Cloning, expression and subcellular distribution of a *Rana grylio* virus late gene encoding ERV1 homologue

Fei Ke · Zhe Zhao · Qiya Zhang

Abstract An essential for respiration and viability (ERV1) homologue, 88R, was cloned and characterized from *Rana grylio* virus (RGV). Database searches found its homologues in all sequenced iridoviruses, and sequence alignment revealed a highly conserved motif shared by all ERV1 family proteins: Cys-X-X-Cys. RT-PCR and western blot analysis revealed that *88R* begins to transcribe and translate at 6 h postinfection (p.i.) and remains detectable at 48 h p.i. during RGV infection course. Furthermore, using drug inhibition analysis by a de novo protein synthesis inhibitor and a viral DNA replication inhibitor, RGV *88R* was classified as a late (L) viral gene during the in vitro infection. 88R-EGFP fusion protein was observed in both the cytoplasm and nucleus of pEGFP-N3-88R transfected EPC cells. Although result of immunofluorescence is similar, 88R protein was not detected in viromatrix. Moreover, function of RGV *88R* on virus replication were evaluated by RNAi assay. Nevertheless, effect of knockdown of RGV *88R* expression on virus replication was not detected in cultured fish cell lines. Collectively, current data indicate that RGV *88R* was a late gene of iridovirus encoding protein that distributed both the cytoplasm and nucleus.

Keywords *Rana grylio* virus (RGV) · Iridovirus · ERV1 · Late viral gene · RNAi

F. Ke · Z. Zhao · Q. Zhang (✉)
State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, Wuhan 430072, China
e-mail: zhangqy@ihb.ac.cn

Introduction

Rana grylio virus (RGV) is a pathogenic agent that results in high mortality in cultured pig frog *R. grylio* [1]. RGV is a large DNA virus and is closely related to *frog virus 3* (FV3), the type species of genus *Ranavirus* (family *Iridoviridae*) [2–4]. Iridoviruses display a complex gene regulation strategy in which genes are expressed in three main temporal stages: immediate early (IE) genes, early (E) genes and late (L) genes, which can be defined experimentally by using DNA replication and protein synthesis inhibitors [5–8].

To date, the genomes of twelve iridoviruses, including FV3, have been completely sequenced [8, 9], and 26 core genes have been predicted to exist in all iridoviruses [10]. One of these is a gene belonging to ERV1 (essential for respiration and viability) family. However, we need experimental evidence to declare the existence of this gene.

ERV1 homologues appear to be widespread in nature, present in organisms as diverse as human, mice, roundworms, insects, and protozoa [11–13]. These homologues are multifunctional, performing roles in DNA replication, protein synthesis, protein folding, and photosynthesis [14]. Interestingly, ERV1 gene homologues are present in a number of other DNA viruses, including poxvirus, chlorella virus, and African swine fever virus [15, 16].

RNA interference (RNAi) is a natural biological mechanism for silencing genes in most cells of living organisms [17]. It is a process of sequence-specific gene silencing in the cytoplasm of a eukaryotic cell, in which double-stranded small interfering RNAs (siRNAs) of 21–23 nucleotides (nts) are associated with a multiprotein complex known as the RNA-induced silencing complex (RISC) to target homologous mRNA for degradation based on complementary base pairing. Targets of siRNAs may

include mRNAs of cellular genes or genes of an invading virus [18–20]. Indeed, siRNA has been used to silence exogenous viral gene expression, such as genes from iridoviruses, in several cell lines [21, 22].

In this study, we are attempting to clone and characterize the ERV1 homologue in RGV genome, and to understand its role in iridovirus replication by RNAi.

Materials and methods

Virus and cells

The RGV isolates RGV9506 which was isolated from the cultured pig frog *R. grylio* was used in this study. Propagation of RGV and isolation of the virus genomic DNA were performed as described previously [3]. *Epithelioma papulosum cyprini* (EPC) cell and Grass carp ovary (GCO) cells were maintained in TC199 medium supplemented with 10% fetal bovine serum (FBS) at 25°C.

Gene cloning, amino acid sequence comparisons and phylogenetic analysis

The complete *88R* gene was amplified by PCR from RGV genomic DNA using the primers, p1, (5′ GGGCGTGGAT GGACTTGA 3′) and p2, (5′ TCGTCCCAGTCGGCGTAT 3′), which were designed using the flanking sequence of the putative *88R* gene in FV3 [23]. The amplified fragment was cloned into PMD18-T vector (TaKaRa) and sequenced. Database similarity searches were carried out using the BLAST server [24], alignment of amino acid sequences was carried out using ClustalX 1.83 [25] and edited by GeneDoc program.

Phylogenetic analysis was performed on the basis of 25 ERV1 homologues containing all known 12 iridovirus genomes and some other species, such as human, rat, drosophila, and other viruses. Briefly, alignment of amino acid sequences was carried out using Muscle 3.6 [26]. The result was cured and phylogenetic analysis was done by maximum likelihood program using PHYML online web server [27].

Cloning into expression vectors

The fragments in *88R* gene were amplified by PCR from RGV genomic DNA using the designed primers, p3/p4, (p3, 5′ ACACGGATCCATAAAAATGCACG 3′, *Bam*HI; p4, 5′ TG CAGCTCGAGTTAGTCTCAGTTAA 3′, *Xho*I); p5/p6, (p5, 5′ ACAAAGCTTCATAAAAATGCACG 3′, *Hin*dIII; p6, 5′ TTAGGATCCGTTAAAAGTGCTC 3′, *Bam*HI); p7/p4, (p7, 5′ ACACGGATCCATAAAAATGG ACG 3′, *Bam*HI; p4, 5′ TGCAGCTCGAGTTAGTCT-CAGTTAA 3′, *Xho*I), with restriction enzymes sites, respectively. The amplified products were, respectively, cloned into prokaryotic vector pET32a (Novagen), eukaryotic vectors pEGFP-N3 (clontech) and pcDNA3.1 (+) (Invitrogen) that were previously cleaved by corresponding restriction enzymes. Three different constructs, named pET32a-88R, pEGFP-N3-88R, pcDNA3.1-88R, were confirmed by restriction enzyme digestion and DNA sequencing.

Prokaryotic expression, protein purification and antibody preparation

pET32a-88R was transformed into *E. coli* BL21 (DE3), and then protein expression was induced using 1 mM IPTG at 37°C for 6 h. The recombinant protein was purified using the HisBind Purification Kit (Novagen) according to the manufacturer's instructions. To generate antibody to RGV 88R, the purified recombinant 88R protein was mixed with equal volume of Freund's adjuvant (Sigma). Mice were immunized by intraperitoneal injection (IP) weekly for 5 weeks at a dose of 50 μg every mouse every times, and the antiserum was collected at 7 days post the fifth immunization. Freund's complete adjuvant was used in the first injection and Freund's incomplete adjuvant was used in all subsequent injections.

RT-PCR and western blot analysis

EPC monolayer cells were infected with RGV at an MOI of 1 and harvested at 0, 2, 4, 6, 8, 12, 16, 24, 36, 48 h p.i. Total RNA was extracted from the harvested samples with TRIzol reagent (Invitrogen) and digested with RNase-free DNase I (TaKaRa) before carrying out RT-PCR. The first strand cDNA was synthesized using random primers and MMLV reverse transcriptase (Promega) by incubation for 1 h with 2 μg of RNA at 37°C. RT-PCR was performed with specific primers, p8/p9, (p8, 5′ TGGACCCTCC ATGTGGTTCA 3′; p9, 5′ TTCAACCGAGCGTTGACC AT 3′). The PCR reaction was performed in a volume of 25 μl, containing 1 μl cDNA, 0.2 μM of each primer, 0.5 U of Taq polymerase (MBI), 0.5 μl of 10 mM dNTP, 2.5 μl of 10× Taq buffer and 2.5 μl of 25 mM MgCl2 (MBI). PCR conditions were as follows: 4 min at 94°C and then 30 s at 94°C, 40 s at 60°C, 40 s at 72°C for 30 cycles, followed by 72°C for 10 min. The amplification of β-actin gene with primers, Actin-F/Actin- R, (Actin-F, 5′ CAC-TGTGCCCATCTACGAG 3′; Actin-R, 5′ CCATCTCCT GCTCGAAGTC 3′) served as an internal control and PCR procedure was the same as 88R except for the annealing temperature of 55°C.
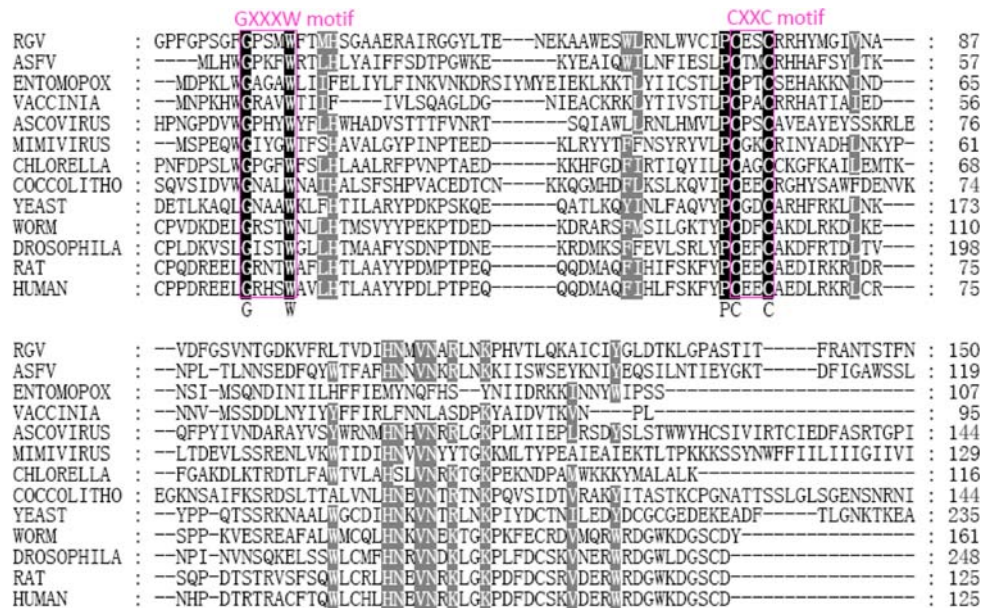
Fig. 1 Alignment of the predicted amino acid sequence of the RGV 88R ORF product (RGV) with ERV1 homologues found in some viruses and eukaryotes. Homologues shown are as follows (with the GenBank accession number of the nucleotide sequence given in parentheses following each): ASFV, African fever virus ORF 9GL product (AAF27970); ENTOMOPOX, *M. sanguinipes* entomopoxvirus ORF MSV093 product (AF063866); VACCINIA, Vaccinia virus ORF E10R product (NC_006998); ASCOVIRUS, Spodoptera frugiperda ascovirus 1a ORF 061 product (YP_762416); MIMIVIRUS, *A. polyphaga* mimivirus ORF R368 product (YP_142722); CHLORELLA, Chlorella virus FR483 ORF N668R product (YP_001426300); COCCOLITHO, *E. huxleyi* virus 86 ORF EhV128 product (YP_293881); YEAST, Yeast *pichia stipitis* CBS 6054 ERV1 (XP_001382524); WORM, *C. elegan*s gene product F56C11 (AF043697); DROSOPHILA, Drosophila pseudoobscura (XP_001354903); RAT, Rattus norvegicus ALR (S72606); HUMAN, Homo sapiens ALR (AF146394). Invariant amino acids in all proteins are shown on a *solid background* and also repeated under the alignment, while conservative substitutions are *shaded*. Conserved C-X-X-C motif and GXXXW motif are shown in a *box*. Periods in the sequence denote gaps introduced by the alignment program

Western blot was performed on the protein extracts from the samples described above. Equivalent amounts of the cell extracts were electrophoresis in 15% SDS–PAGE and subsequently transferred to PVDF membrane (Millipore) according to the method of Zhao et al. [28]. The following were performed as described previously [28].

Drug inhibition assay

De novo protein synthesis inhibitor cycloheximide (CHX) and DNA replication inhibitor cytosine arabinoside (AraC) were utilized to classify the transcript of RGV 88R gene. Firstly, the suitable concentrations of CHX and AraC were estimated according to the previous report [7]. Briefly, EPC monolayer cells was pretreated by CHX or AraC for 1 h prior to and throughout the RGV infection. 50 µg/ml CHX-pretreated cells were mock-infected or infected with approximately 0.1 MOI RGV and then harvested at 6 h p.i. 100 µg/ml AraC-pretreated cells were mock-infected or infected with approximately 0.1 MOI RGV and then harvested at 48 h p.i. RNA isolation and *88R* transcription RT-PCR analysis were performed as described above. As

control, two known temporal kinetic genes including immediate-early (IE) transcription gene *ICP18* [29] and late (L) transcription gene *MCP* (major capsid protein) [30] were included in the assay. Western blot was performed on the protein extracts from AraC-pretreated samples as described above.

Distribution of RGV 88R assay

Determining the distribution of RGV 88R were performed by EGFP fusion protein expression and immunofluorescence. For EGFP fusion protein expression, EPC cells were transfected with pEGFP-N3-88R or empty vector using Lipofectamine reagent (Invitrogen). After 48 h incubation, the cells were fixed and stained with DAPI as described previously [31]. For immunofluorescence localization, EPC cells were mock or infected with approximately 1 MOI RGV for 16 h and then fixed as described previously [31]. After the cells were blocked in 10% normal goat serum at room temperature for 1 h, they were incubated with anti-RGV 88R serum in 1% normal goat serum for 2 h, rinsed three times for 10 min with PBS containing 1% normal
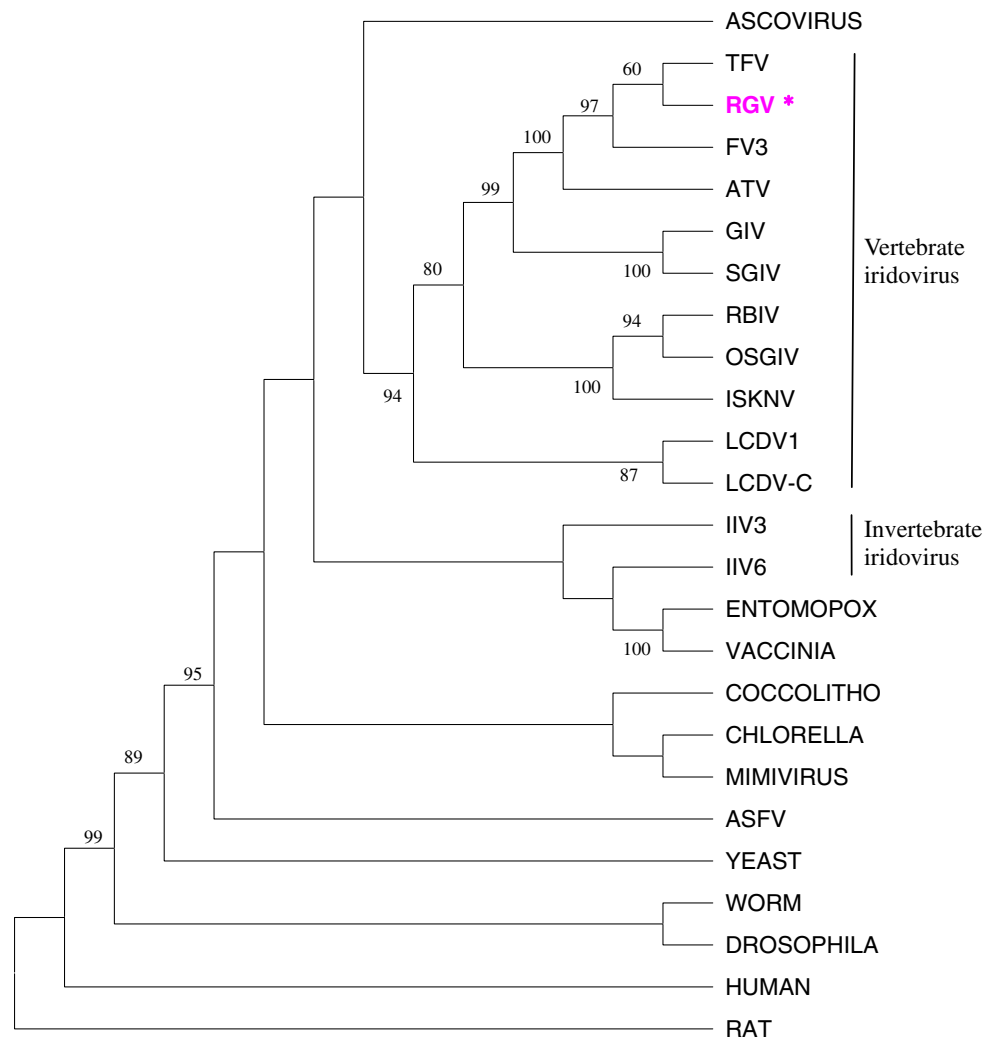
Fig. 2 Phylogenetic tree of the ERV1 family proteins based on 25 ERV1 homologues from all known iridovirus genomes and other species. The numbers given are frequencies (%) at which a given branch appeared in 500 bootstrap replications and only bootstraps greater than 50% were shown for convenience. RGV 88R is indicated by an *asterisk*. Homologues shown are as follows (with the GenBank accession number of the nucleotide sequence given in parentheses following each): TFV, Tiger frog virus thiol oxidoreductase (ABB92342.1); FV3, Frog virus 3 ERV1 homologue (AAT09748.1); ATV, Ambystoma tigrinum virus thiol oxidoreductase (YP_003787.1); SGIV, Singapore grouper iridovirus thiol oxidoreductase (YP_164165.1); GIV, Grouper iridovirus thiol oxidoreductase (AAV91064.1); ISKNV, Infectious spleen and kidney necrosis virus ORF043L (NP_612265.1); OSGIV, Orange-spotted grouper iridovirus thiol oxidoreductase (AAX82354.1); RBIV, Rock bream iridovirus 043.5L (AY532606); LCDV-C, Lymphocystis disease virus-isolate China ERV1 family protein (AAU10986.1); LCDV1, Lymphocystis disease virus 1 thiol oxidoreductase (NP_078699.1); ASCOVIRUS, Spodoptera frugiperda ascovirus 1a ORF 061 product (YP_762416); MIMIVIRUS, *A. polyphaga* mimivirus ORF R368 product (YP_142722); CHLORELLA, Chlorella virus FR483 ORF N668R product (YP_001426300); ASFV, African fever virus ORF 9GL product (AAF27970); COCCOLITHO, *E. huxleyi* virus 86 ORF EhV128 product (YP_293881); YEAST, Yeast *pichia stipitis* CBS 6054 ERV1 (XP_001382524); DROSOPHILA, Drosophila pseudoobscura (XP_001354903); WORM, *C. elegans* gene product F56C11 (AF043697); RAT, Rattus norvegicus ALR (S72606); HUMAN, Homo sapiens ALR (AF146394); IIV6, Invertebrate iridescent virus 6 347L (NP_149810.1); IIV3, Invertebrate iridescent virus 3 96R (YP_654668.1); ENTOMOPOX, *M. sanguinipes* entomopoxvirus ORF MSV093 product (AF063866); VACCINIA, Vaccinia virus ORF E10R product (NC_006998). The vertebrate iridovirus and invertebrate iridovirus were marked on the *right*

goat serum, and then incubated with FITC-conjugated goat anti-mouse antibodies (pierce), Nuclei were visualized by staining with 1 µg/ml DAPI. Anti-88R serum used in this assay was adsorbed with normal cell lysates before use. All samples were examined under a Leica DM IRB fluorescence microscope. EGFP-transfected and FITC-stained cells were visualized with a blue filter block (excitation range 450–480 nm), while DAPI-stained cells were visualized with a UV filter block (excitation range 340–380 nm).
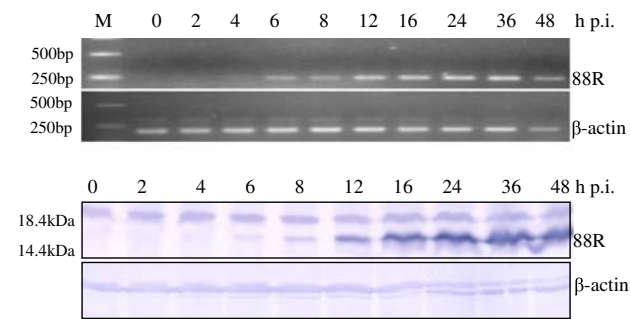
Fig. 3 RT-PCR and western blot detections of RGV 88R gene expression in the infected EPC cells during RGV infection course. β-actin was amplified under the same conditions as a positive control. Hours postinfection (h p.i.) are indicated *above* the lanes. DNA markers is in lane M in the *left*. The top of the figure is an RT-PCR experiment, and the *bottom* is a western blot
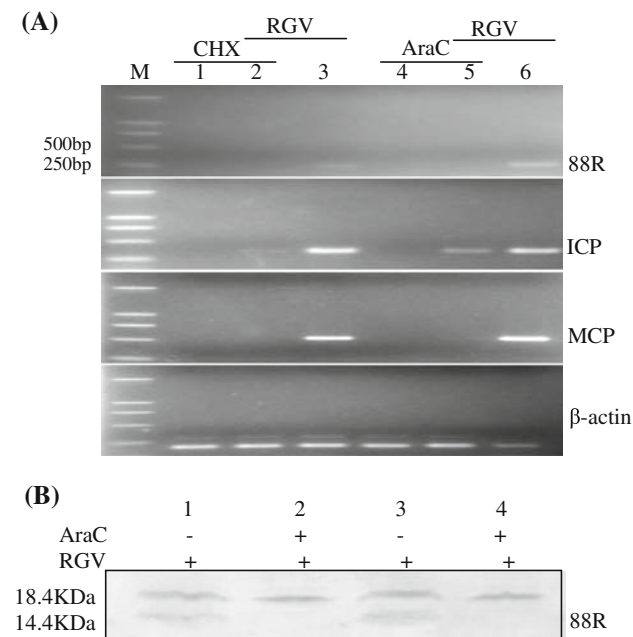

Fig. 4 RGV *88R* gene expression under drug treatments. **a** RT-PCR detection of RGV *88R* transcripts under drug treatments: lane M, DL2000 ladder; lane 1, CHX (Cycloheximide)-treated uninfected at 6 h p.i.; lane 2, CHX-treated RGV-infected at 6 h p.i.; lane 3, RGV-infected at 6 h p.i.; lane 4, AraC (cytosine arabinoside)-treated uninfected at 48 h p.i.; lane 5, AraC-treated RGV-infected at 48 h p.i.; and lane 6, RGV-infected at 48 h p.i. **b** Western blot analysis of RGV *88R* gene expression under AraC treatment: lane 1, RGV-infected at 24 h p.i.; lane 2, AraC-treated RGV-infected at 24 h p.i.; lane 3, RGV-infected at 48 h p.i.; lane 4, AraC-treated RGV-infected at 48 h p.i

Knockdown of *88R* gene expression by RNAi

Four duplex siRNAs (contain three duplex siRNAs targeted to *88R* and a negative control) were chemically synthesized for using in this study (GenePharma, Shanghai, China).

Considering the transfection efficiency, Grass carp ovaries (GCO) cell lines was used in this RNAi assay. GCO cells were transfected with four siRNAs using Lipofectamine reagent (Invitrogen) at a final concentration of 120 nM, respectively. Five hours after transfection, cells were infected with approximately 5 MOI RGV and then harvested 24 h p.i. The silence effect of different siRNAs was detected by western blot analysis. Compared the silence effect of different siRNAs, siRNA-6 (targeted sequence: 5′ CGGUUGCAAUUGUAACAGATT 3′, position in *88R* gene sequence 6–24) was chosen for use in next study to suppress RGV 88R expression. And siRNA-NC (targeted sequence: 5′ UUCUCCGAACGUGUCACGUTT 3′) served as a negative control.

GCO cells were transfected with siRNA-6 or NC using Lipofectamine reagent (Invitrogen) at a final concentration 120 nM. 5 h later, transfected or untransfected cells were infected with approximately 5 MOI RGV and then harvested at 24 h, 30 h, 48 h p.i. Samples at 30 h and 48 h p.i. were used to detect the silencing effect of *88R* expression by western blot analysis. Samples at 24 h p.i. was diluted from 10^{-1} to 10^{-10} and used to infect GCO cells with four repetitions per dilution to perform the $TCID_{50}$ assay.

Results

Identification and sequence analysis of RGV *88R* gene

To obtain complete *88R* gene, a fragment with a length of 861 bp was amplified from RGV genomic DNA by the designed primers p1/p2. Sequence analysis showed that the fragment contained complete ORF of RGV *88R* gene (GenBank accession no. EU239358), which is 453 bp long and encodes a peptide of 150 aa with a predicted molecular mass of 16.5 kDa. An alignment of RGV 88R amino acids sequence with other ERV1 homologues is shown in Fig. 1. The 88R protein has a similarity to the yeast ERV1 homologues (26% identity over 109 residues; *E* value, 0.091) and more similarity to the ERV1 homologues found in other viruses, such as Chlorella virus, Ascovirus and African swine fever virus (Fig. 1). They all contain two highly conserved motifs: Gly-X-X-X-Trp (GXXXW) at residue 32 and Pro-Cys-X-X-Cys (CXXC) at residue 73 of 88R. The pair of conserved cysteine residues is similar to the glutaredoxin and thioredoxin redox active center motif (pfam00085), however, the glycine-tryptophan motif is unique to 88R and ERV1 homologue.

To better understand the position of RGV 88R in evolutionary history, a phylogenetic tree was constructed with all known iridovirus genomes and other species. Because the similarity between some homologues was little, Muscle was selected to do the alignment. And phylogeny analysis

Fig. 5 Distribution of RGV 88R: **a–f** distribution of 88R protein detected by EGFP fusion protein under fluorescence microscopy. EPC cells were transfected with pEGFP-N3-88R (**a–c**) or empty vector pEGFP-N3 (**d–f**). The blue images show the nucleus stained by DAPI. **g–l**, distribution of 88R protein in RGV-infected EPC cells by immunofluorescence. EPC cells infected (**g–i**) or mock infected (**j–l**) detected by anti-88R serum. The *arrows* indicate viromatrix. The *blue* images show the nucleus and viromatrix stained by DAPI
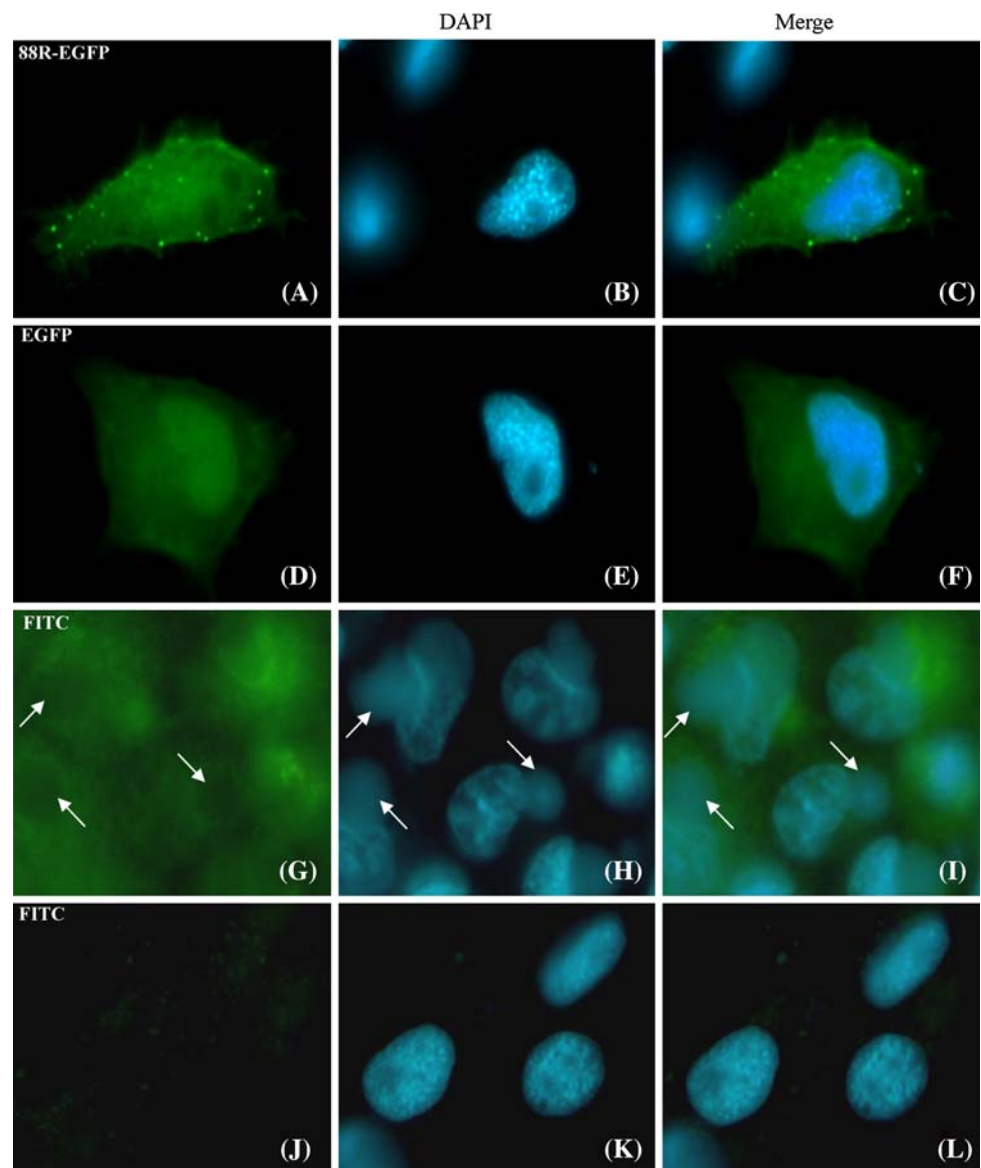
was developed with maximum likelihood program. As shown in Fig. 2, RGV 88R is more closed to homologue in TFV than to homologue in FV3, although they all isolated from frog. And all ERV1 homologues in iridovirus that isolated from vertebrate animals were clustered within a clade with more than 50% bootstrap and appears more divergent to the other two ERV1 homologues from invertebrate animals.

Expression pattern of RGV 88R in the infection course

The temporal expression pattern of RGV *88R* was characterized during RGV infection by RT-PCR and western blot analysis. As shown in Fig. 3, the 252 bp RGV *88R* gene specific-fragment was detected at 6 h postinfection by the specific primers p8/p9, and its content increased to high

level at 36 h p.i. For internal control, the transcripts of cellular β-actin were consistent with each other. At the protein level, a specific protein band was also observed from 6 h p.i. and increased to high level at 36 h p.i.

Identification of RGV 88R gene as a late viral gene

To further classify the transcripts of RGV *88R* gene, the de novo protein synthesis inhibitor CHX and the viral DNA replication inhibitor AraC inhibition assay were performed. Two known temporal kinetic genes including immediate-early (IE) transcription gene *ICP18* [29] and late (L) transcription gene *MCP* (major capsid protein) [30] were chosen for control. As shown in Fig. 4a, *ICP18* gene was expressed in all RGV-infected cell samples regardless of absence or presence of CHX and AraC, which is consistent

with the known fact that IE gene was not dependent on de novo protein synthesis or viral DNA replication. MCP was inhibited in the presence of CHX or AraC, because L gene transcription requires de novo protein synthesis and viral DNA replication. Expression of RGV *88R* gene is similar to the *MCP* gene, which is inhibited in the presence of CHX and AraC and only presented in the sample infected with RGV at 48 h p.i.

Because the signal from RT-PCR is weak even at 48 h p.i., we also examined the AraC treated samples by western blot analysis. The result is identical to RT-PCR. 88R protein band was not detected in samples pretreated by AraC but can be detected in samples untreated by AraC (Fig. 4b). Therefore, the data confirmed that RGV *88R* gene is L gene during the in vitro infection.

Distribution of RGV 88R

Distribution of RGV 88R was determined by 88R-EGFP fusion protein expression and anti-88R antibody immunofluorescence. Firstly, a recombinant plasmid pEGFP-N3-88R, which could express RGV 88R with a C-terminal EGFP tag, was constructed and transfected into EPC cells. Observation under fluorescence microscope showed that the green fluorescence distributed in both the cytoplasm and the nucleus except the nucleolus, and could aggregated into spots (Fig. 5a), indicating that 88R-EGFP fusion protein was distributed in EPC cells entirely except the nucleolus. As control, the vector-expressed EGFP was distributed in both the cytoplasm and the nucleus including nucleolus in EPC cells, and mainly in nucleus (Fig. 5d). Secondly, the anti-88R antibody immunofluorescence gave the similar result. The FITC green fluorescence was scattered in the cytoplasm and nucleus after RGV-infected EPC cells were immunostained by anti-88R antibody (Fig. 5g), whereas no special fluorescence signal was detected in mock-infected cells (Fig. 5j). Moreover, some compartments were not immunostained by anti-88R antibody but stained by DAPI in RGV-infected EPC cells (Fig. 5g, h, I). The compartments were viromatrix in which a large number of RGVs were replicated and assembled [32].

Effect of RGV *88R* silence on RGV replication cannot be detected

To study the effect of RGV *88R* silencing on the replication of RGV, a specially silencing siRNA, siRNA-6 which was selected from three chemically synthesized siRNAs targeted to *88R* gene, was used to reduce *88R* gene expression. As shown in Fig. 6a, western blot revealed that siRNA-6 suppressed RGV 88R expression most effectively at 24 h p.i. for the band is the weakest among these four siRNAs. As shown in Fig. 6b, siRNA-6 can suppress the

expression of RGV 88R gene at 30 h p.i. but the effect of suppression is diminished at 48 h p.i. So, samples at 24 h p.i. were used to do $TCID_{50}$ assay. As shown in Fig. 6c, NC and un-transfected samples had similar viral titers with siRNA-6 transfected samples. Differences of cytopathic effect among these samples were difficult to distinguish.

Discussion

In this study, an *ERV1* homologue in RGV, *88R*, was cloned, sequenced, and expressed in EPC cells. Its expression pattern, transcription kinetics and distribution were determined. It was found that RGV 88R has homologues in all iridoviruses sequenced to date. And sequence analysis showed that RGV 88R also contains the conserved CXXC motif with potential redox activity which is the characteristic of ERV1 homologues. The conserved cysteine residues indicate redox-active centers found in glutaredoxin and thioredoxin [33, 34]. Thus, 88R protein could conceivably perform an important redox function
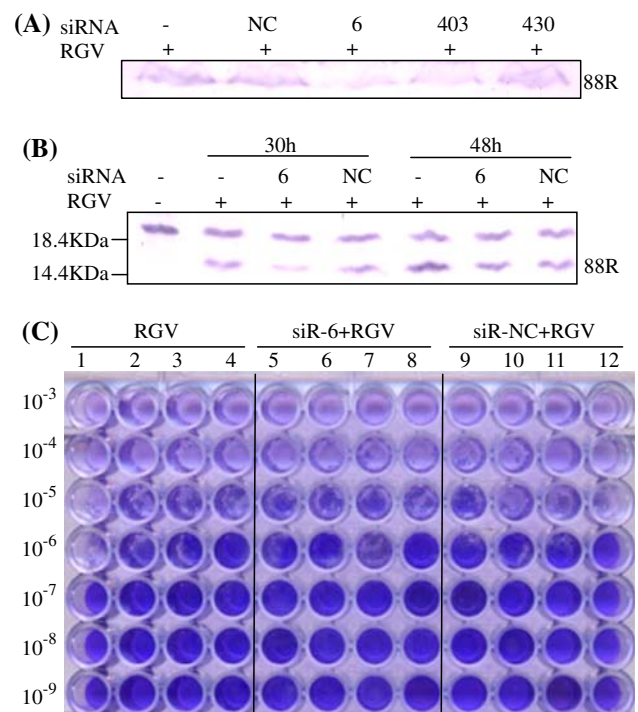

Fig. 6 Viral titers with RGV 88R suppressed by siRNA. a Western blot analysis of 88R gene expression after transfected with four chemically synthesized siRNAs at 24 h p.i. GCO cells were transfected or not transfected with 120 nM siRNAs for 5 h prior to infection with RGV (MOI of 5); b Western blot analysis of 88R gene expression after transfected with siRNA-6. GCO cells were transfected or not transfected with 120 nM siRNA-6 or siRNA-NC for 5 h prior to infection with RGV (MOI of 5) and then harvested at 30 h p.i. or 48 h p.i. c cytopathic effects of RGV on GCO cells when RGV 88R was suppressed by siRNA-6 at 24 h p.i. by $TCID_{50}$ assay

that is critical to efficient virion morphogenesis, like the redox function of ERV1 homologue in vaccinia virus [35, 36]. Interestingly, a GXXXW motif was found existed in all ERV1 homologues in our alignment. This motif also existed in ERV1 homologue from ASFV [16], but function of this motif remains unknown.

ERV1 homologues are widespread in nature, but the evolutionary position of these homologues remains unknown. Here, phylogenetic analysis was done with maximum likelihood program. It revealed that ERV1 homologues in iridovirus from vertebrates clustered appears more divergent to the other two ERV1 homologues from invertebrate animals (Fig. 2). And it implicated that the evolutionary position of these homologues is consistent with its resources or taxonomic status.

As described above, iridoviruses displays a complex gene regulation strategy in which genes are expressed in three main temporal kinetics stages: IE genes, E genes and L genes [5–8]. L genes are expressed after the onset of viral DNA replication, and can be blocked by DNA replication and protein synthesis inhibitor [7, 8]. In this study, RGV *88R* transcripts and expression were blocked by both DNA replication inhibitor and protein synthesis inhibitor (Fig. 4a, b). It confirmed that RGV *88R* was a late gene during in vitro infection. However, RGV 88R was not distributed in viromatrix which is a factory for viral genome replication and particle assembly [32]. It revealed that 88R protein might not be a structural protein in RGV, and Lewis et al. has confirmed that a ERV1 homologue, 9GL, existed in African swine fever virus was also a late protein but not a structural protein [16]. In general, late genes of large DNA virus encode mainly structural proteins. In addition to the structural proteins, there are also some nonstructural proteins encoded by late gene in large DNA virus. These proteins play an important role in virus assembly, transporting process, virus–host interactions [37, 38]. Giving that RGV 88R is not distributed in viromatrix, it seems that this protein was not participated in virus assembly and transporting process directly and suggested that RGV 88R may have a function in virus–host interactions.

We also examined the distribution of RGV 88R in another fish cell line, fathead minnow (FHM) cell line. The result was similar (data not shown). This is thought to be the first report about subcellular distribution of ERV1 family proteins in virus-infected fish cells. Furthermore, we observed the effect of silencing of 88R on viral replication. Out of anticipation, the effect of reducing of RGV *88R* expression on virus replication cannot be detected in cultured GCO cells (Fig 6c). Three possible reasons could lead to this result: (i) RGV *88R* may be a gene that functioned in vivo. (ii) Expression of RGV *88R* was not eliminated completely by siRNA. A small quantity of 88R protein may be enough to RGV replication. (iii) Other

homologue of 88R may exist in the RGV genome that able to circumvent its function. And knockout ERV1 homologue in ASFV affects viral growth in vitro and viral virulence in vivo [16]. Consequently, gene knockout techniques may be needed to understand RGV 88R function in further studies.

References

1. Zhang QY, Li ZQ, Jiang YL, Liang SC, Gui JF (1996) Preliminary studies on virus isolation and cell infection from disease frogs *Rana grylio*. Acta Hydrobiol Sin 20:390–392
2. Zhang QY, Li ZQ, Gui JF (1999) Studies on morphogenesis and cellular interactions of *Rana grylio* virus in an infected fish cell line. Aquaculture 175:185–197. doi:10.1016/S0044-8486(99)00041-1
3. Zhang QY, Xiao F, Li ZQ, Gui JF, Mao JH, Chinchar VG (2001) Characterization of an iridovirus from the cultured pig frog (*Rana grylio*) with lethal syndrome. Dis Aquat Organ 48:27–36. doi:10.3354/dao048027
4. Zhang QY, Zhao Z, Xiao F, Li ZQ, Gui JF (2006) Molecular characterization of three *Rana grylio* virus (RGV) isolates and *Paralichthys olivaceus* lymphocystis disease virus (LCDV-C) in iridoviruses. Aquaculture 251:1–10. doi:10.1016/j.aquaculture.2005.05.012
5. Chinchar VG (2002) Ranaviruses (family *Iridoviridae*): emerging cold-blooded killers. Arch Virol 147:447–470. doi:10.1007/s007050200000
6. D'Costa SM, Yao H, Bilimoria SL (2001) Transcription and temporal cascade in *Chilo* iridescent virus infected cells. Arch Virol 146:2165–2178. doi:10.1007/s007050170027
7. Lua DT, Yasuike M, Hirono I, Aoki T (2005) Transcription program of red sea bream iridovirus as revealed by DNA microarrays. J Virol 79:15151–15164. doi:10.1128/JVI.79.24.15151-15164.2005
8. Williams T, Barbosa-Solomieu V, Chinchar VG (2005) A decade of advances in iridovirus research. Adv Virus Res 65:173–248. doi:10.1016/S0065-3527(05)65006-3
9. Delhon G, Tulman ER, Afonso CL, Lu Z, Becnel JJ, Moser BA et al (2006) Genome of invertebrate iridescent virus type 3 (Mosquito iridescent virus). J Virol 80:8439–8449. doi:10.1128/JVI.00464-06
10. Eaton HE, Metcalf J, Penny E, Tcherepanov V, Upton C, Brunetti CR (2007) Comparative genomic analysis of the family *Iridoviridae*: re-annotating and defining the core set of iridovirus genes. Virol J 4:11. doi:10.1186/1743-422X-4-11
11. Hagiya M, Francavilla A, Polimeno L, Ihara I, Sakai H, Seki T et al (1994) Cloning and sequence analysis of the rat augmenter of liver regeneration (ALR) gene: expression of biologically active recombinant ALR and demonstration of tissue distribution. Proc Natl Acad Sci USA 91:8142–8146. doi:10.1073/pnas.91.17.8142
12. Lisowsky T (1992) Dual function of a new nuclear gene for oxidative phosphorylation and vegetative growth in yeast. Mol Gen Genet 232:58–64. doi:10.1007/BF00299137

13. Lisowsky T, Weinstatsaslow DL, Barton N, Reeders ST, Schneider MC (1995) A new human gene located in the PKD1 region of chromosome 16 is a functional homologue to ERV1 of yeast. Genomics 29:690–697. doi:10.1006/geno.1995.9950

14. Holmgren A (1985) Thioredoxin. Annu Rev Biochem 54:237–271. doi:10.1146/annurev.bi.54.070185.001321

15. Afonso CL, Tulman ER, Lu Z, Oma E, Kutish GF, Rock DL (1999) The genome of *Melanoplus sanguinipes* entomopoxvirus. J Virol 73:533–552

16. Lewis T, Zsak L, Burrage TG, Lu Z, Kutish GF, Neilan JG et al (2000) An African swine fever virus ERV1-ALR homologue, 9GL, affects virion maturation and viral growth in macrophages and viral virulence in swine. J Virol 74:1275–1285. doi:10.1128/JVI.74.3.1275-1285.2000

17. Fjose A, Ellingsen S, Wargelius A, Seo HC (2001) RNA interference: mechanisms and applications. Biotechnol Annu Rev 7:31–57. doi:10.1016/S1387-2656(01)07032-6

18. Agami R (2002) RNAi and related mechanisms and their potential use for therapy. Curr Opin Chem Biol 6:829–834. doi:10.1016/S1367-5931(02)00378-2

19. Carmichael GG (2002) Silencing viruses with RNA. Nature 418:379–380. doi:10.1038/418379a

20. Huelsmann PM, Rauch P, Allers K, John MJ, Metzner KJ (2006) Inhibition of drug-resistant HIV-1 by RNA interference. Antivir Res 69:1–8. doi:10.1016/j.antiviral.2005.10.001

21. Dang LT, Kondo H, Hirono I, Aoki T (2008) Inhibition of red seabream iridovirus (RSIV) replication by small interfering RNA (siRNA) in a cell culture system. Antivir Res 77:142–149. doi:10.1016/j.antiviral.2007.10.007

22. Xie J, Lü L, Deng M, Weng S, Zhu J, Wu Y et al (2005) Inhibition of reporter gene and Iridovirus-tiger frog virus in fish cell by RNA interference. Virology 338:43–52. doi:10.1016/j.virol.2005.04.040

23. Tan WG, Barkman TJ, Gregory Chinchar V, Essani K (2004) Comparative genomic analyses of frog virus 3, type species of the genus *Ranavirus* (family *Iridoviridae*). Virology 323:70–84. doi:10.1016/j.virol.2004.02.019

24. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W et al (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. doi:10.1093/nar/25.17.3389

25. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882. doi:10.1093/nar/25.24.4876

26. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797. doi:10.1093/nar/gkh340

27. Guindon S, Lethiec F, Duroux P, Gascuel O (2005) PHYML Online – a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res. 33(Web Server issue):W557–W559

28. Zhao Z, Ke F, Gui JF, Zhang QY (2007) Characterization of an early gene encoding for dUTPase in *Rana grylio* virus. Virus Res 123:128–137. doi:10.1016/j.virusres.2006.08.007

29. Willis DB, Foglesong D, Granoff A (1984) Nucleotide sequence of an immediate-early FV3 gene. J Virol 53:905–912

30. Mao J, Tham TN, Gentry GA, Aubertin AM, Chinchar VG (1996) Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. Virology 216:431–436. doi:10.1006/viro.1996.0080

31. Du CS, Zhang QY, Li CL, Miao DL, Gui JF (2004) Induction of apoptosis in a carp leucocyte cell line infected with turbot (*Scophthalmus maximus* L.) rhabdovirus. Virus Res 101:119–126. doi:10.1016/j.virusres.2003.12.034

32. Huang XH, Huang YH, Yuan XP, Zhang QY (2006) Electron microscopic examination of the viromatrix of *Rana grylio* virus in a fish cell line. J Virol Methods 133:117–123. doi:10.1016/j.jviromet.2005.10.029

33. Coppock DL, Cina-Poppe D, Gilleran S (1998) The quiescin Q6 gene families: thioredoxin and ERV1. Genomics 54:460–468. doi:10.1006/geno.1998.5605

34. Ellis LBM, Saurugger P, Woodward C (1992) Identification of the three-dimensional thioredoxin motif: related structure in the ORF3 protein of the Staphylococcus aureus mer operon. Biochemistry 31:4882–4891. doi:10.1021/bi00135a020

35. Senkevich TG, Weisberg AS, Moss B (2000) Vaccinia virus E10R protein is associated with the membranes of intracellular mature virions and has a role in morphogenesis. Virology 278:244–252. doi:10.1006/viro.2000.0656

36. Senkevich TG, White CL, Koonin EV, Moss B (2002) Complete pathway for protein disulfide bond formation encoded by poxviruses. Proc Natl Acad Sci USA 99:6667–6672. doi:10.1073/pnas.062163799

37. Epifano C, Krijnse-Locker J, Salas ML, Rodríguez JM, Salas J (2006) The African swine fever virus nonstructural protein pB602L is required for formation of the icosahedral capsid of the virus particle. J Virol 80:12260–12270. doi:10.1128/JVI.01323-06

38. Wang D, Zhang CX (2006) HearSNPV orf83 encodes a late, nonstructural protein with an active chitin-binding domain. Virus Res 117:237–243. doi:10.1016/j.virusres.2005.10.019