Molecular characterization of three *Rana grylio* virus (RGV) isolates and *Paralichthys olivaceus* lymphocystis disease virus (LCDV-C) in iridoviruses

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Abstract

Three *Rana grylio* virus (RGV) isolates and lymphocystis disease virus (LCDV-C) were molecularly characterized by antigenicity comparison, Western blot detection of viral polypeptides, restriction fragment length polymorphism analysis of viral genomes, and MCP sequence analysis. Significant antigenicity differences existed among the three RGV isolates and LCDV-C. Western blot detection indicated that the viral polypeptides of the three RGV isolates could be recognized by the anti-RGV9807 serum, whereas no bands were observed in the LCDV-C, and significant differences exist among the band patterns of three RGV isolates. Restriction fragment length polymorphism (RFLP) analysis was performed by digesting genomic DNA of the four iridovirus isolates with restriction endonucleases *Hind*III, *Kpn*I, *Xba*I and *Bam*HI. On the whole, obvious discrepancies existed between LCDV-C and RGV isolates, and some significant band pattern differences were also revealed between RGV9808 and RGV isolates and LCDV-C and other iridoviruses. RGV9506, RGV9807 and RGV9808 are clustered together with other ranaviruses, such as FV3, BJV, TFV and ENHV, although the RGV9808 is more close to EHNV than to other ranaviruses. Additionally, LCDV-C is clustered with LCDV-1, the type species of genus Lymphocystisvirus. The current study provides clear evidence that significant genetic difference exists among the three RGV isolates. Therefore, further work on comparative genomic studies will contribute significantly to understanding of their taxonomic position and pathological mechanism.

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1. Introduction

The family *Iridoviridae* includes the genera *Iridovirus*, *Chloririodovirus*, *Ranavirus* and *Lymphocystis-
Some viruses in the genus *Ranavirus* have been recognized as disease cause of lower vertebrates including fish, amphibians and reptiles, because they have lead to severe necrosis of renal and splenic haematopoietic tissues in the infected animals (Essani and Granoff, 1989; Hedrick et al., 1992; Hedrick and McDowell, 1995; Drury et al., 1995; Ahne et al., 1997; Granzow et al., 1997; Zupanovic et al., 1998). Members of the genus Lymphocystisvirus cause chronic and benign infections and result in hypertrophy of epidermal cells in more than 100 species of fish (Essbauer and Ahne, 2001). Although ranaviruses had been viewed as study models for their specific molecular features before the mid-1980s (Williams, 1996), systemic diseases caused by them have produced high mortalities in the world aquaculture with economic and environmental impacts in the last 10 years (Chinchar, 2002). By comparing their morphology, serology, structural polypeptides, MCP sequences and nucleic acid profiles (restriction profile length patterns, RFLP) of about 30 iridoviruses collected from different areas in the world, Hyatt et al. (2000) divided the viruses into six groups according to their geographical origins and hosts. They were group 1—isolates from Australian fish, group 2—isolates from Australian frogs, group 3—isolates from South East Asian fish, group 4—isolates from European fish, group 5—isolates from South American frogs, and group 6—isolates from European and North American frogs.

In recent years, epizootic outbreaks of some lethal diseases usually occurred in densely stocked fish or frog ponds, and became restricting factors for aquaculture development in China. Since 1995, we have initiated a project for identifying the pathogens (Zhang, 2002). Up to now, two species of iridoviruses have been isolated from the diseased aquatic animals. One is *Rana grylio* virus (RGV). It was first isolated from diseased frog (*R. grylio*) in 1995 (isolate RGV9506) (Zhang et al., 1996a,b). Then, another two isolates of RGV9807 and RGV9808 were identified from frogs with lethal syndrome in different areas of China. Based on the studies on morphogenesis, cellular interaction and characterization in the infected EPC (*Epithelioma papulosum cyprini*) cell line, the three isolates were suggested to be an iridovirus similar to frog virus 3 (FV3), the type species of *Ranavirus* (Zhang et al., 1999, 2001). The second iridovirus belongs to the genus Lymphocystisvirus. Because of morphological similarities in viral structures, clinical disease signs (Sun et al., 2000; Zhang et al., 2003a) and genetic divergence in genome size, gene organization and gene product identity (Zhang et al., 2004) with LCDV-1 (the type species in the genus *lymphocystisvirus*) (Tidona and Darai, 1999), the lymphocystis disease virus isolated from the cultured flounder (*Paralichthys olivaceus*) in China has been identified as LCDV-C (LCDV isolated in China) and suggested as a separate species different from LCDV-1 (Zhang et al., 2004). In this study, we are further attempting to analyze molecular characterization of the three RGV isolates to determine whether there exist genetic divergences among them, and to compare them with FV3, the type species of *Ranavirus*. In the same way, the LCDV-C is also characterized and compared by molecular studies.

### 2. Materials and methods

#### 2.1. Cell infection, virus propagation and virus purification

Three *R. grylio* virus isolates (RGV9506, RGV9807 and RGV9808) were preserved in our laboratory (Zhang et al., 2001), and the LCDV-C isolate was isolated from the lymphocystis tissue of diseased flounder (*P. olivaceus*) (Sun et al., 2000; Zhang et al., 2003a, 2004). Because GCO (grass carp ovary) cell line was found to be susceptible to the RGV isolates (Zhang et al., 1999, 2001) and the LCDV-C isolate (Zhang et al., 2003a), the cultured GCO cells were used to propagate the viruses. The cells were grown in TC199 medium supplemented with 10% fetal bovine serum. For virus propagation, the cell cultures were infected with different isolates respectively, and incubated at 25 °C. Until cytopathic effects (CPE) were complete, cell cultures were harvested and stored at −20 °C.

The stock virus suspensions were frozen and thawed three times and centrifuged at 5000 × g for 20 min, and the resulted supernatants were ultracentrifuged at 110,000 × g (Beckman rotor type, SW41) for 90 min. The pellets were resuspended in PBS buffer (NaCl 0.8%, KCl 0.02%, KH₂PO₄ 0.02%, Na₂HPO₄ 0.289%, pH 7.4), and then further puri-
fied by discontinuous sucrose 30%, 40%, 50% and 60% gradient centrifugation at 90,000 × g for 40 min. The viral bands were collected, and sucrose was removed by further centrifugation. Then the pellets were dissolved in TE buffer (10 m mol/L Tris–HCl, pH 7.4; 1 m mol/L EDTA, pH 7.4), and stored at −20 °C.

2.2. Restriction endonuclease analysis of viral genomic DNA

Viral genomic DNAs were isolated as described previously (Zhang et al., 2000, 2001) and digested with restriction endonucleases HinDIII, XbaI, KpnI and BamHI, respectively. The digested DNAs were separated on 0.6% agarose gels, visualized by staining with ethidium bromide, and photographed under UV illumination.

2.3. SDS-PAGE analysis of viral polypeptides

SDS-PAGE analyses were used to compare viral polypeptides among three RGV isolates and LCDV-C isolate. The purified viruses were dissolved in loading buffer (50 mM Tris–Cl, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8) and boiled at 100 °C for 5 min. Polypeptides were separated by 12% SDS-PAGE on Mini-Protein III electrophoresis system (Bio-Rad), and visualized using 0.25% Coomassic brilliant blue.

2.4. Anti-serum preparation

Purified RGV9807 was mixed with Freund’s complete adjuvant (1:1), and used to immunize a New Zealand white rabbit (2.5 kg) by injecting intramuscularly with 2 ml of the mixture emulsion six times at intervals of 5 weeks. The anti-serum was collected and stored at −20 °C until use.

2.5. Antigen capture ELISA assay

The anti-RGV9807 serum was used to capture the four iridovirus isolates in the GCO cell culture. First, the 96-well plates with GCO cell monolayer were coated with anti-RGV9807 serum (1:1000) in carbonate–bicarbonate coating buffer (pH 9.6) overnight at 4 °C. Then, they were rinsed three times with PBS-Tween (PBS with 0.05% Tween 20), and blocked with 5% skim milk resolved in PBS-Tween at 37 °C for 1 h. After rinsing 3 times, each purified virus isolate was added to the plates, and incubated at 37 °C for 90 min. Mock infected GCO cells were used as negative control antigen. The plates were rinsed and incubated with sheep anti-rabbit-IgG HRP (IgG conjugated to horseradish peroxidase) conjugate (streptavidin–biotin complex, SABC) at 37 °C for 90 min. After rinsing, the peroxidase enzyme substrate was added. The color reaction was terminated after 30 min using 1 M H2SO4. Absorbance was measured by spectrophotometer at a wavelength of 490 nm (BIO-RAD Module 550).

2.6. Western blot

Polypeptide proteins of the iridovirus isolates were separated by 12% SDS-PAGE, and transferred to nitrocellulose using a BIO-RAD transfer apparatus for 90 min at 100 V. The nitrocellulose membranes were blocked overnight in 5% skim milk in TBST (150 mM NaCl, 10 mM Tris–HCl pH 7.5, 0.05% Tween-20). Then, the diluted (1:1000) anti-RGV9807 serum was applied for 1 h at room temperature (RT). After rinsing 3 times for 15 min in TBST, the membranes were incubated with sheep-anti-rabbit IgG alkaline phosphatase conjugate (SABC) diluted 1:1000 in 5% skim milk for 1 h at RT. After rinsing in TBST, the membranes were developed with NBT-BCIP substrate system for 20 min in the dark. The reaction was stopped with water (Zhang et al., 2003b).

2.7. PCR amplification and sequence analysis of complete MCP genes

Two pairs of primers were used to amplify major capsid protein (MCP) gene sequences in the three RGV isolates and LCDV-C. One pair of primers was targeted to the 5’ terminus of the iridovirus MCP gene (forward primer: 5’GACTTGGCCACCTTATGAC3’; reverse primer, 5’GTCTCTGGAGAAAGAAGAA3’, Mao et al., 1999b), and other (MCP-F: 5’ACAGTCACCGTGATCTTTGA3’, MCP-R: 5’GGAAAGACTTTTGCGCTGAAGA3’) was designed to amplify the complete MCP gene sequences. Viral DNA samples isolated from the infected cells were respectively used as template. PCR reaction and DNA sequencing were per-
formed as described previously (Zhang et al., 2001). Briefly, 0.5 μl (0.15–0.25 μg) of the viral DNA samples was added to a 20 μl PCR mixture containing 2 μl of 10× PCR buffer (100 mMTris/HCl; 500 mM KCl; 0.8% Nonidet P40), 2 μl of 25 mM MgCl₂, 0.5 μl of 10 mM dNTP, 1 μl of each primer, and 0.5 U Taq DNA polymerase. Cycling conditions were: 94 °C/4 min (1 cycle); 94 °C/40 s, 58 °C/40 s, 72 °C/1 min (34 cycles); 72 °C/2 min (1 cycle). Amplified DNA was fractionated by electrophoresis in a 1.0% agarose gel and visualized by ethidium bromide staining. PCR products were cloned into pMD-18T vector (Takara). After transformation into DH5α competent cells, the recombinant plasmid DNAs were extracted and the cloned viral DNA fragments were sequenced in both directions with M13 universal primers.

Multiple alignment of the deduced amino acid sequences from the three RGV isolates was performed with the corresponding sequences of LCDV-C (Zhang et al., 2004), FV3, TFV (Tiger frog virus) (He et al., 2002), EHV (Marshall et al., 2002), BIV (Marsh et al., 2002), ISKNV (Infectious spleen and kidney necrosis iridovirus) (He et al., 2001), RSIV (Red sea bream iridovirus), LCDV-1 (Tidona and Darai, 1997), and CIV (chilo iridescent virus) (Jakob et al., 2001) using the Clustal W algorithm (Thompson et al., 1994) within MegAlign (DNASTAR Software Package, Madison, WI). Based on the alignment, a phylogenetic tree was constructed using the neighbor-joining method of DNASTAR.

3. Results

3.1. Antigenicity comparison

The RGV9807 anti-serum was used to perform antigen capture ELISA assay for determining whether the three RGV isolates and LCDV-C could cross-react with RGV9807. As expected, significant antigenicity differences existed among the three RGV isolates and LCDV-C. As shown in Fig. 1, LCDV-C produced the lowest optical density (OD) value (0.7), only higher than that of the negative control of GCO cells (OD₄₉₀ 0.06). RGV9807 and RGV9506 produced high OD values (2.8 and 2.4, respectively), while RGV9808 gave a relatively low value (1.6).

3.2. Western blot detection of viral polypeptides

SDS-PAGE separation of the purified virus particles revealed about 20 protein bands (data not shown), but the stained gel was of little comparative value because only a few distinct bands were observed among the four isolates. In order to distinguish the viral polypeptides efficiently, the purified virus particles were subjected to Western blot detection. As shown in Fig. 2, the viral polypeptides of three RGV isolates could be recognized by the anti-RGV9807 serum, whereas no bands were observed in the LCDV-C line. Significant differences exist among the band patterns of three RGV isolates. For example, in comparison with RGV9506 and RGV9807, RGV9808 lacks two main bands of 48 kDa and 16 kDa, and adds three main bands of 85 kDa, 44 kDa and 33 kDa. One main band of 36 kDa in RGV9506 is absent in RGV9807. One main band of 39 kDa in RGV9807 is absent in RGV9808, and very weak in RGV9506.

3.3. Restriction fragment length polymorphism analysis of viral genomes

Restriction fragment length polymorphism (RFLP) analysis was performed by digesting genomic DNA of the four iridovirus isolates with restriction endonucleases HindIII, KpnI, XbaI and BamHI. On the whole, obvious discrepancies existed between LCDV-C and RGV isolates. As shown in Fig. 3,
HindIII digestion resulted in more than 30 fragments in LCDV-C, whereas only 6 fragments were observed in the three RGV isolates (Fig. 3A). KpnI digestion produced near 20 fragments in three RGV isolates, while only 2 fragments were seen in the LCDV-C (Fig. 3C). Interestingly, some significant differences were also revealed among the three RGV isolates. For example, RGV9808 was obviously distinct from RGV9506 and RGV9807 in the profiles of restriction endonucleases XbaI, KpnI and BamHI (Fig. 3), and only about 50% common fragments were shared among them. In comparison with RGV9808, RGV9506 and RGV9807 contained more than 80% common fragments, and possessed identical band patterns in the profiles of HindIII and BamHI digestion (Fig. 3A,D).

3.4. Phylogenetic relationship analysis of three RGV isolates, LCDV-C and other iridoviruses based on their MCP sequences

PCR amplification and sequence analysis further revealed their phylogenetic relationship among the three RGV isolates, LCDV-C and other iridoviruses. As shown in Fig. 4, the 5’ end primers of MCP gene generated a specific fragment of about 500 bp in FV3, RGV-9506, RGV-9807, RGV-9808 and LCDV-C, and the complete primers of MCP
gene produced an about 1.5 kb fragment from the three RGV isolates and FV3, whereas no product was produced from LCDV-C genome. Multiple alignments (Fig. 5) of the deduced amino acid sequences showed that the MCP sequence in RGV9506 is 99.4% identical to that in RGV9807, while the sequence in RGV9808 is respectively 97.6% and 97.8% identical to that in RGV9506 and RGV9807. In comparison with FV3 MCP, the identity (97.4%) of RGV9808 is lower than that of RGV9506 (99.4%) and RGV9807 (99.1%). In contrast to the high homology between the RGV isolates and FV3, the LCDV-C MCP has only 51.1% identity to the RGV isolates, although it possesses 87.6% identity to that of LCDV-1. Based on the alignments of MCP amino acid sequences, a phylogenetic tree was constructed. As shown in Fig. 6, RGV9506, RGV9807 and RGV9808 are clustered together with other ranaviruses, such as FV3, BIV, TFV and ENHV, although the RGV9808 is more close to EHNV than to other ranaviruses. Additionally, LCDV-C is clustered with LCDV-1, the type species of genus Lymphocystivirus.

4. Discussion

Viruses in the genus of Ranavirus and Lymphocystivirus have a wide host range, which have lead to severe diseases in lower vertebrates including fish, amphibians and reptiles (Ahne et al., 1997; Essbauer and Ahne, 2001). Previous studies have proved that a minimally pathogenic iridovirus in one species can cause serious diseases in other specie (Hengstberger et al., 1993; Zupanovic et al., 1998). And, some similar iridoviruses have also been isolated from wild sympatric fishes and amphibian (Mao et al., 1999b). In this study, we observed apparent genetic divergences among the three RGV isolates by comparative studies on molecular characterization. All data obtained from antigenicity comparison, Western blot detection of viral polypeptides, restriction fragment length polymorphism analysis of viral genomes, and MCP sequence analysis indicated that RGV9808 was obviously distinct from RGV9506 and RGV9807. In earlier studies on morphogenesis, cellular interaction and preliminary characterization, we have suggested that the three RGV isolates might belong to the genus Ranavirus, and might be similar to frog virus 3 (FV3), the type species of Ranavirus (Zhang et al., 1999, 2001). The current studies confirmed the previous suggestion, and further revealed their genetic differences among the three RGV isolates. Besides the type species FV3, some putative species, including BIV, EHNV, Redwood Park virus (RPV), Regnia Ranavirus (RRV) and Santee-Cooper Ranavirus (SCRV), have been listed in the genus Ranavirus (van Regenmortel et al., 2000). According to the phylogenetic analysis of MCP amino acid sequences (Fig. 6), the three RGV isolates were
clustered together with other ranaviruses, such as FV3, BIV, TFV and ENHV, but they were obviously divided into two subgroups. RGV9506 and RGV9807 were clustered together with FV3, BIV and TFV, whereas RGV9808 was clustered together with EHNV.

MCP is highly conserved in iridoviruses, and is predominant structural component of the virus particles comprising 40–45% of total particle polypeptides (Tham et al., 1986). Therefore, it has been widely used for their phylogenetic analysis. Mao et al. (1997, 1999a,b) and Webby and Kalmakoff (1998) characterized a number of iridoviruses isolated from various fish and insect species by comparing 5′ end sequences of MCP. Using this approach, novel isolates can more readily be sorted into distinct viral species based on the amino acid multiple alignments, and many iridoviruses isolated in different aquatic animal hosts from Australian, European, American and Asian have been classified into the genus Ranavirus (Mao et al., 1999a). In a previously study, we amplified and sequenced about 500 nucleotides of the MCP 5′end from RGV9506 and RGV9807, and found that their amino acid sequences were identical to each other, and differed from FV3 at only 1 position (Zhang et al., 2001). In the current study, we further amplified and sequenced the corresponding sequence of RGV9808 and the complete MCP sequences of RGV9506, RGV9807 and RGV9808, and revealed significant sequence difference in RGV9808. Originally, RGV9506 was prepared in diseased young frogs after metamorphosis from a frog culture farm located in Wuhan, Hubei province (Zhang et al., 1996a,b), whereas RGV-9807 and RGV-9808 were isolated in 1998, respectively, from diseased adult frogs (weight, about 300 g) and tadpoles (weight, about 10 g) from 2 different frog culture farms. One farm was located in Yueyang, Hunan province, and the other in Wuhan, Hubei province (Zhang et al., 2001). Obviously, RGV9808 was the viral isolate from tadpoles. Therefore, complete genome sequence will be needed to clarify their taxonomic position in ranaviruses.

Recently, the complete genome sequence of LCDV-C has been reported, and an unexpected level of divergence in their genomes in size, gene organization, and gene product identity has been revealed between LCDV-C and LCDV-1, the type species of lymphocystisviruses, and between LCDV-C and other iridoviruses (Zhang et al., 2004). In the current study, we amplified the about 500 bp fragment of MCP gene in LCDV-C by the 5′ end primers of conserved region, but could not obtain the complete MCP sequence similar to the RGV isolates. The constructed phylogenetic tree (Fig. 6) again confirmed that LCDV-C and LCDV-1 are clustered together, but the amino acid sequences of LCDV-C and LCDV-1 MCPs have only 87.6% identity.

In summary, variations in iridoviruses are ubiquitous. This study provides clear evidence that significant genetic difference exists among different RGV isolates. Therefore, further work on comparative genomic studies will contribute significantly to under-
standing of their taxonomic position and pathological mechanism.

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