An insulin-like growth factor homologue of Singapore grouper iridovirus modulates cell proliferation, apoptosis and enhances viral replication

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Insulin-like growth factors (IGFs) play crucial roles in regulating cell differentiation, proliferation and apoptosis. In this study, a novel IGF homologue gene (IGF-like) encoded by Singapore grouper iridovirus (SGIV) ORF062R (termed SGIV–IGF), was cloned and characterized. The coding region of SGIV–IGF is 771 bp in length, with a variable number of tandem repeats (VNTR) locus at the 3′-end. We cloned one isoform of this novel gene, 582 bp in length, containing the predicted IGF domain and 3.6 copy numbers of the 27 bp repeat unit. SGIV–IGF was an early transcribed gene during viral infection, and SGIV–IGF was distributed predominantly in the cytoplasm with a diffused granular appearance. Intriguingly, overexpression of SGIV–IGF was able to promote the growth of grouper embryonic cells (GP cells) by promoting G1/S phase transition, which was at least partially dependent on its 3′-end VNTR locus. Furthermore, viral titre assay and real-time quantitative PCR (RT-qPCR) analysis proved that SGIV–IGF could promote SGIV replication in grouper cells. In addition, overexpression of SGIV–IGF mildly facilitated apoptosis in SGIV-infected non-host fathead minnow (FHM) cells. Together, our study demonstrated a novel functional gene of SGIV which may regulate viral replication and cellular processes through multiple mechanisms that appear to be cell type-dependent.

INTRODUCTION

The insulin-like growth factors family (IGFs), including IGF-I and IGF-II, and their receptors are implicated in modulating a variety of cellular processes, such as cell proliferation, tissue differentiation and apoptosis in a multitude of cell types (Laviola et al., 2007). IGF-I and IGF-II are polypeptide hormones structurally related to insulin, all of which contain the conserved IIGF domain, and belong to an integrated growth factor system that includes these three hormones, at least four cell surface receptors and six IGF binding proteins (Cohick & Clemmons, 1993). In contrast to insulin, IGFs are not exclusively synthesized in specialized cells within specific tissues, but are produced by a wide variety of cell types in the body (Dupont et al., 2003). In mammals, IGF-I is thought to be more important for postnatal growth and development (Baker et al., 1993), whereas IGF-II plays a critical role in embryonic and fetal development, and its role in the postnatal period is substituted by IGF-I (DeChiara et al., 1990).

Mechanistically, IGFs were proved to be implicated in regulating cell cycle progression and proliferation of a number of cell types, including both normal cells and cancer cells (Dupont et al., 2000; Hellawell et al., 2002; Jones & Clemmons, 1995; Lai et al., 2001; Ren et al., 2009; Vanderburg et al., 1990). IGF-I is a mitogenic factor for cortical precursors during embryonic development, by increasing the phosphorylation of Akt at 30 min, increasing cyclins D1 and E protein levels by 6 h, and downregulating
expression of the cyclin-dependent kinase (CDK) inhibitors p27^KIP1^ and p57^KIP2^ (Mairet-Coello et al., 2009). In addition, IGF-I can stimulate breast cancer cell MCF-7 replication by inducing cyclin D1 expression through the phosphatidylinositol-3 kinase–Akt pathway and triggering cyclin D1 nuclear accumulation (Dupont et al., 2000; Hamelers et al., 2002). Like IGF-I, IGF-II is also a positive mediator of the G1/S transition by increasing cyclin D1 expression. In mammary epithelial cells, IGF-II directly increases cyclin D1 expression and thus stimulates cell proliferation (Brisken et al., 2002).

Another layer of cellular regulation by IGFs, which is complementary to stimulation of cell proliferation, is their capacity to protect cells from a wide range of apoptotic injuries. A number of studies have concluded that both IGF-I and IGF-II inhibit apoptosis in certain cell types. IGF-I was able to inhibit the mitochondrial apoptosis programme in both normal human mesangial cells and SV40-transformed murine mesangial cells after exposure to high glucose, through the recruitment of both Akt/PKB and ERK subfamilies of mitogen-activated protein kinases (Kang et al., 2003). Furthermore, Hill et al. (2012) found that villous cytotrophoblast cells isolated from first trimester and term human placenta underwent spontaneous–TNFα-villous cytotrophoblast cells isolated from first trimester and term human placenta underwent spontaneous–TNFα-induced apoptosis of human MG63 osteosarcoma cells (Raile et al., 1998). Kawakami et al. (1998) reported that Fas-mediated apoptosis in human osteoblasts was augmented by IGF-I. All of the above cases indicate that IGFs serve as co-activators of both pro- and anti-apoptotic pathways in a cell type- and/or condition-dependent manner.

Multiple isoforms of IGFs have been identified in human, mouse, bovine, fish, etc. (Dupont et al., 2003; Jones & Clemmons, 1995; Laviola et al., 2007). However, to our knowledge, no IGF homologue has yet been functionally revealed in viruses. Iridoviruses are DNA viruses with large genomes that infect only invertebrates and poikilothermic vertebrates (Chinchar, 2002). In recent years, iridoviruses have gained more attention because of the high mortality and serious systemic diseases they can cause in aquaculture practice. Singapore grouper iridovirus (SGIV) is a newly identified member of the genus Ranavirus, family Iridoviridae (Qin et al., 2001, 2003), and has caused significant economic losses in grouper aquaculture in China and South-East Asian countries. The SGIV genome sequence has been determined, and in silico genomic analysis of SGIV suggested that a number of potential viral gene products may be involved in virus–host interactions (Song et al., 2004). In this study, we demonstrate a putative insulin-like growth factor homologue gene encoded by SGIV ORF062R, and term it SGIV–IGF. SGIV–IGF shares a conserved IIGF domain with other reported IGF genes of different species from fish to mammals. We found that SGIV–IGF was an early gene during viral infection and SGIV–IGF was distributed mainly in the cytoplasm. Furthermore, SGIV–IGF could promote cell proliferation and facilitate viral replication, as well as regulate SGIV-induced cell apoptosis, suggesting that SGIV–IGF could regulate cellular processes through multiple mechanisms.

**RESULTS**

**Cloning and characterization of the SGIV–IGF gene**

The previously annotated SGIV genome shows that SGIV ORF062R (GenBank accession number YP_164157) is 771 bp in length, with a long tandem repeat locus at the 3'-end. In silico analysis demonstrated that this gene encodes an IGF homologue, suggesting it may play important roles during viral infection (Song et al., 2004). We designed cloning primers based on the predicted ORF062R region to amplify the full length of SGIV ORF062R. A variety of fragments with different lengths were amplified from the SGIV genomic DNA. DNA sequencing showed that all of these products were from SGIV ORF062R (data not shown). Thus, we chose the clone with the longest sequence, which was 582 bp in length and contained the predicted IIGF domain and 3.6 copy numbers of the repeat unit, for further characterization and functional analysis. The deduced amino acid sequence of SGIV ORF062R shares 92% similarity with orthologue of grouper iridovirus (GIV, AAV91059), 50% with IGF-II of Siniperca chuatsi (ADO14143) and 34% with IGF-II of Cynoglossus semilaevis (ACM43292). Furthermore, SGIV ORF062R also shares about 40% identity with IGF homologues from frog (AAH72153), mouse (EDL21461.1) and human (ADO21454). Sequence analysis shows SGIV ORF062R contains a conserved IIGF domain at its N-terminal, and shares many conserved residues with other reported IGFs (Fig. S1a, b, available in JGV Online). Given the context of the conserved IIGF domain, we termed SGIV ORF062R the SGIV–IGF gene.

To better delineate the effect of the 3'-end tandem repeat region in SGIV–IGF, we cloned a fragment, 498 bp in length, which contains the predicted IIGF domain without any 3'-end repeat unit, into pcDNA3.1(+) and pEGFP-N3. The resulting constructs were termed as pcDNA-ΔIGF and pEGFP-ΔIGF, respectively.

**Expression pattern of SGIV–IGF in SGIV-infected grouper embryonic cells**

To determine the temporal transcription pattern of SGIV–IGF during in vitro viral infection, we examined its transcriptional kinetics at consecutive time points after SGIV infection. As shown in Fig. 1(a), SGIV–IGF mRNA could be detected as early as 6 h after SGIV inoculation,
with increasing enrichment throughout by the viral infection process, suggesting it is transcribed and expressed at early stages of SGIV infection.

To further determine the expression pattern of SGIV–IGF, we performed CHX and AraC inhibition assays on SGIV-infected grouper embryonic cells (GP cells). Three previously characterized SGIV genes, including immediate-early (IE) transcription gene ICP18 (Xia et al., 2009), early (E) gene dUTPase (Gong et al., 2010) and the late (L) gene encoding major capsid protein (MCP) were chosen as indicative controls. As shown in Fig. 1(b), we can efficiently inhibit protein or DNA synthesis using CHX and AraC, respectively. In detail, after viral infection of the drug-treated cells, SGIV ICP18 was expressed regardless of the absence or presence of CHX and AraC, while SGIV dUTPase transcript was only detected in the AraC-treated cells, and transcription of MCP was inhibited by each of the drugs and only presented in the control cells after SGIV infection for 48 h (Fig. 1b). The transcription pattern of SGIV–IGF is similar to that of SGIV dUTPase, an early transcription gene which can be only inhibited in the presence of CHX but not AraC, suggesting that SGIV–IGF is an early transcribed gene during infection.

Subcellular localization of SGIV–IGF

The intracellular distribution of SGIV–IGF was assessed by detecting the localization of SGIV–IGF-GFP fusion proteins in GP and fathead minnow (FHM) cells. The green fluorescence in pEGFP-IGF transfected cells was predominantly aggregated in the cytoplasm with granular appearance and diffuse patterns (Fig. 2a, b, middle row). Similar fluorescence signal was also observed in pEGFP-AIGF-transfected cells (Fig. 2a, b, lower row). As a control, GFP expressed by the empty vector pEGFP-N3 was distributed in whole cells and mainly in the nucleus of fish cells (Fig. 2a, b, upper row). These results indicate that SGIV–IGF is exclusively localized to and exerts functions in the cytoplasm. Furthermore, the repeat region at its C-terminal is irrelevant to its intracellular distribution.

SGIV–IGF promotes proliferation of fish cells

To evaluate the effect of SGIV–IGF on cell proliferation, we generated GP cell lines that stably express full-length SGIV–IGF (GP/pcDNA-IGF) or a C-terminal-truncated form of SGIV–IGF (GP/pcDNA-DIGF). As a control, we also established a stable line that was transfected with an empty vector pcDNA3.1 (+) (Fig. 3b). Then we compared the proliferation rates of these stable lines. We found SGIV–IGF or SGIV-DIGF expressing cells were not morphologically distinguishable from control cells, but both showed higher density monolayers than the control line (Fig. 3a). In addition, the growth curves showed an obvious alteration between the growth of SGIV–IGF expressing cells and the control cell line. Under the same culture condition for indicated periods, the number of GP/pcDNA-IGF cells was significantly higher than that of control cells from day 3 ($P < 0.01$). Moreover, GP/pcDNA-AIGF cells also showed a higher growth curve than the control line ($P < 0.05$ from day 3), but a relatively lower level than cells that express full-length SGIV–IGF ($P < 0.05$ at day 3) (Fig. 3c). Similar results were also observed with stable FHM cell lines (data not shown).
The impact of SGIV–IGF on cell proliferation was further confirmed by colony formation assays. In agreement with the results shown above, GP cells that stably express SGIV–IGF demonstrated more obvious colonies after 4–5 weeks culturing, as indicated by higher colony numbers, as well as bigger colonies, compared to the control group (Fig. 3d, e). These results suggest that SGIV–IGF can promote cell cycle progression into S phase and consequently enhance cell proliferation.

SGIV–IGF promotes cell cycle S phase entry

To delineate the mechanism by which SGIV–IGF promotes cell proliferation, we analysed the cell cycle profiles of GP/pcDNA-IGF and control cells by flow cytometry. As shown in Fig. 4(a), compared to the control group, SGIV–IGF expressing cells were associated with a decreased proportion of cells in phase G0/G1 which was accompanied by a significantly increased cell proportion in the S phase. However, the population of phase G2/M was not obviously altered. An approximately 1.7-fold higher proportion of S phase cells was detected in SGIV–IGF expressing cells than in the control group (P<0.01) (Fig. 4b). In detail, for the GP/pcDNA-IGF cell line, the populations of G0/G1 phase, S phase and G2/M phase were 64.99 %, 27.56 % and 7.45 %, respectively, whereas they were 79.47 %, 10.07 % and 10.46 % in the control line. These data suggest that SGIV–IGF can promote cell cycle progression into S phase.

Overexpression of SGIV–IGF enhanced SGIV replication during in vitro infection

Given our finding that SGIV–IGF was able to promote cell proliferation, we next determined if it is relevant to the success of SGIV infection. To do this, stable GP/pcDNA-IGF and GP/pcDNA3.1 cells were infected with SGIV individually, and the viral replication kinetic curves were examined and compared. As shown in Fig. 5(a), the replication kinetics of SGIV in GP/pcDNA-IGF was quite different to that in GP/pcDNA3.1. In detail, the viral titres in the two stable lines were quite similar before 12 h post infection (p.i.). After 12 h p.i., the viral titres in GP/pcDNA-IGF began to increase more quickly than that in GP/pcDNA3.1. During the period from 24 to 48 h p.i., the viral titres yielded from SGIV–IGF expressing cells were almost 10-fold higher than that in the control group (P<0.01).

To verify the above results, the transcription kinetics of three indicative SGIV genes, including SGIV ICP18, dUTPase and MCP, were measured by RT-qPCR. As shown in Fig. 5(b), expression levels of all these genes were much higher in GP/pcDNA-IGF cells than that in GP/pcDNA3.1 at 24, 48 and 72 h p.i., individually. Taken together, these data suggest that overexpression of SGIV–IGF could enhance SGIV replication during viral infection in vitro.

Overexpression of SGIV–IGF facilitated apoptosis induced by SGIV

To test whether SGIV–IGF is involved in SGIV-induced apoptosis during viral infection, we first compared the nuclear morphological features of SGIV-infected FHM/pcDNA3.1 and FHM/pcDNA-IGF cells (Fig. 6b). As shown in Fig. 6(a), more apoptotic characteristics, such as nuclear condensation and apoptotic body formation were exclusively observed in FHM/pcDNA-IGF cells after SGIV infection.

We next examined the activity of caspase-3 in these two stable lines after SGIV infection. As shown in Fig. 6(c), caspase-3 activity was detectable at as early as 6 h after viral infection, followed by gradual increases until 18 h p.i., suggesting caspase-3 activation is involved in SGIV-induced FHM apoptosis. Notably, during the whole course of infection, the activity of caspase-3 in FHM/pcDNA-IGF cells was significantly higher than that in FHM/pcDNA3.1 cells (P<0.01).
of SGIV infection, especially from 6 to 24 h p.i., the caspase-3 activity remained >1.6-fold higher in SGIV–IGF expressing cells in comparison to the control group. In addition, we performed flow cytometry to assess the percentage of apoptotic cells in two SGIV-infected stable lines. At 24 h p.i., the percentage of apoptotic cells in the FHM/pcDNA-IGF line was 32.78 %, while it was only 18.94 % in the control line (Fig. 6d). These results indicate that SGIV–IGF may serve as a positive mediator in SGIV-induced apoptosis in non-host cells.
DISCUSSION

IGFs can bind with high affinity to their cognate membrane receptors, IGF-I receptor and IGF-II receptor, and thus transduce external signals to distinct intracellular pathways leading to the activation of multiple cellular processes such as proliferation, differentiation and apoptosis (Laviola et al., 2007). In the past two decades, the complex IGF system has been widely studied and found to be highly conserved from teleost to mammalian species (Planas et al., 2000). Some viruses, especially the DNA viruses, can encode a repertoire of homologues of host genes which may regulate the host immune response, cell proliferation, and apoptosis (Holzerlandt et al., 2002). However, no IGF homologue has been functionally identified in viruses based on our current knowledge. In the present study, we identify a novel viral IGF homologue encoded by SGIV, a recently identified iridovirus belonging to genus Ranavirus, family Iridoviridae.

Based on the previous genomic annotation of SGIV (Song et al., 2004; Teng et al., 2008), we analysed in detail the sequences of the putative 162 SGIV ORFs. Intriguingly, we found that SGIV ORF062R encodes a putative protein containing a typical IGF-like domain that shares high similarity with other known IGF genes, including IGFs of fish, frog, as well as mammals such as mouse and human. This suggests that the product of SGIV ORF062R may exert similar functions to vertebrate IGFs, and prompted the speculation that SGIV ORF062R may be a viral homologue of the IGF family. We thus termed this gene SGIV–IGF.

Notably, we did not find any SGIV–IGF homologue gene from other reported iridovirus family members, except for another grouper iridovirus (GIV) isolated from yellow grouper, Epinephelus awoara (Tsai et al., 2005), indicating SGIV–IGF is a unique gene of grouper fish iridoviruses.

In human, repetitive elements may comprise over two-thirds of the genome (de Koning et al., 2011), and among these a large proportion is referred to as variable number of tandem repeats (VNTR) with repeat unit sizes ranging from 6 bp to more than 100 bp (Tamaki & Jeffreys, 2005). Similar VNTR loci have also been described in a number of
prokaryotic species (Hardy et al., 2004). Increasing evidence shows that VNTR polymorphisms in mammals are associated with gene regulation (Fuke et al., 2001; Paquette et al., 1998; Yoon et al., 2010). Moreover, a recent study found that the numbers of repeat units in VNTR regions of white spot syndrome virus, a large dsDNA virus that mainly infects penaeid shrimp, are correlated with viral disease outbreaks (Hoa et al., 2012). Generally, there are 17 repetitive regions distributed throughout the SGIV genome, occupying 2.6% of the genome, varying from 31 to 1,119 bp in size (Song et al., 2004). Among them, one repetitive region consisting of 10.6 copy numbers of a 27 bp repeat unit is found at the 3′-terminal region of SGIV ORF062R (SGIV–IGF). In our experiments, during cloning of this gene, a variety of fragments with different lengths were amplified from the SGIV genomic DNA. DNA sequencing showed all these products were from SGIV ORF062R, with variable numbers of the repeat units in the 3′-terminal region. All these features suggest the 3′-terminal repetitive region of SGIV–IGF is a novel VNTR locus in the SGIV genome.

According to the temporal kinetic expression profile, genes of iridoviruses can be categorized into three classes: immediate-early (IE) genes, early (E) or delayed-early (DE) genes, and late (L) genes (D’Costa et al., 2001). By assessing the expression profiles of SGIV genes during in vitro viral infection, we found that SGIV–IGF transcripts can be detected as early as 6 h after viral inoculation. Drug inhibitor assays showed only treatment by CHX, a protein synthesis inhibitor, could block SGIV–IGF expression. These data suggest that SGIV–IGF is an early gene and may exert functions at the early stage during viral infection. Furthermore, SGIV–IGF was predominantly distributed in the cytoplasm and exhibited a punctate and diffuse cytoplasmic pattern, indicating it may colocalize with specific organelles such as the Golgi apparatus and exocytic vesicles, a phenomenon that is observed in mammals (Duguay et al., 1998). Notably, deletion of the SGIV–IGF C-terminal repetitive region showed no effect on its intracellular distribution, indicating the intracellular localization signals of SGIV–IGF protein do not lie in the repetitive region.

It has been widely proven that the IGF system plays crucial roles in regulating proliferation of a variety of cell types (Fernández et al., 2012; Ren et al., 2009). In mice, Fernández et al. (2012) found IGF-I promotes murine pheochromocytoma development by stimulating cell proliferation, motility and the ability to grow unattached. Besides, IGF-I displays critical roles in controlling brain growth and cell number (Beck et al., 1995; Cheng et al., 1998), and transgenic mice overexpressing IGF-1 appeared to have upregulation of cortical volume and cell number (Popken et al., 2004). To elucidate the effect of SGIV–IGF
Fig. 6. SGIV–IGF facilitates virus induced apoptosis in FHM cells. (a) Observation of apoptotic bodies in SGIV-infected stable FHM cell lines. Cells were stained with Hoechst 33342 and observed under fluorescence microscopy. White arrows indicate the apoptotic bodies, and red arrowheads indicate the virus assembly sites. (b) Validation of the stably transfected FHM cells by RT-qPCR. (c) Examination of caspase-3 activity during SGIV infection in SGIV–IGF expressing cells and a control line. n=3; error bars represent the mean ± SD. The experiments were performed three times with similar results. (d) Flow cytometry analysis of DNA content in two stable lines after SGIV infection. The percentage of apoptotic cells in the hypodiploid DNA peak (M4 phase) was calculated and indicated in each plot. A second experiment provided similar results.
on cell proliferation, we generated a stable grouper cell line that constitutively expresses SGIV–IGF. The growth curve analysis and colony formation assays showed GP cells with overexpression of SGIV–IGF demonstrated a much higher proliferation rate than the control line, and notably, the 3’-end VNTR of SGIV–IGF plays a relevant, but not dominant role during this process. In murine thyroid cells and cerebral cortical precursors, IGF-I plays a mitogenic role by accelerating cell cycle progression from G0/G1 to S phase, which is responsible for DNA replication during the cell cycle (Fernández et al., 2012; Ren et al., 2009). To ascertain the underlying mechanism of SGIV–IGF mediated cell growth promotion, the cell distribution among cell cycle phases was analyzed and compared between SGIV–IGF expressing cells and a control cell line. After a 24 h subculture, the percentage of S phase cells in the SGIV–IGF expressing population was about 1.7-fold higher, accompanied by a lesser portion of cells in G0/G1 phase, than that in the control cell line. Together, our observations indicate that SGIV–IGF exhibits a mitogenic rather than a trophic effect on cell proliferation.

Increasing evidence shows that manipulation of the host cell cycle is a frequent virus strategy for subverting host cell function, presumably in order to achieve a favourable cellular environment and benefit virus replication (Emmett et al., 2005; Nascimento et al., 2012). For example, the Epstein–Barr virus transactivators Zta and Rta, two IE viral proteins, could influence the checkpoint factors of cell cycle progression and promote G1 to S phase progression to support efficient viral replication and assembly in Raji cells (Guo et al., 2010). The transcriptional transactivator Tax of human T-cell leukaemia virus type I (HTLV-I) could interact with Cdk4 and Cdk6, resulting in an accumulation of phosphorylated pRB and accelerating S phase entry, which is thought to be vital for proliferation of the HTLV-I infected T cells (Iwanaga et al., 2008). Since we have confirmed that SGIV–IGF can accelerate cell cycle progression from G0/G1 to S phase, we next asked if the expression of SGIV–IGF is essential or beneficial to viral replication. To address this question, we examined and compared the SGIV replication kinetic curves of SGIV–IGF expressing cells and a control cell line during the time-course of infection. We found that the viral titres in SGIV–IGF expressing cells increased much more quickly from 12 h p.i., and during 24 to 48 h p.i. the viral titres yielded from SGIV–IGF expressing cells were almost 10-fold higher than that of the control group. Meanwhile, the transcript levels of SGIV functional genes such as SGIV ICP18, dUTPase, and MCP were higher in samples from the SGIV–IGF expressing cell line. These data suggest that the expression of SGIV–IGF is beneficial to SGIV replication, presumably through promoting cell cycle progression. However, it is important to state that the relationship between SGIV replication and cell cycle regulation is complex, and further studies await to be performed to uncover this complex network.

Although IGFs predominantly act as powerful mitogenic factors, their relevance to inhibition of cell apoptosis has also been described in a number of studies (Gooch et al., 1999; Hills et al., 2012; Holly et al., 1999; Kang et al., 2003). However, the effect of IGFs on apoptosis is more controversial. In some cases, IGFs were found to mildly induce apoptosis (Kawakami et al., 1998; Raile et al., 2003; Yang et al., 1996), suggesting IGFs may be involved in apoptosis regulation in a cell type and/or condition-dependent manner. Recent work in our laboratory demonstrated that SGIV infection in non-host FHM cells evoked typical apoptosis, characterized by apoptotic bodies and caspase activation (Huang et al., 2011). Whether SGIV–IGF is involved in SGIV-induced FHM apoptosis is another interesting question to be answered. In our study, we found more apoptotic bodies in SGIV–IGF expressing FHM cells than in the control line after SGIV infection. Meanwhile, SGIV–IGF expressing cells showed higher SGIV activated caspase-3 activity, a hallmark of cell apoptosis. In addition, flow cytometry analysis further confirmed SGIV–IGF expressing FHM cells demonstrated higher apoptosis after SGIV infection. Notably, we did not find any effects of SGIV–IGF on apoptosis in SGIV-infected host cells, such as GP cells and grouper spleen cells (data not shown), and these results are concordant with our previous findings that SGIV infection in host cells evoked non-apoptotic programmed cell death, characterized without DNA fragmentation, apoptotic bodies and caspase activation (Huang et al., 2011). These data suggest SGIV–IGF could act as a pro-apoptosis mediator during viral infection of non-host cells.

Taken together, we identified a novel IGF homologue gene, SGIV–IGF, encoded by SGIV ORF062R. SGIV–IGF is an early transcribed gene during viral replication and predominantly distributed in the cytoplasm. SGIV–IGF could modulate cell proliferation through promoting G1 to S phase cell cycle transition. Furthermore, expression of SGIV–IGF was beneficial to viral replication during SGIV infection of host cells, while it mildly facilitates apoptosis in non-host FHM cells after viral infection. Our present study provides additional evidence-based information on SGIV functional genes, which may help us better understand the mechanisms of SGIV infection and pathogenesis, as well as empower researchers to develop new control strategies to combat this aquatic animal virus.

**METHODS**

**Cells and viruses.** Grouper embryonic (GP) cells and fathead minnow (FHM) cells were cultured as previously described (Cui et al., 2011; Gravel & Malsberger, 1965). SGIV (strain A3/12/98) was originally isolated from diseased brown-spotted grouper, *Epinephelus tauvina*, and the propagation of SGIV was performed as described previously (Qin et al., 2003).

**Cloning, plasmid construction, and computer-assisted analysis.** The SGIV ORF062R gene was cloned from SGIV genomic DNA using two pairs of primers: pDNA-IGF-F/pDNA-IGF-R and pEGFP-IGF-F/pEGFP-IGF-R (Table S1). The target PCR products were purified and subcloned into pcDNA3.1 (+) vector and
pEGFP-N3 to generate the recombinant plasmid pcDNA-IGF and pEGFP-IGF, respectively.

The similarity of SGIV–IGF with other IGFs was analysed using the BLAST search program from NCBI (http://www.ncbi.nlm.nih.gov/blast). The conserved domains were predicted using SMART (http://smart.embl-heidelberg.de/). Multiple-sequence alignment of reported IGF amino acid sequences was performed with CLUSTAL_X version 1.83 (http://www.ebi.ac.uk/clustalw/) and edited using the GeneDoc 2.6 program.

Temporal transcription analysis and drug inhibition assays. To determine the temporal transcription of SGIV–IGF after viral infection, GP cells were either infected or mock-infected with SGIV at an m.o.i. of −0.1 for the indicated time. Total RNA was extracted using TRIzol reagent (Invitrogen) and digested with RNase-free DNase I (TaKaRa). The first-strand cDNA was synthesized with a ReverTra Ace qPCR RT kit (TOYOBO), and real-time quantitative PCR (RT-qPCR) was performed using primers RT-IGF-F/RT-IGF-R (Table S1). β-Actin was used as a reference gene.

To examine the temporal kinetic class of SGIV–IGF during viral infection in vitro, cycloheximide (CHX) and cytosine arabinoside (AraC) (Sigma-Aldrich) were used as a de novo protein synthesis inhibitor and a DNA synthesis inhibitor, respectively. Briefly, GP monolayer cells were either pretreated with 50 μg CHX ml⁻¹ or 100 μg AraC ml⁻¹ for 1 h prior to and throughout the SGIV infection. Mock treated cells were used as the control group. Total RNA was extracted and subjected to RT-qPCR as described above. Three previously characterized SGIV genes, including immediate-early gene (IE) ICP18, early gene (E) dUTPase, and late gene (L) MCP were used as indicative controls for definition of the expression kinetic class of SGIV–IGF.

Intracellular localization analysis. GP or FHM cells were plated on coverslips and cultured in 24-well plates. Cells were transiently transfected with 800 ng of pEGFP-IGF or pEGFP-N3 empty vectors using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s protocol. Two days after transfection, cells were fixed with 4 % paraformaldehyde for 30 min, rinsed with PBS and permeabilized with 0.2 % Triton X-100 for 15 min. Finally, cells were stained with 1 μg DAPI (Sigma-Aldrich) and observed under fluorescence microscopy (Leica).

Generation of stable cell lines. To obtain cells that stably express SGIV–IGF, GP or FHM cells were transfected with pcDNA-IGF or empty vector and selected with 800 μg genetin ml⁻¹ (G418; Gibco) for 4 weeks. The stable line that expresses SGIV–IGF was confirmed by RT-qPCR. The stable GP lines were termed as GP/pcDNA-IGF and GP/pcDNA3.1, and stable lines of FHM were termed FHM/pcDNA-IGF and FHM/pcDNA3.1, respectively.

Cell proliferation and colony formation efficiency assay. To detect the impact of SGIV–IGF on GP cell growth, GP/pcDNA3.1 and GP/pcDNA-IGF cells were seeded in 12-well plates (3 × 10³ cells per well). Cell numbers from each line were counted daily for 1 week using a haemocytometer under a light microscope.

To determine the colony formation efficiency, 5000 stable cells of GP/pcDNA3.1 and GP/pcDNA-IGF were placed in 6-well plates in parallel and maintained in growth medium for about 4 weeks until colonies could be visualized. Colonies were fixed in methanol/acetic acid (3 : 1, v/v) and stained with Giemsa (Sigma-Aldrich) for picture taking.

Cell cycle analysis by flow cytometry. Nuclear DNA content was measured by propidium iodide (PI) staining and flow cytometry as previously described (Yu et al., 1993). Briefly, GP/pcDNA3.1 and GP/pcDNA-IGF cells were seeded into 6-well plates in triplicate (5 × 10⁵ cells per well) and cultured for 36 h. Cells were trypsinized into single cell suspensions and fixed with 70 % ice-cold ethanol overnight at −20 °C. The cells were then collected by centrifugation and resuspended in PBS containing 100 μg DNase-free RNase A ml⁻¹ (Sigma-Aldrich) and 50 μg PI ml⁻¹ (Sigma-Aldrich) for 30 min. PI fluorescence was measured with a FACScan flow cytometer (Becton–Dickinson), and cell cycle analysis was done using the CellQuest program by manually setting regions for G0/G1, S and G2/M phases. Ten thousand cells were counted per sample and the data were processed using CellQuest software (Becton–Dickinson).

Viral replication kinetics assay. To investigate the effect of SGIV–IGF on SGIV infection in vitro, viral replication kinetics were evaluated based on SGIV replication in GP/pcDNA3.1 and GP/pcDNA-IGF cells, respectively. Briefly, the stable lines were separately seeded in 24-well plates and infected with SGIV at an m.o.i. of −0.1. The virus-infected cell lysates were harvested at the indicated time points (0, 6, 12, 24, 36 and 48 h p.i.), and viral titres were determined using a 50 % tissue culture infectious dose (TCID₅₀) assay (Reed & Muench, 1938). Cytopathic effect was observed daily under a light microscope (Leica).

Meanwhile, the expression profiles of SGIV ICP18, dUTPase and MCP in GP/pcDNA3.1 and GP/pcDNA-IGF cells after SGIV infection were examined by RT-qPCR using β-actin as reference gene.

Apoptosis analysis. The impact of SGIV–IGF on viral infection-induced apoptosis was evaluated by Hoechst staining, caspase-3 activity analysis, as well as PI staining plus flow cytometry, individually.

FHM/pcDNA3.1 and FHM/pcDNA-IGF cells were seeded in 24-well plates, following inoculation of SGIV for 12 and 24 h. Cells were washed twice with PBS, and then stained with 1 μg Hoechst 33342 ml⁻¹ (Sigma-Aldrich). Nuclear morphology was visualized by fluorescence microscopy. Additionally, parallel samples were collected for PI staining and flow cytometry to assess the percentage of apoptotic cells in SGIV-infected FHM/pcDNA3.1 and FHM/pcDNA-IGF populations. A minimum of 10 000 cells was examined for each sample.

To test the caspase-3 activity, FHM stable lines were inoculated with SGIV for indicated time points (0, 6, 12, 18, 24, 36 h p.i.). Cells were collected and caspase-3 activity was assessed using a Fluorometric Protease Assay kit (BioVision), according to the manufacturer’s instructions. The reactions were measured on a PerkinElmer 2030 Multilabel Reader (PerkinElmer Life and Analytical Science).

Statistical analysis. The two-tailed Student t-test was used for two-group analyses. One-way ANOVA followed by the Holm–Sidak or Tukey–Kramer test was performed for multiple group comparisons. A P-value of less than 0.05 was considered statistically significant.

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