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Monoclonal antibody against a putative myristoylated membrane protein encoded by grouper iridovirus 59L gene

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ABSTRACT: Groupers (*Epinephelus* spp.) are economically important fish species worldwide, and ranaviruses are major viral pathogens causing heavy economic losses in grouper aquaculture. In this study, the 59L gene of grouper iridovirus (GIV-59L) was cloned and characterized. This gene is 1521 bp and encodes a protein of 506 amino acids with a predicted molecular mass of 53.9 kDa. Interestingly, GIV-59L and its homologs are found in all genera of the family *Iridoviridae*. A mouse monoclonal antibody specific for the C-terminal domain (amino acid positions 254–506) of the GIV-59L protein, GIV-59L(760-1518)-MAb-21, was produced and proved to be well suited for use in a number of GIV immunoassays. RT-PCR, Western blotting, and cycloheximide and cytosine arabinoside drug inhibition analyses indicated that GIV-59L is a viral late gene in GIV-infected grouper kidney cells. Immunofluorescence analysis revealed that GIV-59L protein mainly accumulates in the cytoplasm of infected cells and is finally packed into a whole virus particle. The GIV-59L(760-1518)-MAb-21 characterized in this study could have widespread application in GIV immunodiagnostics and other research on GIV. In addition, the results presented here offer important insights into the pathogenesis of GIV.

KEY WORDS: Grouper iridovirus 59L · Monoclonal antibody · C-terminal domain · Immunodiagnostics

INTRODUCTION

Iridoviridae comprises a family of large icosahedral viruses containing linear double-stranded DNA packed into a viral particle with diameters ranging from 120 to 350 nm (Williams 1996). The family contains 5 genera: *Ranavirus*, *Chloriridovirus*, *Iridovirus*, *Lymphocystivirus*, and *Megalocytivirus*, whose members can infect invertebrates and poikilothermic vertebrate species such as fish, amphibians, and reptiles (Chinchar et al. 2005, Williams et al. 2005). Ranaviruses have been isolated from a wide variety of fish, including freshwater and saltwater species. Groupers (*Epinephelus* spp.) are among the most economically important cultured marine saltwater fish in many Asian countries. Viral disease is a major constraint in the hatchery production of groupers (Lai et al. 2000), and ranaviruses are among the most important viral pathogens in these fish, particularly at the fry and fingerling stages. Groupers infected by ranaviruses might show darkened body color, pale gills, and an enlarged spleen, and often become lethargic and have a reduced appetite (Langdon & Humphrey 1987, Schuh & Shirley 1990, Chua et al. 1994, He et al. 2000, Lai et al. 2000). Microscopic pathological signs can include enlargement of cells and necrosis of the renal

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and splenic hematopoietic tissues (Qin et al. 2003, Brunner et al. 2005, Hoverman et al. 2010, Miller et al. 2011, Bayley et al. 2013, Cheng et al. 2014).

Grouper iridovirus (GIV), originally isolated from southern Taiwan and belonging to the genus *Ranavirus*, has caused heavy economic losses in the grouper aquaculture industry for years (Murali et al. 2002, Tsai et al. 2005). GIV-susceptible cell lines have been established from yellow grouper (Lai et al. 2000, 2003), and the complete genome of GIV has been sequenced. The GIV genome consists of 139 793 bp with a 49% G + C content and is predicted to encode 120 open reading frames ranging in size from 62 to 1268 amino acids (Tsai et al. 2005). The host gene expression patterns in response to GIV infection (Yeh et al. 2008, Hu et al. 2014), and GIV-induced host cell apoptosis characteristics have also been investigated (Lai et al. 2008, Chiou et al. 2009, Pham et al. 2012). However, our understanding of molecular aspects of GIV pathogenicity remains limited.

Antibodies are an efficient tool for diagnosis (Crane et al. 2000, Luo et al. 2014) and characterization (Shi et al. 2003, Lin et al. 2014) of viral pathogens. The production of monoclonal antibodies (MAbs) was pioneered by Köhler & Milstein (1975), and MAbs now serve a variety of academic, medical, and commercial uses (Carter et al. 1992, Waldmann 2003, Hudis 2007). The development and use of specific MAbs against viral gene products will in all likelihood lead to a better understanding of the molecular mechanism of GIV pathogenesis.

Protein myristoylation has been associated with various biological functions, including the assembly of viruses (Bryant & Ratner 1990, Martin et al. 1997, Andrés et al. 2002, Capul et al. 2007). In iridoviruses, all known myristoylated proteins are viral envelope membrane proteins (Tsai et al. 2005, Zhou et al. 2011). In addition, the myristoylated membrane protein is essential for replication of frog virus 3 (FV3), type species of the genus *Ranavirus* in the *Iridoviridae* family (Whitley et al. 2010).

Viral envelope membrane proteins are critical to virus infection and virus–host interaction, and antibodies specific to these proteins can serve as valuable tools for virus detection. In this study, we cloned the GIV-59L gene, which encodes a putative myristoylated envelope membrane protein, from GIV genomic DNA and produced a mouse MAb specific to the C-terminal domain of GIV-59L protein for use in various GIV-59L protein immunoassays. This investigation marks the first examination of GIV-59L gene expression and protein subcellular location during GIV infection *in vitro*, and the specificity of the GIV-59L MAb for the C-terminal domain of GIV-59L makes it a valuable tool for GIV diagnostics and studies of GIV pathogenesis.

MATERIALS AND METHODS

Cells and viruses

Grouper kidney (GK) cell culture and GIV propagation have been described previously (Lai et al. 2000). Briefly, GK cells were grown at 28°C in Leibovitz's L15 medium with 10% fetal bovine serum (FBS), 100 IU ml^{-1} penicillin, and 100 µg ml^{-1} streptomycin. GK cells (n = 10^7) grown in a 175-cm^2 flask were infected with GIV at a multiplicity of infection (MOI) of 0.01. The GIV was then allowed to multiply for 7 d at 28°C. The culture mediums of 10 flasks were collected and centrifuged at 2000 × *g* (30 min at 4°C). After centrifugation, the supernatant and cell pellet (cell-associated virus) were collected separately, and the supernatant was mixed with polyethylene glycol 1500 (Sigma) and stirred at 4°C overnight and then centrifuged at 4000 × *g* (1 h at 4°C). The pellet was resuspended in 2 ml of TNE buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.3), mixed with an equal volume of Freon (1,1,2-trichlorotrifluoroethane, Sigma) and centrifuged at 1800 × *g* (10 min). The cell pellet (cell-associated virus) was also extracted with Freon in the same way. Aqueous solutions of both virus samples were collected and combined, layered over a 3-step cesium chloride gradient (4 ml 40%, 3 ml 30%, 2 ml 20%), and centrifuged at 13 000 × *g* (16 h at 4°C). The virus band was collected and diluted with 5 ml TNE buffer, and re-centrifuged at 23 000 × *g* (2 h at 4°C). The purified virus pellet was resuspended in 1 ml TNE buffer and used for all analyses. The virus titer was calculated by the $TCID_{50}$ method, as described by Reed & Muench (1938).

pET expression plasmid construction

The full length and C-terminal domain (762–1518 bp) of GIV-59L were isolated from GIV genomic DNA by polymerase chain reaction (PCR) using primer pairs GIV-59L-F1(1) + 59L-R and GIV-59L-F2(760) + 59L-R (Table 1). PCR was carried out under the following thermal conditions: initial denaturation for 10 min at 95°C, followed by 40 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C

Table 1. Sequences of primers used in this study. Restriction enzyme (*Eco*RI or *Xho*I) cleavage sites are underlined

Primer name	Sequence (5′–3′)
GIV-59L-F1(1)	CCGGAATTCATGGGCGCAGCGCAATCC (*Eco*RI)
GIV-59L-F2(760)	CCGGAATTCGCCGTGGTGCCCGTGGCC (*Eco*RI)
GIV-59L-R	CCGCTCGAGCTTTGCAGCTTCTCC (*Xho*I)
GIV-97L-F	CGCGGATCCATGGAATGTTTATAC
GIV-97L-R	CCGCTCGAGCACAAACCCAAATTT
GIV-45R(MCP)-F	CCGGAATTCATGACTTGTACAACGGGTGCT
GIV-45R(MCP)-R	CCGCTCGAGCAAGATAGGGAACCCCATGGA
β-Actin-F	CCGGAATTCATGGCCATTCAACTGACACTT
β-Actin-R	CCGCTCGAGAACCCCTTGGATGATAAATTC

for 1 min; and then final elongation at 72°C for 10 min. The PCR amplification products were digested with restriction enzymes, then cloned into the pET23a (Novagen) expression vector using T4 DNA ligase (Roche). These constructs were transformed into *Escherichia coli* XL1-Blue, and the transformation was confirmed by restriction enzyme digestion and DNA sequencing.

Bioinformatic sequence analysis

GIV-59L homology analyses were performed using the BLAST network server of the National Center for Biotechnology Information. Multiple alignment of the GIV-59L amino acid sequence and 29 other sequences was performed with GeneDoc. The phylogenetic tree was constructed based on neighbor-joining MEGA 6.

Preparation of recombinant His-tag protein

The recombinant fusion constructs, pET23a-GIV-59L and pET23a-GIV-59L(760-1518) expression vectors, were transformed into *E. coli* BL21(DE3)-competent cells, and recombinant His-tag proteins were induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) for 24 h at 37°C. The recombinant fusion protein was purified by Ni-NTA resin affinity column (GE Healthcare) and dialyzed with S100 buffer (25 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, pH 7.9). The concentration of the purified protein was determined by the Bradford assay, and protein purity was confirmed by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Samples were stored at –20°C until use.

Generation of MAbs directed against GIV-59L protein

Three female (8 wk old) BALB/c mice (National Laboratory Animal Breeding and Research Center, National Science Council, Taiwan) were used for immunization. All animal experiments were performed in accordance with the guidelines of the Ethics Review Committee for Animal Experiments at National Ilan University. The protocols for mouse immunization and cell fusion were similar to those previously described (Hu et al. 2015). The selection of hybridoma clones producing MAbs specific against GIV-59L proteins was accomplished by ELISA and Western blotting.

ELISA

The protocol for ELISA was similar to that previously described (Hu et al. 2015), whereby 96-well plates (Nunc) were coated with recombinant GIV-59L(760-1518)-His proteins and washed with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBST) and blocked with 5% skim milk in PBS for 2 h at 37°C. The mouse antiserum or hybridoma culture supernatant as the first antibody was added and incubated for 1 h at 37°C. After the plates were washed 3 times with PBST, they were incubated for 1 h at 37°C with the second antibody, a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Santa Cruz) diluted 1:5000 in PBST. The plates were then washed 3 times and the reaction visualized with tetramethylbenzidine (Invitrogen) substrate, followed by analysis at 650 nm with an ELISA plate spectrophotometer reader (SpectraMax M2, Molecular Devices).

Isotype determination and purification of mouse MAbs

Isotyping was achieved using the mouse MAb isotyping ELISA kit (Sigma) according to the manufacturer's recommendations. Subsequently, following National Laboratory Animal Center on Institutional Animal Care and Use Committee guidelines, the cloned hybridoma-21 cells were injected into the peritoneal cavity of BALB/c mice to produce ascites. The GIV-59L-MAb-21 was purified from the ascitic

fluids using saturated ammonium sulfate solution and the NAb™ protein G spin kit (Thermo Scientific) according to the manufacturer's protocol.

Reverse transcription (RT)-PCR

GK cells were infected by GIV at an MOI of 5, and cells were harvested at 0, 3, 6, 9, 12, 18, and 24 h post-infection (hpi) in the presence or absence of cycloheximide (CHX, 50 μg ml^{-1}; Sigma) or cytosine arabinoside (AraC, 40 μg ml^{-1}; Sigma). Total RNA was isolated from the collected cells at each time point using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The concentration was quantified by measuring the OD_{260} in a UV spectrophotometer. After treatment with the RNase-free DNase (Promega), a total of 2 μg total RNA from each time point was used for first-strand cDNA synthesis with a random primer following the manufacturer's protocol for the SuperScripIII Reverse Transcriptase kit (Invitrogen). The cDNAs from each time point were then subjected to PCR with specific primers for each gene (Table 1). The PCR reagents were 2 μl cDNA, 2 μM forward primer, 2 μM reverse primer, 2.5 μM of each dNTP, 1× PCR buffer, and 2.5 U *Taq* DNA polymerase (Viogene) to a final volume of 50 μl. The PCR cycles were as follows: 10 min at 95°C for 1 cycle; 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C for 35 cycles; and 10 min at 72°C for 1 cycle. The GIV-97L was used as an immediate early gene, but GIV-45R(MCP) was used as a late gene and β-actin as the internal control gene. After amplification, the PCR products were electrophoresed in a 1.2% agarose-Tris-acetate-EDTA (TAE) buffer gel and stained with ethidium bromide.

Western blot analysis

The expression pattern of the GIV-59L protein was evaluated by Western blotting following SDS-PAGE. GK cells were infected by GIV at an MOI of 5 and harvested at 0, 3, 6, 9, 12, 18, 24, and 30 hpi. Equivalent amounts of cell extracts were separated by electrophoresis using 12% SDS-PAGE at 120 V for 1.5 h and subsequently transferred to a polyvinylidene difluoride membrane (Pall) at 400 mA for 2 h. After electrophoresis, the membrane was blocked at room temperature with a blocking reagent (PBST containing 5% non-fat milk) for 2 h. After the membrane was incubated with GIV-59L-MAb-21 (0.1 μg ml^{-1}), GIV-45R(MCP)-MAb-21 (0.1 μg ml^{-1}) (Lin et al. 2014), GIV-97L-MAb-3 (0.1 μg ml^{-1}) (Hu et al. 2015), or β-actin (0.6 μg ml^{-1}, Sigma) for 2 h at 37°C, it was exposed to alkaline phosphatase-conjugated goat anti-mouse immunoglobulins (0.08 μg ml^{-1}) (Santa Cruz) at 37°C for 1 h. Finally, the color band was developed in the dark using the substrates 4-nitro-blue tetrazolium chloride (NBT; 0.3 mg ml^{-1}) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 0.15 mg ml^{-1}) (Sigma).

Immunofluorescence assay for GIV-59L protein location

To analyze the location of the GIV-59L protein in GIV-infected cells, GK cells were seeded on coverslips in a 12-well plate and cultured at 28°C for 1 d before infection with GIV at an MOI of 5. At 24 hpi, the cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed 3 times with PBS. Fixed cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min and washed 3 times with PBS. Cells were incubated with GIV-59L-MAb-21 (5 μg ml^{-1}) for 1 h at 37°C. After 3 washes with PBS, fluorescein-labeled goat anti-mouse immunoglobulin G (H+L) (10 μg ml^{-1}) (KPL) was added and incubated for 1 h at 37°C. Cellular and GIV DNA were labelled with 4,6-diamidino-2-phenylindole (DAPI) for 10 min. The coverslips were washed with PBS 3 times, mounted, and observed under a fluorescence microscope (Axio Observer, Zeiss).

RESULTS

Bioinformatic sequence analysis of GIV-59L

GIV-59L (GenBank accession no. AAV91076) is 1518 bp in length and encodes a putative protein of 506 amino acids with a predicted molecular mass of 53.9 kDa. The deduced amino acid sequence of GIV-59L was aligned with the published sequences of another 29 iridoviruses, revealing no conserved domains, and the percentage similarity of GIV-59L was only 54 to 55% within the ranavirus sequences (Table 2). To understand the position of GIV-59L in the evolutionary process, a neighbor-joining phylogenetic tree was constructed with all amino acid sequences of the 29 published sequences and GIV-59L. As illustrated in Fig. 1, GIV-59L showed the closest relationship to Singapore grouper iridovirus (SGIV), and the phylogenetic tree was consistent

Table 2. GenBank accession numbers of homologs of the 59L gene of grouper iridovirus (GIV-59L) used in this study. ORF: open reading frame

Virus	Abbrev.	Accession no.	ORF	Length (aa)	Identity (%)
Ranaviruses					
Grouper iridovirus	GIV	AAV91076	59L	506	
Singapore grouper iridovirus	SGIV	YP_164183	88L	506	97
Tiger frog virus	TFV	ABB92314	55R	522	55
Andrias davidianus ranavirus isolate 1201	ADRV-I	AGV20589	58L	522	55
Andrias davidianus ranavirus isolate 2010SX	ADRV-2	AHA42326	58L	522	55
Chinese giant salamander iridovirus	CGSIV	AHA80904	60R	522	55
Frog virus 3	FV3	YP_031631	53R	522	55
Soft-shelled turtle iridovirus	STIV	ACF42273	55R	522	55
European catfish virus	ECV	YP_006347665	73L	523	55
Rana grylio virus 9506	RGV-9	ABY71546	53R	522	55
Rana grylio iridovirus	RGV	AFG73095	53R	522	55
Epizootic hematopoietic necrosis virus	EHNV	ACO25243	53L	523	55
Ambystoma tigrinum virus	ATV	YP_003824	51L	526	54
Ambystoma tigrinum stebbensi virus	ATSV	AAP33230	51L	526	54
Common midwife toad ranavirus	CMTV	AFA44962	56L	522	54
Megalocytiviruses					
Infectious spleen and kidney necrosis virus	ISKNV	NP_612229	7L	485	25
Red seabream iridovirus	RSIV	BAC66967	374R	485	25
Orange-spotted grouper iridovirus	OSGIV	AAX82317	8L	485	25
Turbot reddish body iridovirus	TRBIV	ADE34352	7L	485	25
Rock bream iridovirus	RBIV	AAT71823	8L	485	25
Lymphocystiviruses					
Lymphocystis disease virus 1	LCDV-I	NP_078665	67L	459	23
Lymphocystis disease virus — isolate China	LCDV-C	YP_073663	158R	457	22
Iridoviruses					
Invertebrate iridovirus 25	IIV-25	YP_009010607	74R	518	21
Armadillidium vulgare iridescent virus	AVIV	YP_009046668	54R	535	20
Invertebrate iridescent virus 6	IIV-6	NP_149581	118L	515	20
Invertebrate iridescent virus 30	IIV-30	YP_009010366	72R	513	20
Invertebrate iridovirus 22	IIV-22	YP_008357365	67R	513	20
Invertebrate iridescent virus 22	IIV-22A	YP_009010832	71R	508	20
Wiseana iridescent virus	WIV	YP_004732788	5R	515	19
Chloriridovirus					
Invertebrate iridescent virus 3	IIV-3	YP_654578	6R	494	15

with the relationship of 5 genera within the family *Iridoviridae*.

Expression and purification of recombinant GIV-59L(760-1518)-His protein

The full length and C-terminal domain (760–1518 bp) of GIV-59L were amplified by PCR from GIV genomic DNA and cloned into the pET-23a vector, respectively (Fig. 2A). After transformation into the *E. coli* XL1-Blue strain, the recombinant plasmids of positive clones were extracted and identified by restriction enzyme digestion and DNA sequencing (data not shown), and results showed that the recombinant genes were present. The correct expression plasmids were transformed into an *E. coli* BL21(DE3) strain with only the recombinant GIV-59L(760-1518)-His protein expressed in the presence of IPTG. As shown in Fig. 2B, the band with an expected molecular weight of approximately 30.22 kDa (277 amino acids) was identified by SDS-PAGE in the cell lysate after a 24 h induction with 1 mM IPTG when compared with non-induced negative control. The recombinant GIV-59L(760-1518)-His protein was purified and verified by SDS-PAGE (Fig. 2B).

Production and characterization of GIV-59L(760-1518)-MAb-21

Mice were immunized with GIV-59L(760-1518)-His protein to induce an immune response. To identify the antigenicity of the recombinant GIV-59L(760-

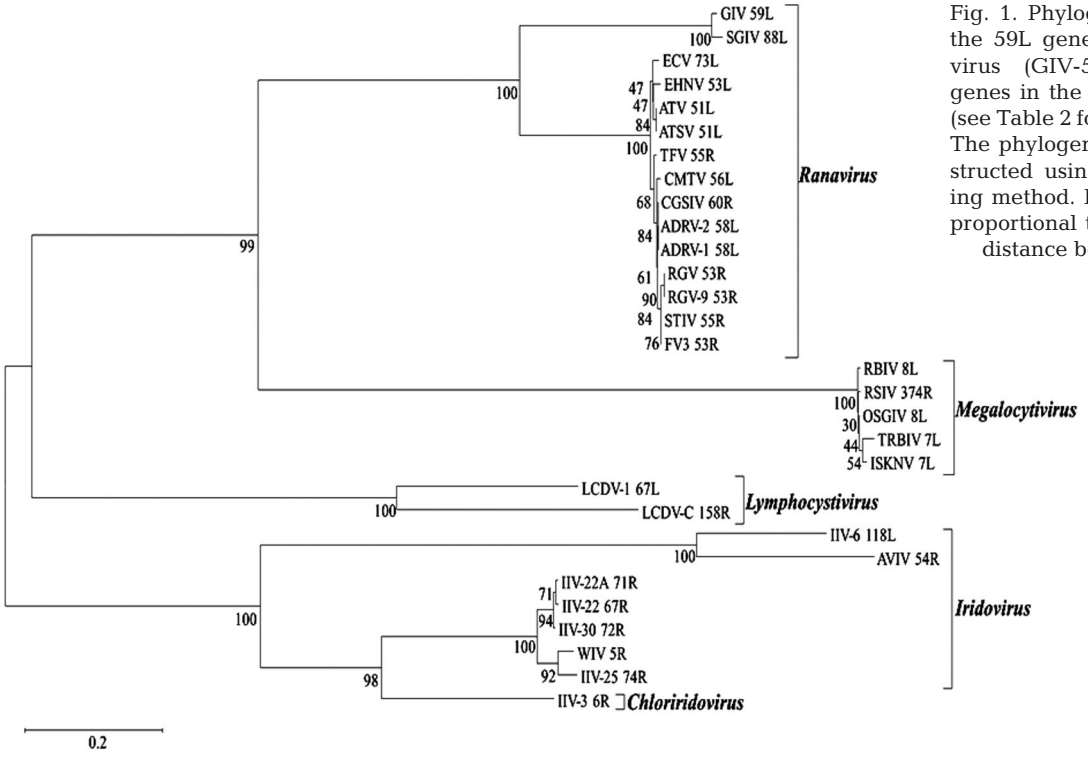

Fig. 1. Phylogenetic analysis of the 59L gene of grouper iridovirus (GIV-59L) and related genes in the family *Iridoviridae* (see Table 2 for full virus names). The phylogenetic tree was constructed using a neighbor-joining method. Branch lengths are proportional to the evolutionary distance between the taxa

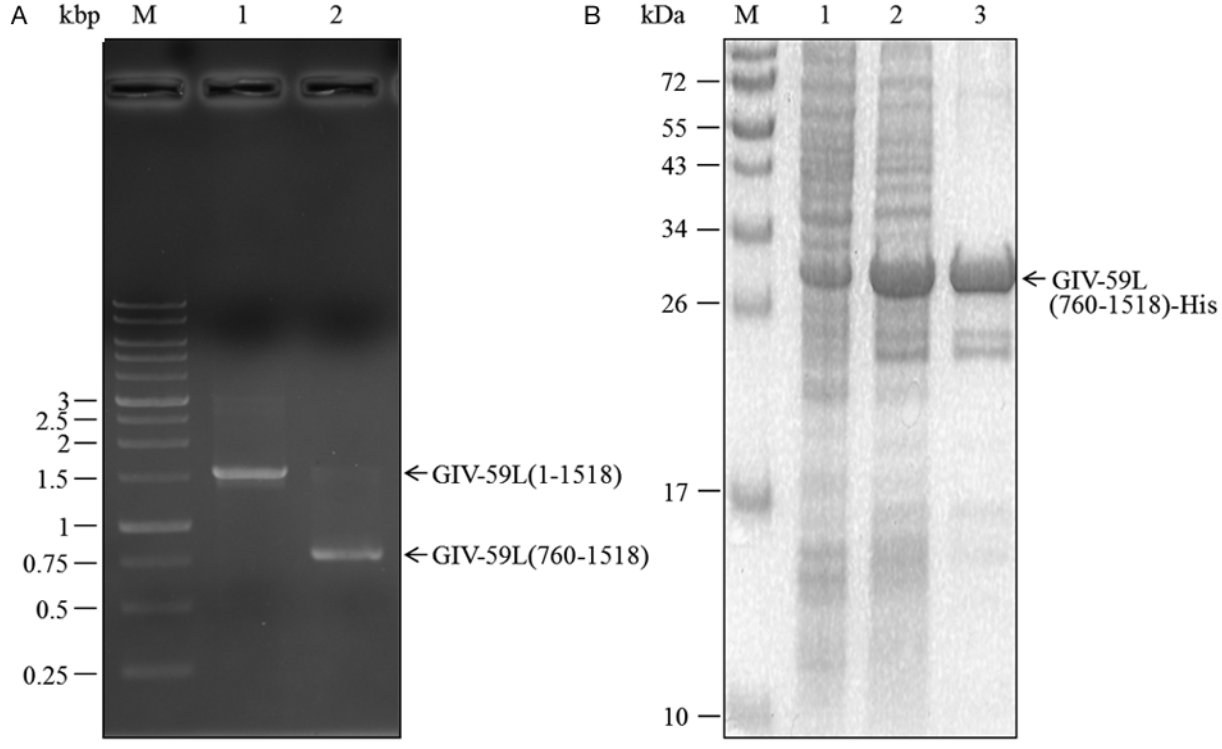

Fig. 2. Isolation of the 59L gene of grouper iridovirus (GIV-59L) and recombinant GIV-59L(760-1518)-His protein purification. (A) PCR amplification of GIV-59L(1-1518) and GIV-59L(760-1518) fragments from GIV genomic DNA. M: DNA marker; Lane 1: GIV-59L(1-1518); Lane 2: GIV-59L(760-1518). (B) Expression and purification of recombinant GIV-59L(760-1518)-His protein. The expressed and purified GIV-59L(760-1518)-His protein was separated by SDS-PAGE. The gel was stained with Coomassie blue after electrophoresis. M: pre-stained protein marker; Lane 1: non-isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced cell lysate; Lane 2: IPTG-induced cell lysate; Lane 3: purified GIV-59L(760-1518)-His protein

1518)-His, Western blotting was performed, with results showing that recombinant GIV-59L(760-1518)-His and purified GIV particle (pGIVP) were recognized by mouse polyclonal antibodies. This finding demonstrated that the recombinant GIV-59L(760-1518)-His protein possessed good immunoreactivity and that the GIV-59L protein was assembled into the whole virus particle (Fig. 3A). Subsequently, at 3 wk after cell fusion, 1 mouse MAb against GIV-59L(760-1518) was produced; the hybridoma clone was screened by ELISA against GIV-59L(760-1518)-His protein. Out of 33 clones, only 1 (GIV-59L(760-1518)-MAb-21) reacted with GIV-59L (760-1518)-His protein and was determined to be a secreted MAb belonging to the immunoglobulin G1 isotype (data not shown). Western blot analysis showed that the GIV-59L(760-1518)-MAb-21 could specifically recognize pGIVP (Fig. 3B). Finally, the GIV-59L(760-1518)-MAb-21 hybridoma cells were injected into mice to produce ascites, and a high concentration of GIV-59L(760-1518)-MAb-21 was prepared after treatments with saturated ammonium sulfate solution and protein G agarose (Fig. 3C).

The detection sensitivity of the prepared GIV-59L(760-1518)-MAb-21 was analyzed by Western blotting. With an antigen concentration of 1 µg pGIVP per lane, GIV-59L(760-1518)-MAb-21 (500 µg ml^{-1}) was applied in a dilution series in PBST. GIV-59L(760-1518)-MAb-21 could detect GIV-59L of pGIVP with an approximate detection limit dilution of 1:50 000 (Fig. 3D).

Temporal expression of the GIV-59L gene in GIV-infected GK cells

To study the temporal transcription of GIV-59L in GIV-infected GK cells, RT-PCR was performed on GIV-infected GK cells at 0, 3, 6, 9, 12, 18, and 24 hpi. The transcripts of GIV-97L and GIV-45R(MCP) were detected at 3 and 9 hpi, respectively, whereas the GIV-59L gene fragment was first detected at 12 hpi, and a high level of transcription continued until 24 hpi (Fig. 4A). To verify the nature of the GIV-59L viral gene, the transcription of GIV-59 was assayed in GIV-infected cells in the presence of CHX or AraC. The transcription of only GIV-45R(MCP) and GIV-59L but not of GIV-97L was inhibited by CHX and AraC in GIV-infected GK cells at 6 and 18 hpi (Fig. 4B). The data support that GIV-59L, like GIV-45R(MCP), is a viral late gene in GIV-infected GK cells.

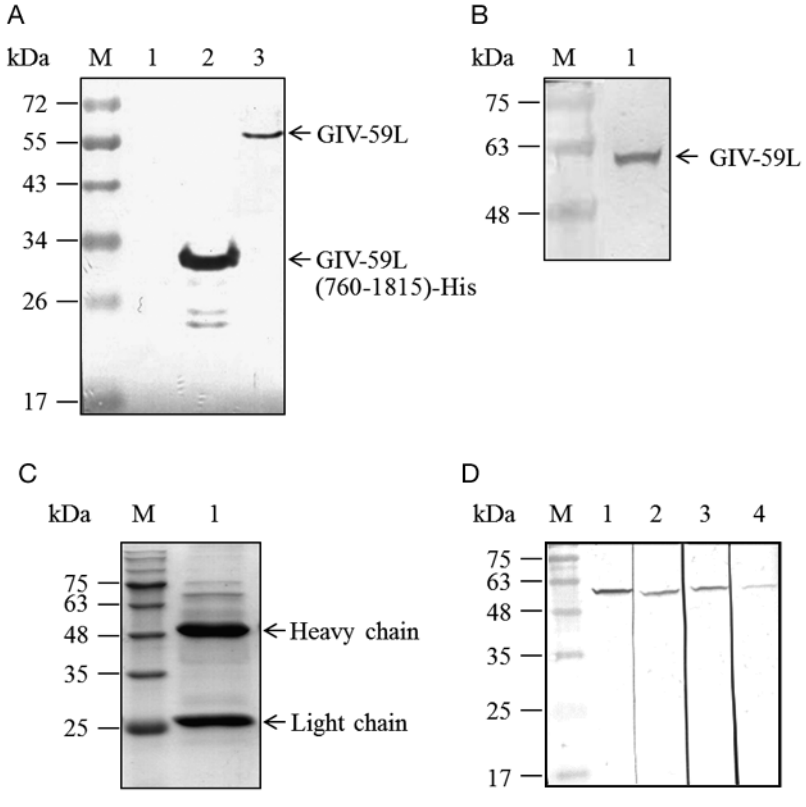

Fig. 3. Production, screening, purification, and affinity assay of mouse antibodies against the grouper iridovirus 59L protein C-terminal domain GIV-59L(760-1518) (amino acid positions 254–506). (A) Production of mouse polyclonal serum against GIV-59L analyzed by Western blotting. M: pre-stained protein marker; Lane 1: cell lysate of expression protein of the pET23a empty vector; Lane 2: purified recombinant GIV-59L(760-1518)-His protein; Lane 3: purified grouper iridovirus particle (pGIVP). (B) Screening of positive hybridoma-21 cells by Western blotting using pGIVP as antigen. M: pre-stained protein marker; Lane 1: pGIVP (1 µg $lane^{-1}$). (C) SDS-PAGE of GIV-59L(760-1518)-MAb-21 purified from ascites resulting from injection of hybridoma-21. The gel was stained with Coomassie blue after electrophoresis. M: pre-stained protein marker; Lane 1: purified GIV-59L(760-1518)-MAb-21 protein. (D) Western blot analysis for measuring GIV-59L(760-1518)-MAb-21 titer against GIV-59L, with 1 µg of pGIVP loaded per lane, and GIV-MCP-MAb-17 (500 µg ml^{-1}) applied in a dilution series ranging from 1:1000 to 1:50 000 in phosphate-buffered saline containing 0.05% Tween 20. Color signals were visualized with a goat anti-mouse secondary antibody conjugated to alkaline phosphatase (1:5000) and 4-nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as substrate. M: pre-stained protein marker; Lane 1: dilution 1:1000; Lane 2: dilution 1:5000; Lane 3: dilution 1:10 000; Lane 4: dilution 1:50 000

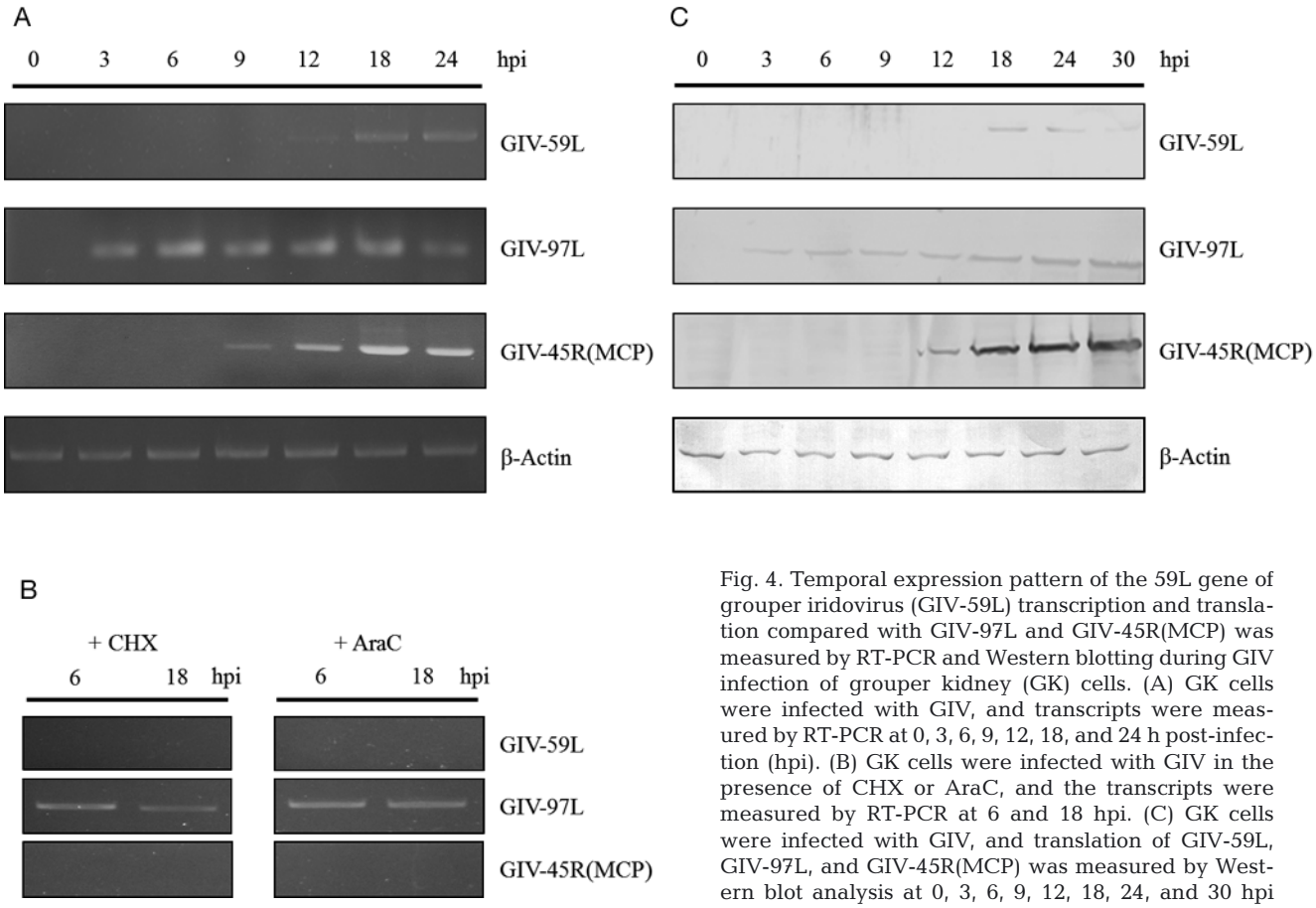

Fig. 4. Temporal expression pattern of the 59L gene of grouper iridovirus (GIV-59L) transcription and translation compared with GIV-97L and GIV-45R(MCP) was measured by RT-PCR and Western blotting during GIV infection of grouper kidney (GK) cells. (A) GK cells were infected with GIV, and transcripts were measured by RT-PCR at 0, 3, 6, 9, 12, 18, and 24 h post-infection (hpi). (B) GK cells were infected with GIV in the presence of CHX or AraC, and the transcripts were measured by RT-PCR at 6 and 18 hpi. (C) GK cells were infected with GIV, and translation of GIV-59L, GIV-97L, and GIV-45R(MCP) was measured by Western blot analysis at 0, 3, 6, 9, 12, 18, 24, and 30 hpi

Similarly, for the temporal translation of GIV-59L in GIV-infected GK cells, Western blotting was performed on GIV-infected GK cells at 0, 3, 6, 9, 12, 18, 24, and 36 hpi. GIV-97L and GIV-45R(MCP) were detected at 3 and 12 hpi, but GIV-59L was not detected until 18 hpi (Fig. 4C). Overall, our results indicate that GIV-59L might be a viral late gene and that GIV-59L(760-1518)-MAb-21 can be used as an effective detection tool in Western blotting.

Subcellular localization of GIV-59L in GIV-infected GK cells

To study the subcellular localization of GIV-59L, GIV-59L(760-1518)-MAb-21 was used in an immunofluorescence assay to examine the intracellular localization of GIV-59L in GIV-infected GK cells. Significant green fluorescence signal of GIV-59L was observed at 24 hpi (Fig. 5B) when compared with mock-infected GK cells (Fig. 5A). Interestingly, the green fluorescence signals of GIV-59L were distributed mainly in the cytoplasm (Fig. 5B,C). This result demonstrates that GIV-59L(760-1518)-MAb-21, in addition to serving as an effective detection tool in Western blotting, can function in immunofluorescence assays.

DISCUSSION

The sequence of the GIV genome is known, but the molecular mechanisms underlying the pathogenicity of iridoviruses are still not well understo od, mainly because of insu fficient gene characterization. In this study, we investigated a late gene, viz. GIV-59L. Our results showed that GIV-59L is 1521 bp in length and can encode a peptide that contains 506 amino acids with a molecular mass of 53.9 kDa. It is predicted that the GIV-59L gene is a myristoylated membrane protein gene. Compared with homologs from other iridovirus isolates, our results showed 98% identity of GIV-59L to 88L of the SGIV (Table 2). Interestingly, the evolutionary relationship of GIV-59L with other homologs was consistent with the relationship of 5 genera within the *Iridoviridae* family (Fig. 1).

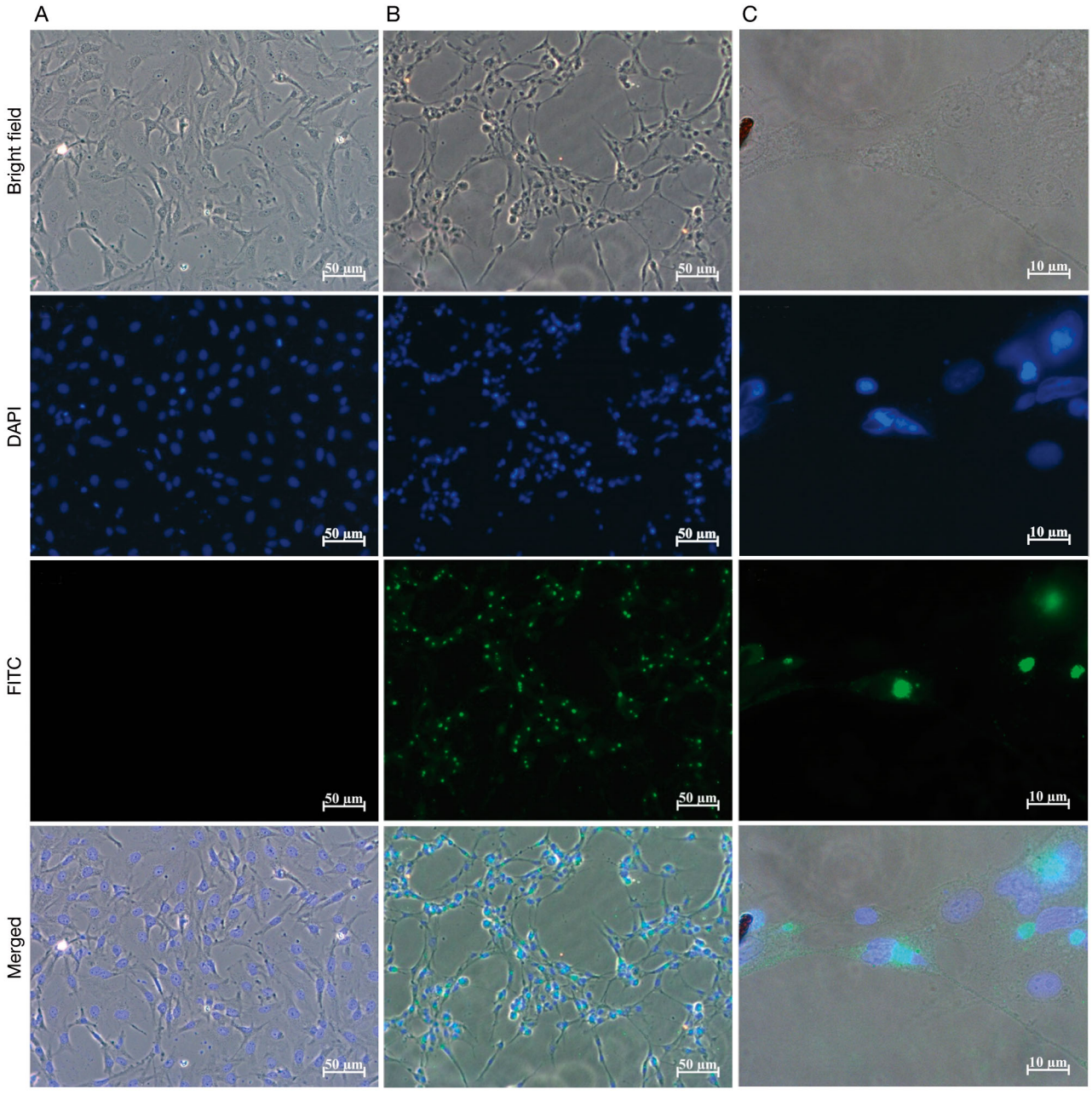

Fig. 5. Localization assay of the 59L gene of grouper iridovirus (GIV-59L) in GIV-infected grouper kidney (GK) cells. Subcellular localization of GIV-59L was analyzed using GIV-59L(760-1518)-MAb-21 against GIV-59L by immunofluorescence assay during GIV infection of GK cells at 24 h post-infection (hpi). The nucleus was counter-stained by DAPI. The fluorescent signal was examined under a fluorescence microscope. (A) Mock-infected GK cells at 24 hpi, (B) GIV-infected GK cells at 24 hpi, (C) higher magnification of GIV-infected GK cells at 24 hpi

In recent years, MAb technology has provided tools for the detection and identification of viral pathogens of aquatic animals, and for research on these viruses (Lai et al. 2001, 2002, Shi et al. 2003, Côté et al. 2009, Hou et al. 2011, Aamelfot et al. 2013, Patil et al. 2013, Siriwattanarat et al. 2013). In previous studies, 3 mouse MAbs against GIV-45R(MCP), -55L, and -97L proteins were produced and used to verify the detection and subcellular localization of target viral proteins in GIV-infected GK cells (Lin et al. 2014, Hu et al. 2015). In this study, we developed and characterized a further mouse MAb (GIV-59L(760-1518)-MAb-21) that is specific for the C-terminal domain (amino acid positions 254–506) of the GIV-59L protein

(Fig. 3). Western blot and immunofluorescence analyses demonstrated that this MAb could serve as a useful tool for investigating the expression and potential role of GIV-59L during GIV infection (Figs. 4 & 5). Our previous and current studies demonstrate that all 4 MAbs could detect their specific antigens by Western blotting. In the previous assays, GIV-97L was detected at as early as 3 hpi, and GIV-45R(MCP) and GIV-55L were detected at 12 hpi, whereas in this study GIV 59L was detected at 18 hpi.

In general, PCR is a more sensitive method than antibody-based assays (e.g. ELISA/Western blotting). This notion is also reflected in the results described here; the transcripts of GIV-97L, -55L, -45R, and -59L were detected by RT-PCR as early as 3–6, 6–12, 9–12, and 12–18 hpi, respectively, and that GIV-97L, -55L, -45R, and -59L proteins were detected later by Western blotting at 3, 12, 12, and 18 hpi, respectively (Lin et al. 2014, Hu et al. 2015). Hence, PCR would be a more sensitive method than Western blotting for detecting the presence of GIV in tissue/cell samples. However, in situations where the DNA or RNA samples are not well preserved or proper laboratory equipment is not available, the antibody-based assays (ELISA/Western blotting) would be alternative options (Omrani et al. 2009).

Viruses usually show a temporal gene expression during infection, and studying this temporal expression can help us to understand the mechanism of infection. The transcription of iridovirus genes can be classified into immediate early (IE), delayed early (DE), and late (L) genes according to their temporal synthesis (Williams 1996, Williams et al. 2005). The IE genes are the first viral genes transcribed by the host polymerase using input virion DNA as a template after infection, and their transcription does not require de novo viral protein synthesis. The IE proteins are essential for the viral life cycle (Willis & Granoff 1985, Xia et al. 2010), and their function might be involved in activating the expression of viral DE and L genes, altering the functions of host genes, and eliminating host immune defense (Buisson et al. 1989, Holley-Guthrie et al. 1990, Williams et al. 2005, Huang et al. 2011). Expression of early (E) genes, dependent on the preceding expression of IE genes, mainly results in enzymes required for viral DNA synthesis and for regulating the expression of L genes, whereas L transcription is a cytoplasmic event catalyzed by a virus-modified or virus-encoded polymerase (Goorha 1981, Williams et al. 2005). L-gene expression is seen only after the onset of DNA replication and results mainly in structural or envelope proteins of viral particles that often are selected as vaccine candidate targets (Ebrahimi et al. 2003, Lua et al. 2005, Sánchez-Paz 2010). In biochemical studies, IE gene expression is usually insensitive to inhibitors of proteins and DNA synthesis such as CHX (which blocks translation and allows only IE viral mRNAs to be synthesized) and AraC (a pyrimidine antimetabolite that can block DNA synthesis), but L-gene expression can be inhibited by AraC. In this study, the temporal expression analysis of GIV-59L was compared with that of GIV-97L and GIV-45R(MCP) by RT-PCR in the presence of inhibitors (CHX or AraC) against DNA or protein synthesis. In our previous study, GIV-97L belonged to the IE genes whereas GIV-45R(MCP) genes were demonstrated to be L genes in GIV infection of GK cells (Lin et al. 2014, Hu et al. 2015). Our data showed that transcription of GIV-59L and GIV-45R(MCP) was inhibited by CHX and AraC. In addition, GIV-45R(MCP) was detected at 9 h, but GIV-59L was detected at 12 h after GIV infection (Fig. 4). The GIV-59L sequence was 98% similar to that of Singapore grouper iridovirus-88L (SGIV-88L), which was classified as a late gene by DNA microarray analysis (Chen et al. 2006) and predicted to belong to a viral envelope myristoylated membrane protein of SGIV by proteomic analysis (Zhou et al. 2011). In the temporal translation assay of GIV-59L, we detected an L gene, GIV-45R(MCP), at 12 h, but GIV-59L was not detected until 18 h after GIV infection (Fig. 4C). Overall, these data support that GIV-59L is a later gene than GIV-45R(MCP) during viral replication.

In our previous studies on the localization of some GIV gene products in GIV-infected GK cells, GIV-45R(MCP) and GIV-97L were found in both the cytoplasm and nucleus, whereas GIV-55L was found only in the cytoplasm during GIV infection of GK cells. In the present work, the immunofluorescence assay with GIV-59L(720-1518)-MAb-21 showed that GIV-59L, like GIV-55L, localized only in the cytoplasm during GIV infection of GK cells.

In conclusion, we have cloned and identified the GIV-59L gene of GIV. Bioinformatic searches revealed that GIV-59L homologs are present in all 5 genera of the family *Iridoviridae*. A mouse MAb was produced specific to the C-terminal domain of the GIV-59L protein, and we have shown that the GIV-59L(760-1518)-MAb-21 can be a valuable tool in the study of the biological function and pathological significance of GIV-59L during viral infection. Finally, GIV-59L was confirmed to be a very late gene and to encode a protein localized in the cytoplasm of infected cells before finally being packed into the whole virus particle.

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