5/P7/P8/P6 and P5/P9/P10/P6 respectively. The mutant VP96-ΔCRD1 and VP96-ΔCRD2 were amplified by overlapping method. The constructed plasmids were confirmed by DNA sequencing.

To prepare the antibody against VP96, *E. coli* BL21 cells containing pET-VP96 were induced by IPTG and purified using the HisBind Purification Kit (Novagen) according to the manufacturer's instruction. The purified VP96 fusion protein was injected into mouse, and the anti-VP96 serum was collected and analyzed by western blotting as described previously (Huang et al., 2008).

4.3. RT-PCR

To detect VP96 transcriptional profile during SGIV infection *in vitro*, total RNA was extracted from mock- and virus-infected GS cells at 2, 4, 6 10, 16, 24, 36, and 48 h post-infection (p.i.) using SV total RNA isolation kit (Promega) according to manufacturer's instructions. Reverse transcription reactions were carried out on 2 μg of total RNA with the ReverTra Ace qPCR RT kit (TOYOBO) (Huang et al., 2011a,b). The resulting cDNA was stored at −80 °C until used. PCR amplification of VP96 was performed using a pair of gene-specific primers P11/P12. Detection of β-actin mRNA was taken as an internal control using the primers actin-PF/actin-PR (Table 1).

4.4. Cell transfection and establishment of stable transfectants

Cells were cultured into 24-well plates overnight. The plasmid pEGFP-N3 and pEGFP-VP96 were transfected using Lipofectamine 2000 (invitrogen) according to the manufacturer's instructions.

To identify the roles of SGIV VP96 *in vitro*, the stable transfectants were established as described previously (Xia et al., 2010). In brief, FHM cells transfected with plasmid pcDNA3.1-HA, pcDNA3.1-HA-VP96 (VP96), pcDNA3.1-HA-VP96-ΔCRD1 (VP96-ΔCRD1) and pcDNA3.1-HA-VP96-ΔCRD2 (VP96-ΔCRD2) were further cultured in complete medium containing 400 μg/ml G418 (Sigma) and selected for 6 weeks. The expression of VP96, VP96-ΔCRD1 and VP96-ΔCRD2 gene was confirmed by RT-PCR using primer P5/P6.

4.5. Fluorescence microscopy

To examine the subcellular localization of SGIV VP96 in fish cells, pEGFP-VP96 or pEGFP-N_3 transfected cells were fixed with 4% paraformaldehyde for 1 h at 4 °C, and then stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min. Finally, fluorescence signal was detected under fluorescent microscopy (Zeiss). The images were processed using the adobe photoshop program.

To analyze the intracellular localization of VP96 during SGIV infection, immune fluorescence assay was performed as Huang et al. (2008). Briefly, mock- and SGIV-infected GS cells were fixed in 4% paraformaldehyde at 24 h p.i. The coverslips were incubated with primary antibodies anti-VP96 (1:50) followed by incubation with FITC-conjugated goat anti-mouse IgG. After staining the nuclei with 1 μg/ml DAPI (Sigma), samples were examined under fluorescence microscope (Zeiss).

4.6. Cell proliferation, cell viability and virus titer determination

For cell proliferation assay, FHM cells stable expressing VP96, VP96-ΔCRD1, VP96-ΔCRD2 or the vector were seeded into 12-well plates at an initial density of 4×10^4 cells per well and cultured at 25 °C for 6 days. Cells from triplicate wells were trypsinized, and harvested daily. The cell numbers were counted by six independent hemocytometer counts under a light microscopy.

To detect the effect of VP96 on the cell viability during SGIV infection, FHM cells stable expressing VP96, VP96-ΔCRD1, VP96-ΔCRD2 or the vector were seeded in 24-wells, and the cells were infected with multiplication of infection (MOI) 2 for 24, 36 and 48 h. At the indicated time points, samples were examined by light microscopy using phase-contrast optics (Zeiss), following which the cell viability was determined using trypan blue dye-exclusion assay. Both floating and adherent cells were harvested by trypsinization, and stained with 0.2% trypan blue. The cell viability was presented as the percentage of live cells over the total number of cells. Cell viability assays were performed in triplicate, and cells were counted by three independent hemocytometer counts for each time.

To determine the effect of VP96 on virus production, virus titer assay was performed on monolayers of cells in triplicate as described previously (Huang et al., 2011a,b).

4.7. Flow cytometric analysis

To determine the effect of VP96 on SGIV induced apoptosis, FHM cells stable expressing VP96 or the vector were infected with SGIV. At 18 h p.i., the percentages of apoptotic cells were determined by flow cytometry as described previously (Huang et al., 2011a,b). Mock- and SGIV-infected cells were harvested and fixed in 70% ice-cold ethanol overnight at −20 °C. After washing with PBS, the cells were centrifuged at 500 × *g*, and then stained for 30 min in PBS containing propidium iodide (PI, 50 μg/ml, Sigma) and RNase A (100 μg/ml, Sigma). The PI fluorescence was measured with a FACScan flow cytometer (Becton–Dickinson), 1×10^4 cells were analyzed for each sample. The obtained data was analyzed using the Cellquest software. All samples were carried out in triplicate, and the data were represented as means ± SD.

4.8. Caspase-3 activity analysis

Caspase-3 activity was determined using caspase-3/CPP32 colorimetric assay kit (BioVision) as described previously (Hu et al., 2004; Zhao et al., 2010). In this assay the capacity of the caspase-3 to cleave the labeled substrate DEVD-*p*-nitroaniline (DEVD-*p*NA) was measured spectrophotometrically. In brief, FHM cells stable expressing VP96 or the vector were seeded in 24-wells for 18 h, and then infected with SGIV at 2 MOI. After infection, cells were harvested by trypsinization at 12 h and 18 h p.i., respectively. The Caspase-3 activity analyses were carried out according to the manufacturer's instruction, and the data were read at 405 nm using a multilabel plate reader (PerkinElmer). All samples were carried out in triplicate, and the relative caspase 3 activity was expressed as the ratio between the absorbance of *p*NA from the treated sample and that from mock-infected cells.

4.9. Statistical analysis

Results were expressed as means ± standard deviations. Statistical comparisons were carried out by using Student's *t* test, and differences between groups were considered significant if the *p* value < 0.05.

Acknowledgements

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