Identification and characterization of a novel envelope protein in *Rana grylio* virus

Zhe Zhao, Fei Ke, You-Hua Huang, Jiu-Gang Zhao, Jian-Fang Gui and Qi-Ya Zhang

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Graduate School of Chinese Academy of Sciences, Wuhan 430072, PR China

Viral envelope proteins have been proposed to play significant roles in virus infection and assembly. In this study, an envelope protein gene, 53R, was cloned and characterized from *Rana grylio* virus (RGV), a member of the family *Iridoviridae*. Database searches found its homologues in all sequenced iridoviruses, and sequence alignment revealed several conserved structural features shared by virus capsid or envelope proteins: a myristoylation site, two predicted transmembrane domains and two invariant cysteine residues. Subsequently, RT-PCR and Western blot detection revealed that the transcripts encoding RGV 53R and the protein itself appeared late during infection of fathead minnow cells and that their appearance was blocked by viral DNA replication inhibitor, indicating that RGV 53R is a late expression gene. Moreover, immunofluorescence localization found an association of 53R with virus factories in RGV-infected cells, and this association was further confirmed by expressing a 53R–GFP fusion protein in pEGFP-N3/53R-transfected cells. Furthermore, detergent extraction and Western blot detection confirmed that RGV 53R was associated with virion membrane. Therefore, the current data suggest that RGV 53R is a novel viral envelope protein and that it may play an important role in virus assembly. This is thought to be the first report on a viral envelope protein that is conserved in all sequenced iridoviruses.

INTRODUCTION

*Rana grylio* virus (RGV) is a pathogenic agent that results in greater than 90% mortality in cultured pig frog (*Rana grylio*) (Zhang et al., 2001). Previous studies have revealed that RGV is a member of the family *Iridoviridae* and is closely related to frog virus 3 (FV3), the type species of the genus *Ranavirus* (Zhang et al., 1999, 2001, 2006). Recently, cellular changes and several viral proteins have also been investigated during RGV infection and replication (Huang et al., 2006, 2007; Sun et al., 2006; Zhao et al., 2007).

Iridoviruses are large, icosahedral, enveloped DNA viruses that contain circularly permuted and terminally redundant double-stranded DNA genomes (Fauquet et al., 2005; Williams et al., 2005). To date, 12 iridovirus genomes have been sequenced completely (Eaton et al., 2007), but the key genes and their functions remain to be elucidated, especially the viral envelope protein genes. Viral envelope proteins are particularly important because of their vital roles in virus assembly and infection (Chazal & Gerlier, 2003). Recently, two proteins were characterized as membrane-tropic structural proteins, but these are conserved only among members of the genus *Megalocytivirus* in the family *Iridoviridae* (Ao & Chen, 2006; Xu et al., 2008). To understand molecular mechanisms for iridovirus assembly and infection, we focused our attention on some of the transmembrane (TM) proteins that are shared by all sequenced iridoviruses. For this purpose, a TM protein gene corresponding to open reading frame (ORF) 53R of FV3 (Tan et al., 2004) was found to be conserved among all sequenced iridoviruses. In this study, we cloned and characterized the TM protein gene from RGV and revealed its possible functional role in virus assembly.

METHODS

Viruses and cells. Virus isolate RGV9506 was used in this study. Fathead minnow (FHM) cells were used to propagate the virus. Cell culture and virus propagation were performed as described previously (Zhang et al., 1999, 2006).

Gene cloning and protein sequence analysis. Using the sequences of the conserved 53R genes of the FV3 and tiger frog virus genomes (He et al., 2002; Tan et al., 2004), a pair of primers (5'-TCCACATAAAATCTACTCGAT-3' and 5'-GAAAGGAATGCAA-GTCTATC-3') located in the 5'- and 3'-flanking regions of the 53R ORF was designed and used to amplify RGV 53R from the genomic DNA. The fragment was cloned and sequenced, and the sequence data
were compiled and analysed using DNAStar software. The non-redundant protein sequence database of the National Center for Biotechnology Information (National Institutes of Health, MD, USA) was searched using BLASTP and iterative searches were performed using PSI-BLAST (Altschul et al., 1997). Multiple sequence alignments were constructed using CLUSTAL_X v1.83 and edited using GeneDoc.

Prokaryotic expression, protein purification and antibody preparation. Because there are two hydrophobic domains in the middle region of RGV 53R, a fragment encoding the C-terminal 279 aa unique to RGV 53R was amplified using primers 5'-GTAGGATCCGAGGGCTTCTCGTCCG-3' and 5'-GTCCTCGAGATCCTTTACCCCTGTGG-3' and ligated into the prokaryotic vector pET-32a (Novagen). The recombinant plasmid, named pET32a/53R, was transformed into *Escherichia coli* BL21(DE3) and the bacteria were induced for 4 h with 1 mM IPTG at 37°C to express the fusion protein. The fusion protein was purified from inclusion bodies under denaturing conditions using a HisBind purification kit (Novagen), mixed with an equal volume of Freund's adjuvant (Sigma) and used to immunize mice by hypodermal injection once every 7 days. After the fifth immunization, anti-RGV 53R serum was collected and tested by Western blotting with lysates from virus-infected cells. Its specificity was validated by a pre-adsorption experiment. Detection of cellular α-tubulin was used as an internal control.

RT-PCR and Western blot analysis. Total RNA and protein were isolated from cells infected with RGV at an m.o.i. of 1 at various times post-infection (p.i.) (0, 4, 8, 12, 16, 24, 36 and 48 h) or mock infected, and were subjected to RT-PCR and Western blot analysis, respectively, as described previously (Zhao et al., 2007). For RT-PCR, the specific primers 5'-CATCAGAACGGGAGGACAGA-3' and 5'-CGCCGTGTCGTCCTTGTAG-3' were used to monitor the transcription of RGV 53R. For Western blot analysis, anti-RGV 53R serum was used as the primary antibody at a dilution of 1:500, followed by alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) antibody at a dilution of 1:1000 (Vector Laboratories) as the secondary antibody. Internal controls were carried out simultaneously by detecting β-tubulin mRNA and protein, respectively. Cytosine β-D-arabinofuranoside (AraC), a viral DNA replication inhibitor, was used to classify the transcription class of RGV 53R. Briefly, 100 μg AraC ml⁻¹ was added to FHM cells for 1 h prior to virus infection and the pre-treated cells were then mock infected or infected with RGV at an m.o.i. of 1. Total protein was extracted at 24 and 48 h p.i. for Western blot analysis.

Immunofluorescence microscopy. FHM cells, grown on coverslips in six-well plates, were mock infected or infected with RGV at an m.o.i. of 0.1 and then fixed in 70% ethanol overnight at −20°C at 24, 36 and 48 h.p.i. After blocking in 10% normal goat serum at
room temperature for 1 h, the cells were incubated with anti-RGV 53R serum in 1% normal goat serum for 2 h, rinsed three times for 10 min each with PBS containing 1% normal goat serum and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (Pierce). 4,6-Diamidino-2-phenylindole (DAPI; Sigma) staining was used to visualize DNA in nuclei and virus factories. All samples were examined under a Leica DM IRB fluorescence microscope.

**Transient transfection and subcellular localization.** A recombinant eukaryotic vector, pEGFP-N3/53R, was constructed by cloning the entire 53R ORF into pEGFP-N3 (Clontech) using primers 5'-CTAAAGCTTTATGTAGGGAAAATGGGA-3' and 5'-ATGGATCCATCCATACCCCTGT-3'. This vector expressed RGV 53R with a C-terminal fusion green fluorescent protein (GFP) tag. Plasmids pEGFP-N3/53R and pDsRed2-ER, an endoplasmic reticulum (ER)-specific marker (Clontech), were transiently co-transfected into FHM cells to evaluate the intracellular location of RGV 53R. The cells were fixed at 12 and 48 h post-transfection and examined by fluorescence microscopy. To track the fate of RGV 53R in transfected cells in more detail, cells were infected with RGV at the same time as transfection. After 48 h, the cells were fixed and stained with DAPI as described above and then examined by fluorescence microscopy.

**Detergent extraction and phase separation of purified virions.** Membrane component was extracted from RGV virions with a non-ionic detergent as described previously (Ojeda et al., 2006b). In brief, purified virions were treated with 50 mM Tris/HCl (pH 7.4) containing 1% NP-40 detergent in the presence or absence of 50 mM dithiothreitol (DTT). The mixture was incubated for 1 h at 37°C, and insoluble and soluble materials were separated by centrifugation at 15 000 g for 1 h. Proteins from the pellet and supernatant were analysed by 12% SDS-PAGE and transferred to PVDF membrane for Western blot analysis.

**RESULTS**

**Conservation of RGV 53R in all sequenced iridoviruses**

Using the designed primers, a 1628 bp fragment was amplified from the RGV genome. Sequence alignment revealed that this fragment contained the complete ORF of RGV 53R (GenBank accession no. EU358954). The ORF was 1569 nt and encoded a peptide of 522 aa with a predicted molecular mass of 54.7 kDa. The RGV 53R gene had homologues in all iridoviruses sequenced to date, but no homologues or orthologues were found among non-iridoviruses using a position-specific iterative BLAST search. Sequence alignment revealed several conserved features, including a consensus sequence M-G-X-X-(S/T/A) for N-terminal glycine myristylation, two invariant cysteines and two predicted TM domains (Fig. 1). In addition, the C-terminal region was less well conserved than the N-terminal region and the C-terminal length was variable.

**Fig. 2.** Expression of RGV 53R C-terminal peptide and specific detection using anti-RGV 53R antibody. (a) SDS-PAGE of expressed and purified tagged fusion protein. Lanes: 1, pET32a/53R, non-induced; 2, pET32a/53R, induced; 3, purified fusion protein. The target protein is indicated by an arrow. M, Protein molecular mass markers. (b) Western blot detection to test anti-RGV 53R antibody specificity. Lanes: 1, lysate from mock-infected cells; 2 and 3, lysate from RGV-infected cells at 48 h p.i. In lanes 1 and 2, the protein was detected using anti-RGV 53R antibody, whilst in lane 3, the antibody was pre-adsorbed with purified RGV 53R prior to blotting. (c) Detection of cellular α-tubulin was used as an internal control.

**Fig. 3.** Temporal expression pattern of the 53R gene and protein in infected cells during RGV infection. (a, b) Total RNA and protein were isolated from mock-infected (lane C) and RGV-infected cells at the times indicated and analysed by RT-PCR (a) and Western blot analysis (b), respectively. α-Tubulin was detected under the same conditions as an internal control. DNA or protein size markers are indicated (lane M). (c) Western blot detection of RGV 53R expression in the presence or absence of AraC. At the indicated times, cells were harvested and analysed as described in (b). Mock, uninfected cells (control).
Specificity of the anti-RGV 53R antibody

To prepare anti-RGV 53R serum, pET32a/53R was transformed into E. coli BL21(DE3) and expression of the fusion protein was induced. The fusion protein was approximately 48.3 kDa, which included the RGV 53R C-terminal fragment (29.1 kDa) and the Trx/His/S tag (19.2 kDa) (Fig. 2a). The fusion protein was purified and used to generate anti-RGV 53R polyclonal antibody in mice. To test the specificity of the antibody, lysates from RGV-infected and mock-infected cells were first subjected to Western blot analysis. As shown in Fig. 2(b), the anti-RGV 53R antibody specifically recognized a 54.7 kDa protein in RGV-infected cell lysate, which corresponded to the theoretical molecular mass of RGV 53R, whereas no protein band was detected in the mock-infected cell lysate. Moreover, when the same antiserum was pre-adsorbed with the purified fusion protein, the specific 54.7 kDa protein in the RGV-infected cell lysate could not be detected using the pre-adsorbed antiserum. As an internal control, the \( \alpha \)-tubulin content was shown to be consistent between the lysates from the RGV-infected and mock-infected cells (Fig. 2c). These data indicated that the antibody was specific to RGV 53R.

Temporal expression pattern of RGV 53R during RGV infection

The temporal expression pattern of RGV 53R was characterized during RGV infection by RT-PCR and Western blot analysis. At the transcriptional level, a 234 bp RGV 53R-specific fragment was detected at 12 h p.i. by using 53R-specific internal primers (see Methods) and the amount of this fragment increased to a high level at 48 h p.i. (Fig. 3a). At the protein level, a 54.7 kDa protein band was detected by Western blotting from 16 h p.i. in RGV-infected cells and its level also increased up to 48 h p.i. (Fig. 3b). As an internal control, the mRNA and protein levels of \( \alpha \)-tubulin expression were consistent throughout the experiments. The 54.7 kDa protein band was not detected when viral DNA replication was inhibited by AraC (Fig. 3c), consistent with the temporal data, indicating that RGV 53R belongs to the late expression class of genes.

![Figure 4](http://vir.sgmjournals.org) 1869

**Fig. 4.** Immunofluorescence localization of RGV 53R. FHM cells were infected with RGV for 24, 36 or 48 h and the cells were fixed, permeabilized and stained with anti-RGV 53R serum and FITC-conjugated anti-mouse antibody, followed by DAPI. The arrows indicate virus factories stained with DAPI. Mock-infected cells were used as a negative control. Magnification \( \times 100 \) (oil-immersion objective).
**Intracellular localization and distribution of RGV 53R**

Immunofluorescence localization revealed the intracellular distribution and dynamic changes in RGV 53R during infection. As shown in Fig. 4, when FHM cells were infected with RGV for 24 h, RGV 53R initially appeared in the cytoplasm and aggregated mainly at the two poles of the cells. At later times p.i., the protein converged at the perinuclear region and co-localized with the virus factories for virus assembly. At 48 h p.i., the distribution of the protein was more compacted around the virus factories than at 36 h p.i.

The intracellular localization and dynamic distribution of RGV 53R were also evaluated by expressing a 53R–GFP fusion protein in pEGFP-N3/53R-transfected cells. Fluorescence microscopy showed that fluorescence was initially distributed in the cytoplasm and co-localized with the ER marker (pDsRed2-ER) at 12 h post-transfection (Fig. 5a), indicating that RGV 53R was targeted to ER membranes in transfected cells. However, as the time post-transfection increased, the synthesized 53R–GFP became more compacted and accumulated into spots at 48 h post-transfection. No co-localization with the ER marker was observed at that time (Fig. 5a). Interestingly, GFP fluorescence and DAPI staining revealed that the accumulated spots of 53R–GFP fluorescence were the sites of virus factories for virus assembly (Fig. 5b). In addition, ER marker labelling and DAPI staining also showed that the virus factory sites excluded the tested ER marker components, but they were closely surrounded by the ER components (Fig. 5c).

**Identification of RGV 53R as viral envelope protein**

The presence of two TM regions suggested that RGV 53R might be associated with the viral envelope. To determine whether RGV 53R was present in the membrane fraction of

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**Fig. 5.** Intracellular localization of RGV 53R detected by expression of a 53R–GFP fusion protein. (a) Localization relationship between the 53R–GFP fusion protein and the ER. Green fluorescence shows the localization of 53R–GFP (left panel), whilst red fluorescence shows the localization of the ER (middle panels). (b) Determination of the fate of 53R–GFP in transfected cells following infection with RGV. Left panel: 53R–GFP fluorescence signals; middle panel: DNA staining pattern. (c) Distribution of the ER in FHM cells following infection with RGV. Left panel: ER fluorescence signals; middle panel: DNA staining pattern. The arrows indicate virus factories stained with DAPI. Magnification ×100 (oil-immersion objective).
RGV virions, we purified virions from RGV-infected FHM cells and extracted the membrane proteins using 1% NP-40 with or without 50 mM DTT. The soluble protein components (supernatant) and the insoluble core components (pellet) were subjected to SDS-PAGE separation and Western blot analysis. As shown in Fig. 6, when the purified RGV virions were extracted using buffer without 1% NP-40 or 50 mM DTT, no RGV 53R was detected in the soluble components. However, when the purified RGV virions were extracted using buffer with 1% NP-40, RGV 53R could be detected equally in the supernatant and in the pellet. Moreover, if the purified RGV virions were extracted using buffer with 1% NP-40 and 50 mM DTT, the protein was dissolved more efficiently in the supernatant. These results indicated that RGV 53R is associated with the virion membrane and is a viral envelope protein.

**DISCUSSION**

In this study, we cloned and characterized the RGV 53R gene and found that it has homologues in all iridoviruses sequenced to date, whereas no homologues or orthologues were found among non-iridoviruses. In addition, some conserved structural features shared by virus capsid or envelope proteins (Ravanello et al., 1993) were revealed in the RGV 53R protein, implying that RGV 53R may be involved in viral infection and assembly (Ravanello & Hruby, 1994; Wolfe et al., 1995; Martin et al., 1997; Ojeda et al., 2006a, b). Therefore, the RGV 53R gene was selected for further characterization to understand its possible functional role in virus infection and assembly.

Iridoviruses have been found to display a complex gene regulation strategy in which genes are expressed in three main temporal kinetic stages: immediate-early, early and late (Nalcacioglu et al., 2003, 2007; Lua et al., 2005; Chen et al., 2006). Late genes are expressed after the onset of viral DNA replication and their expression can be blocked by viral DNA replication inhibitors (Chambers et al., 1999; Ebrahimi et al., 2003). In this study, our data showed that the transcriptional and translational products of RGV 53R were not produced prior to 12 h p.i. or in the presence of AraC, indicating that this gene belongs to the late gene expression class.

Replication and assembly of iridoviruses often take place in specific intracellular compartments known as the ‘viromatrix’ or ‘virus factories’ where viral components concentrate, including structural proteins and genomic DNA, as well as different types of membranous structure (Novoa et al., 2005; Huang et al., 2006; Netherton et al., 2007). Significantly, the association of RGV 53R with virus factories was not only revealed by immunofluorescence localization in RGV-infected cells, but also confirmed by expressing the 53R-GFP fusion protein in pEGFP-N3/53R-transfected cells. Finally, the RGV 53R protein was confirmed as being associated with the virion membrane by detergent extraction and Western blot detection. Therefore, the current data suggest that RGV 53R is a viral envelope protein.

In addition, the intracellular distribution and dynamic changes of RGV 53R in pEGFP-N3/53R-transfected cells revealed an interesting phenomenon. RGV 53R initially co-localized with the ER components at an early stage post-transfection, but as the post-transfection time increased, the ER marker components were excluded from the virus factories. Why? A possible explanation is that RGV 53R may play a role in recruiting ER-derived membranes into virus factories as the precursors of the virus inner envelope. Moreover, the process of recruitment appeared to modify or damage the cellular ER components so that the ER marker components were excluded from the virus factories. African swine fever virus (ASFV), the only member of the family Asfarviridae, shares close similarities in morphology and morphogenesis with iridoviruses (Cobbold et al., 2001). Previously, it has been reported that the ASFV envelope protein p54 is critical for the recruitment and transformation of collapsed ER membranes as the precursors of the inner viral envelope (Rodrı´guez et al., 2004). Therefore, another key aspect of future work is to clarify the function of RGV 53R and its interaction with cellular ER proteins.

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