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Rana grylio virus *43R* encodes an envelope protein involved in virus entry

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Abstract
Rana grylio virus (RGV), a member of genus *Ranavirus* in the family *Iridoviridae*, is a viral pathogen infecting aquatic animal. RGV 43R has homologues only in *Ranavirus* and contains a transmembrane (TM) domain, but its role in RGV infection is unknown. In this study, 43R was determined to be associated with virion membrane. The transcripts encoding 43R and the protein itself appeared late in RGV-infected EPC cells and its expression was blocked by viral DNA replication inhibitor, indicating that 43R is a late expressed protein. Subcellular localization showed that 43R-EGFP fusion protein distributed in cytoplasm of EPC cells and that TM domain is essential for its distribution in cytoplasm. 43R-EGFP fusion protein colocalized with viral factories in RGV-infected cells. A recombinant RGV deleting *43R* (Δ43R-RGV) was constructed by homologous recombination to investigate its role in virus infection. Compared with wild type RGV, the ability of Δ43R-RGV to induce the cytopathic effect and its virus titers were significantly reduced. Furthermore, it is revealed that *43R* deletion significantly inhibited viral entry but did not influence viral DNA replication by measuring and comparing the DNA levels of RGV and Δ43R-RGV in the infected cells at the early stage of infection. RGV neutralization with anti-43R serum reduced the virus titer. Therefore, these data showed that RGV *43R* is a late gene that encodes an envelope protein involved in RGV entry.

Keywords Iridovirid · Ranavirus · *Rana grylio* virus envelope protein 43R · Recombinant virus · Virus entry

Introduction

Ranaviruses, the members in the family *Iridoviridae*, are enveloped DNA viruses containing double-stranded, nucleocytoplasmic large DNA genomes. Ranaviruses are promiscuous pathogens infecting a wide variety of cold-blooded vertebrate hosts including fish, amphibians, and reptiles, leading to great economic losses in the aquaculture industry worldwide [1, 2]. Sequence determination and bioinformatic analysis reveal several recent host shifts among ranaviruses [3]. To date, 22 ranavirus genomes have been sequenced completely [4], but many viral genes remain to be explored, including viral envelope protein genes.

Viral envelope proteins have been reported to play important roles in virus cycle, especially virus entry [5]. Some envelope proteins of ranaviruses have been identified and functionally analyzed by recombinant technology [6, 7]. For example, the first identified envelope protein 53R of *Rana grylio* virus (RGV) is associated with virus assembly [8, 9]. Another envelope protein RGV 2L is involved in virus infectivity and colocalizes with RGV 53R [10]. These two envelope proteins are both core proteins that are conserved in family *Iridoviridae*, and contain one or two transmembrane (TM) domains [11, 12]. However, there are still some envelope proteins that have not been identified.

Rana grylio virus (RGV), a member of genus *Ranavirus*, is a viral pathogen infecting pig frogs (*Rana grylio*) [2]. The complete genome of RGV was sequenced, showing that RGV contained 106 open reading frames (ORFs) [11]. Some

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RGV-encoded proteins involved in gene transcription, viral infection, and assembly have been characterized [13–15]. Recently, the transcriptome response on RGV interspecies infection was also analyzed [16]. Bioinformatic analysis of RGV genome showed that RGV ORF 43R has homologues only in *Ranavirus*. Interestingly, 43R was predicted to possess a TM domain and an arginyl-glycyl-aspartic (RGD) motif. However, whether 43R was an envelope protein and its possible role in RGV infection remain unclear. In this study, we cloned and characterized RGV *43R* and investigated its possible role in RGV infection.

Materials and methods

Virus and cell line

Rana grylio virus (RGV) was used in the study. RGV propagation and purification were performed as described previously [11]. *Epithelioma papulosum cyprinid* (EPC) cells were maintained in Medium 199 supplemented with 10% fetal bovine serum (FBS) at 25 °C.

Sequence analysis

43R was obtained from the RGV genome (Genbank accession number JQ654586). So far, there are 22 known ranaviruses whose complete genomes were available [4], 17 of which have the homologues of 43R. Ten ranaviruses were randomly selected from the 17 ranaviruses including soft-shelled turtle iridovirus (STIV), frog virus 3 (FV3), a FV3 isolate (SSME), Bohle iridovirus (BIV), German gecko ranavirus (GGRV), tortoise ranavirus (ToRV1), tiger frog virus (TFV), pike perch iridovirus (PPIV), common midwife toad ranavirus (CMTV/E), and *Ambystoma tigrinum* virus (ATV). Multiple sequence alignment of RGV 43R with its homologues from the selected ranaviruses was made using ClustalX 1.83, following edited by GENEDOC [17]. Sequence identities were calculated using the MegAlign program. The TM domain was predicted by SMART (http://smart.embl-heidelberg.de/) and functional sites were predicted by SOSUI server (http://www.expasy.org/tools/).

Plasmid construction

RGV genomic DNA were extracted from the RGV-infected EPC cells according to the instructions of TaKaRa MiniBEST Universal Genomic DNA Extraction Kit. Since the 30 amino acids (aa) in the N-terminal of 43R were hydrophobic, they might impede the induced expression of fusion protein. To construct the plasmid for prokaryotic expression of 43R 31 ~ 167 aa, a 411 bp fragment (+91 to +501 relative to translation initiation codon of *43R*) was amplified from RGV genomic DNA with the primers $43R_{\Delta 30aa}$-F/R, and cloned into pET32a to construct pET32a-$43R_{\Delta 30aa}$.

To analyze the subcellular localization of 43R, the ORF 43R was cloned into pEGFP-N3 to produce pEGFP-*43R*. To analyze whether TM domain has any effect on the subcellular localization of 43R, the ORF 43R lacking the TM domain ($43R_{\Delta}$TM) was cloned into pEGFP-N3 to produce pEGFP-$43R_{\Delta}$TM.

To further analyze subcellular localization of 43R during RGV infection, two plasmids expressing 43R-EGFP fusion protein or enhanced green fluorescent protein (EGFP) under the control of the native promoter of *43R* were constructed. The *43R* gene fragment, including its native promoter region but lacking a termination codon (−327 to +501 relative to translation initiation codon of *43R*), was amplified with primers p43R-F/*43R*-R2 from RGV genomic DNA. The fragment EGFP containing the EGFP ORF with SV40 polyadenylation signals was amplified with primers EGFP-F/R from plasmid pEGFP-N3. The fragment 43R-EGFP was amplified by overlap PCR using above amplified two fragments as templates, and cloned into plasmid pMD18-T to produce pMD-*43R-EGFP*. As described above, primers p43R-F/R and EGFP-F2/R were used to construct the plasmid pMD-p43R-*EGFP*.

To generate the plasmid for construction of a recombinant RGV deleting *43R* (Δ43R-RGV), two fragments 43RL (−456 to +244 relative to translation initiation codon of *43R*) and 43RR (+505 to +1,204 relative to translation initiation codon of *43R*) were amplified with primers 43RL-F/R and 43RR-F/R from RGV genomic DNA, respectively. The fragment p50-EGFP which contains the ORF EGFP with SV40 polyadenylation signals under the control of promoter p50 was amplified with primers p50-EGFP-F/R from previously constructed plasmid pcDNA3.1-p50-EGFP [18]. The fragment 43RL-p50-EGFP-43RR was amplified via overlap PCR using 43RL, p50-EGFP and 43RR as templates, and cloned into plasmid pMD18-T to produce pMD-43RL-p50-EGFP-43RR.

To generate the plasmid for standard curve of quantitative real-time PCR (qPCR), a 163 bp fragment of RGV ORF 97R (encoding major capsid protein, MCP) was amplified with primers MCP-F/R via PCR from RGV genomic DNA and cloned into pMD18-T to produce pMD-*MCP*. All primers used in this study are listed in Table 1. All constructs used in this study were confirmed by DNA sequencing.

Prokaryotic expression, protein purification, and antibody preparation

The *Escherichia coli* BL21(DE3) transformed with plasmid pET32a-$43R_{\Delta 30aa}$ was induced for 4 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C to express fusion protein. The fusion protein was purified using the HisBind

Table 1 Primers used in this study

Name	Sequence(5′to 3)[a]	Usage
43R$_{\Delta 30aa}$-F	TACGAATTCGTTGGTGTCCCAG (*EcoRI*)	pET32a-*43R*$_{\Delta 30aa}$/ antibody
43R$_{\Delta 30aa}$-R	CATGGATCCTACGTCTGGAGCC (*BamHI*)	
43R-RT-F	CTGGCTTACTGCTCTCACCGG	RT-PCR
43R-RT-R	CACGATCCCTTGCATGTCC	
β-actin-F	CACTGTGCCCATCTACGAG	Internal control
β-actin-R	CCATCTCCTGCTCGAAGTC	
43R-F	CCGCTCGAGGGTATTGCAATGATACGAGC (*Xho I*)	pEGFP-*43R*
43R-R	CGCGGATCCTACGTCTGGAGCCGCTATGG (*BamHI*)	
*43R*ΔTM-F	CCGCTCGAGATGATACGAGCATACAAGAGCGTTGGTGTCCCAGACG (*Xho I*)	pEGFP-*43R*ΔTM
p43R-F	GTTTTCAGACGCCTCCCTGAG	pMD-*43R-EGFP*
43R-R2	TCCTCGCCCTTGCTCACCAT	
EGFP-F	CCATAGCGGCTCCAGACGTAATGGTGAGCAAGGGCGAGGA	
EGFP-R	CCACAACTAGAATGCAGTGA	
p43R-R	TCCTCGCCCTTGCTCACCATTGCAATACCTTTTTTGTACG	pMD-p43R-*EGFP*
EGFP-F2	CGTACAAAAAAGGTATTGCAATGGTGAGCAAGGGCGAGGA	
43RL-F	ACATTTTATCTGCATATGGT	pMD-43RL-p50-EGFP-43RR/ recombinant virus
43RL-R	CTTGCGCTTTCTCAGAGGTTTTGTAGTCGGTTTCAACGTT	
p50-EGFP-F	AACGTTGAAACCGACTACAAAACCTCTGAGAAAGCGCAAG	
P50-EGFP-R	TAGTCTTACACGTCCATCTTCCACAACTAGAATGCAGTGA	
43RR-F	TCACTGCATTCTAGTTGTGGAAGATGGACGTGTAAGACTA	
43RR-R	ACCCCATCATGCCCCTGTGA	
MCP-F	ATGGTTGTGGAGCAGGTG	pMD-*MCP*/qPCR
MCP-R	TGACGCAGGTGTAATTGGAG	

[a]Sequences of restriction sites are underlined

purification kit (Novagen) and then used to immunize mice by hypodermal injection once every 7 days. The mouse antibody against 43R (anti-43R) serum was collected after fifth immunization. Mouse antibody against RGV envelope protein 2L (anti-2L) and mouse antibody against RGV MCP (anti-MCP) have been prepared previously [10].

This experiment was carried out in strict accordance with the recommendations in the Regulations for the Administration of Affairs Concerning Experimental Animals of China. The protocol was approved by Animal Center of Disease Control and Prevention of Hubei Province (Approval ID: SCXK 2015-0018). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Detergent extraction and phase separation of purified virions

RGV 43R was predicted to contain a TM domain, implying that it might be an envelope protein. To confirm whether 43R is an envelope protein, the envelope proteins were extracted from purified RGV particles as described previously [10]. In brief, purified RGV particles were treated with a solution containing 1% Triton X-100, 150 mM NaCl, and 50 mM Tris/HCl (pH 7.5) for 1 h at room temperature. The insoluble and soluble materials were separated by centrifugation. The envelope fraction and nucleocapsid fraction from purified RGV were in the supernatant and pellet, respectively, and analyzed by 12% SDS–PAGE followed by western blot analysis. The anti-43R (1:1000) was prepared as described in antibody preparation. As control, the known envelope protein RGV 2L and the capsid protein MCP were analyzed in the same ways using anti-2L (1:1000) and anti-MCP (1:1000), respectively.

Detection of temporal expression profile

To analyze the transcriptional and expressional profile of *43R*, total RNAs isolated from EPC cells infected with RGV at multiplicity of infection (MOI) of 0.1 at 0-, 4-, 8-, 12-, 16-, 24-, 36-, and 48-h postinfection (hpi) or mock infected, were analyzed by RT-PCR, as described previously [13]. Proteins from EPC cells infected with RGV at MOI of 0.5 at the same times were subjected to western blot analysis. Internal control was performed simultaneously by detecting β-actin protein. Cytosine β-D-arabinofuranoside (AraC),

a viral DNA replication inhibitor, was used to classify the transcription class of *43R*. Briefly, 100 μg AraC ml^{-1} was added to EPC cells for 1 h prior to virus infection and the pre-treated cells were then mock infected or infected with RGV at MOI of 0.1. Total protein was extracted at 24 and 48 hpi for western blot analysis.

Western blot analysis

Western blot analysis was performed as described previously [10]. Protein samples were resolved by SDS–PAGE, followed by electroblotting to polyvinylidene difluoride (PVDF) membrane. The blots were probed with the anti-43R (1:1000) or other designated antibody. Peroxidase-conjugated goat anti-mouse IgG (H + L) antibody was used as the secondary antibody. The signals were detected with chemiluminescent horseradish peroxidase (HRP) substrate (Millopore).

Fluorescence observation of 43R

EPC cells separately transfected with pEGFP-N3, pEGFP-*43R*, or pEGFP-*43R*ΔTM were fixed, permeabilized with 0.2% Triton X-100, stained by Hoechst 33342 and observed at 24-h post-transfection (hpt) under a Leica DM IRB fluorescence microscope (objective 100×), as described previously [19]. To further analyze subcellular localization of 43R during RGV infection, EPC cells separately transfected with pMD-p43R-*EGFP* or pMD-*43R-EGFP* for 24 h were mock infected or infected with RGV at a MOI of 0.5, then the cells were fixed and observed at 16, 24, 36, and 48 hpi.

Generation of recombinant virus

To investigate the role of *43R* in virus infection, the recombinant virus Δ43R-RGV was constructed by homologous recombination. EPC cells were transfected with pMD-43RL-p50-EGFP-43RR and then infected with RGV. The virus suspension was harvested at 48 hpi and diluted to infect fresh EPC cells. The infected cells were covered with 0.75% melted soft agar and observed under a fluorescence microscope. The plaques emitting green fluorescence were marked and selected to infect fresh EPC cells. In this way, the recombinant virus Δ43R-RGV was purified by ten successive rounds of plaque isolation. *43R* deletion was further verified by PCR and western blot analysis. EPC cells infected with RGV or Δ43R-RGV at a MOI of 1 were harvested at 36 hpi, and subjected to western blot analysis using anti-43R.

Titration of virus

EPC cells were infected with RGV or Δ43R-RGV at a MOI of 0.1 and harvested at 0, 6, 12, 24, 36, 48, and 72 hpi. The harvested cells were subjected to three freeze–thaw cycles. The virus titers were determined using a 50% tissue culture infectious dose ($TCID_{50}$) assay. Virus infection was determined by cytopathology. Data for virus growth curve were presented as means ± SD. *P* values were calculated by using Student's *t* test and $P < 0.05$ was considered as significantly different.

Quantitative analysis of viral entry and replication

To evaluate the effect of *43R* deletion on viral entry and replication, the DNA levels of RGV and Δ43R-RGV in the infected cells at the early stage of infection were measured by qPCR. 5×10^6 EPC cells were infected with RGV or Δ43R-RGV at a MOI of 0.01, then the supernatant from the infected cell culture was collected respectively when cytopathic changes reach about 50%, and centrifuged at 5867 × g for 5 min at 4 °C to remove the cell debris. Subsequently, 180 μl supernatant was transferred to a new tube and incubated with additional DNaseI (NEB, 50 units/ml) for 20 min at 37 °C. After digestion, the DNaseI was inactivated at 75 °C for 10 min. After eliminating the DNA contamination, viral DNA was extracted as described in plasmid construction. Quantification of viral copy numbers was determined by qPCR with specific primers MCP-F/R for RGV MCP gene. qPCR was carried out using Fast SYBR Green Master Mix with the StepOneTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as described previously [20].

Since each infectious RGV virion or Δ43R-RGV virion contains a copy of genome, the genome copy number contained in RGV or Δ43R-RGV in the supernatants represents the virion number. 200 μl supernatant containing 10^7 of RGV virions or Δ43R-RGV virions was separately added to 10^6 of EPC cells grown in six-well plates, then the supernatant was removed after 1 h and the cells were gently washed with Medium 199 for one time. The infected cells at 0, 2, 4, 6, and 8 hpi were harvested and washed with phosphate-buffered saline (PBS) for three times. Then the viral genomic DNA was extracted as described above. Each DNA sample was dissolved in 100 μl of elution buffer, and 1 μl of each DNA sample was used for each qPCR reaction. qPCR was carried out as described above.

Antibody neutralization assay

To further determine if 43R encodes an envelope protein and is important in virus entry, antibody neutralization assay was performed as described previously [21]. Briefly, three groups of RGV suspensions (7.24×10^5 $TCID_{50}$/ml) were incubated with anti-43R serum, normal serum at final concentrations 9 μg/μl or without antibody for 1 h at 25 °C, respectively. After incubation, EPC cells were separately infected with the

three groups at a MOI of 0.01 and harvested at 12, 24, 36, 48, 72, and 96 hpi. The virus titers were determined using a $TCID_{50}$ assay as described above.

Results

Sequence analysis of RGV 43R

The full ORF of RGV *43R* comprises 504 bp encoding 167 amino acids (aa). 43R protein was predicted to contain a TM domain (aa 5 ~ 27) and a RGD motif (aa 101 ~ 103). BLAST searches showed that 43R has homologues only in *Ranavirus*. Multiple alignment of 43R with its homologues from ten ranaviruses showed that the TM domain and the RGD motif are highly conserved and that the highest identity reaches 100.0% (Fig. 1). 43R was predicted to contain seven putative post translational modification sites, including three N-myristoylation sites (aa 14 ~ 19, 32 ~ 37, 106 ~ 111) and four phosphorylation sites: two protein kinase C phosphorylation sites (aa 54 ~ 56, 76 ~ 78), a tyrosine kinase phosphorylation site (aa 46 ~ 53), and a cAMP- and cGMP- dependent protein kinase phosphorylation site (aa 146 ~ 149).

Envelope protein RGV 43R, and its temporal expression profile

The envelope fractions and nucleocapsid fractions of purified RGV were separated using detergent Triton X-100 treatment, and detected by western blot analysis. As a result, a clear band was detected by anti-43R in viral particles and the supernatant but not in the pellet. As control, the known envelope protein RGV 2L was mainly detected in the supernatant and little was detected in the pellet. The major capsid protein (MCP) was detected in both the pellet and the supernatant (Fig. 2a). This result suggested that RGV 43R was an envelope protein.

The temporal transcription pattern of *43R* showed a weak band was first detected until 8 hpi. Its brightness increased over time, and remained stable at 36 hpi and 48 hpi (Fig. 2b). The temporal expression pattern of 43R protein showed a specific band of about 38 kDa was first detected at 12 hpi, and that the expression level remained stable at 36 hpi and 48 hpi. No band was detected in mock-infected cells (Fig. 2c). The 38 kDa protein band was not detected when viral DNA replication was inhibited by AraC (Fig. 2d), consistent with the temporal data, indicating that *43R* belongs to the late expression class of genes.

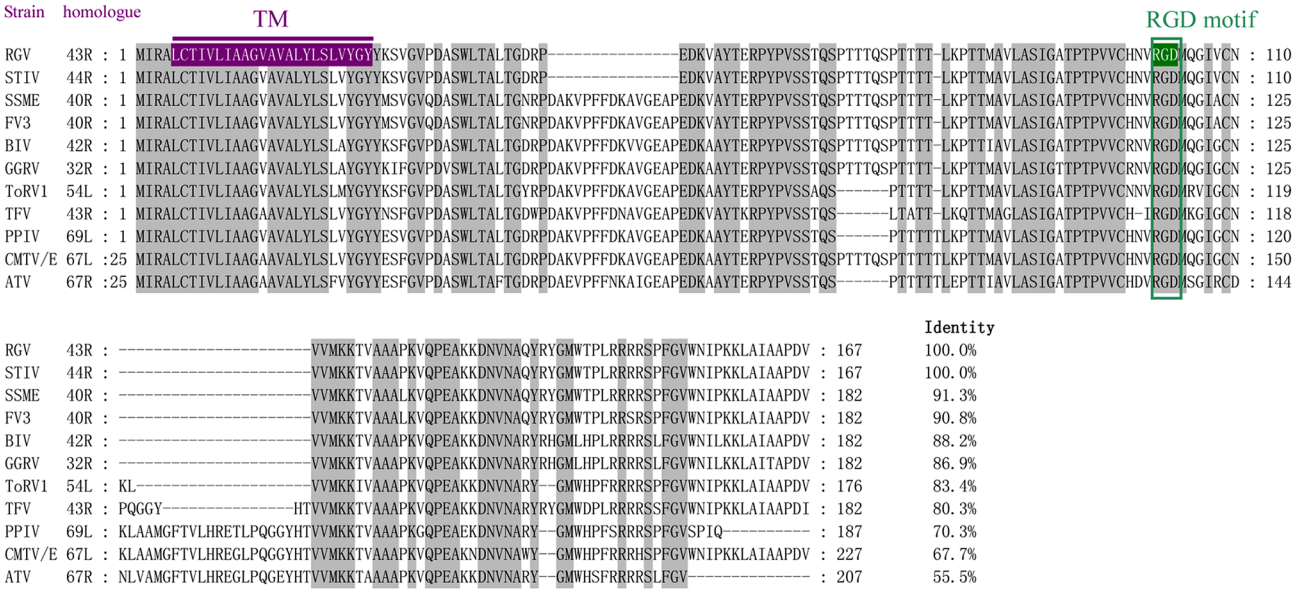

Fig. 1 Multiple amino acid sequence alignments of RGV 43R with its homologues from ten strains of ranaviruses and identity. Amino acid highlighted in light gray and white are 100% and below 100% similar, respectively. The TM domain and RGD motif were conserved in the homologues of RGV 43R. RGV 43R showed high identities with its homologues. The Genbank accession numbers of ranaviruses were listed, RGV (JQ654586), STIV (EU627010), SSME (KJ175144), FV3 (AY548484), BIV (KX185156), GGRV (KP266742), ToRV1 (KP266743), TFV (AF389451), PPIV (KX574341), CMTV/E (JQ231222), and ATV (AY150217)

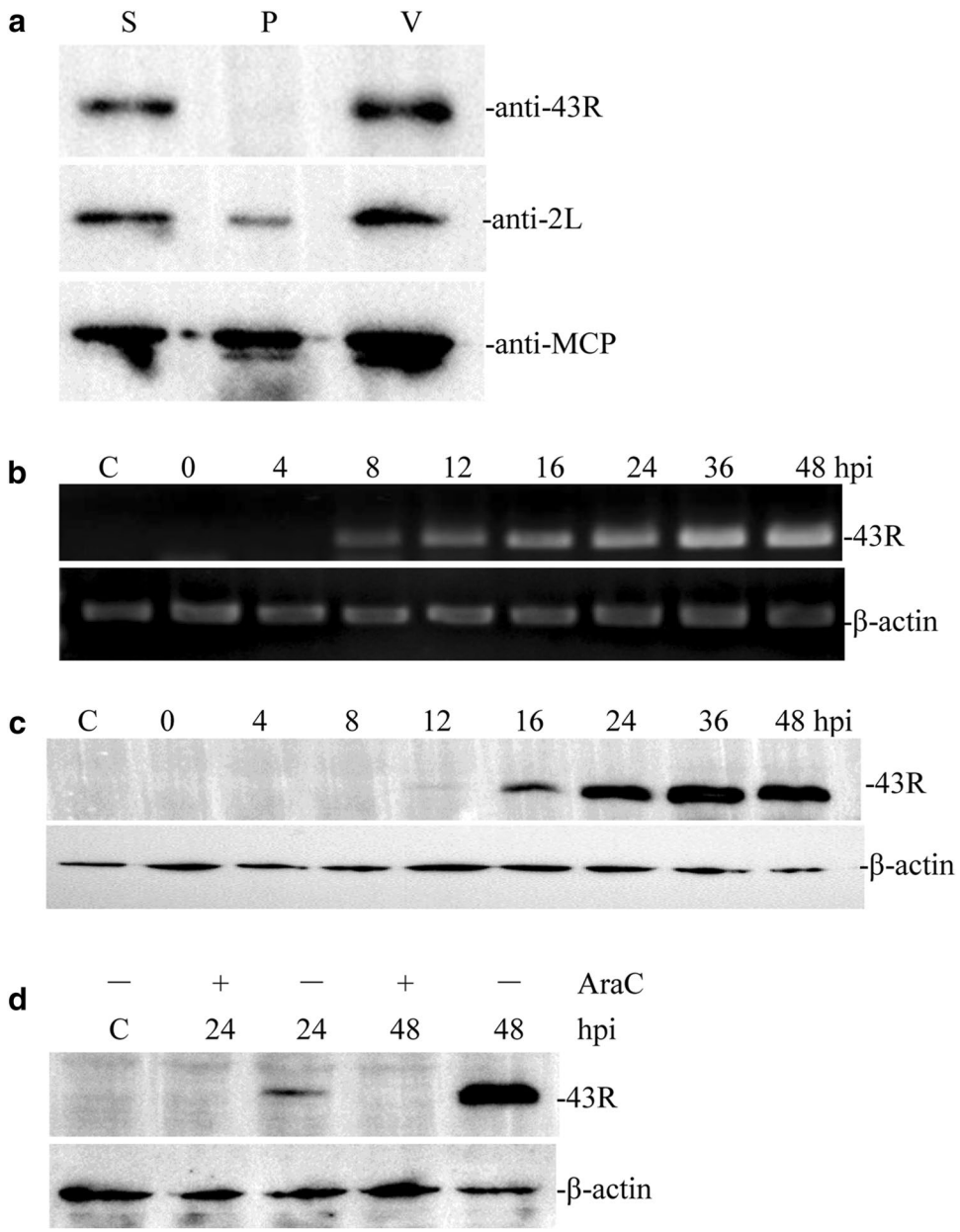

Fig. 2 Identification of envelope protein, and transcriptional and expressional pattern. **a** *S* supernatant, *P* pellet, *V* purified RGV particles. RGV 43R was detected in the supernatant and the viral particles. The known envelope protein RGV 2L and the capsid protein MCP were used as control. **b** RT-PCR detection of temporal transcription pattern of RGV *43R*. The transcript of *43R* was first detected at 8 hpi. **c** Western blot analysis of temporal expression pattern of 43R. RGV 43R protein was first detected at 12 hpi. **d** Western blot detection of RGV 43R expression in the presence or absence of AraC. Lane C, uninfected cells (control). + and − represent presence and absence of AraC, respectively. 43R protein was not detected when viral DNA replication was inhibited by AraC

Subcellular localization of RGV 43R

Subcellular localization of 43R without RGV infection was determined by detecting the fluorescence distribution of 43R-EGFP fusion protein. As shown in Fig. 3a, green fluorescence presented aggregate distribution only in cytoplasm of the EPC cells transfected with pEGFP-*43R* plasmid, indicating that 43R-EGFP fusion protein was localized in the cytoplasm. Interestingly, green fluorescence diffusely distributed in both cytoplasm and the nucleus of EPC cells transfected with pEGFP-*43R*ΔTM plasmid, indicating that the TM domain was essential for the subcellular distribution of 43R-EGFP in cytoplasm. As control, the EGFP protein distributed in both cytoplasm and the nucleus of cells transfected with empty plasmid pEGFP-N3.

The 43R-EGFP fusion protein were further expressed under the control of the native promoter of *43R* to analyze subcellular localization of 43R during RGV infection. As shown in Fig. 3b, when the EPC cells transfected with the plasmid pMD-*43R-EGFP* were infected with RGV for 16 h, green fluorescence diffusely distributed in both cytoplasm and the nucleus. At 24 hpi, most fluorescence signals distributed in the cytoplasm and little fluorescence colocalized with viral factory. At 36 hpi and 48 hpi, fluorescence was highly enriched and colocalized with viral factory. As positive control, when EPC cells transfected with the plasmid pMD-p43R-EGFP were infected with RGV for 16, 24, 36, or

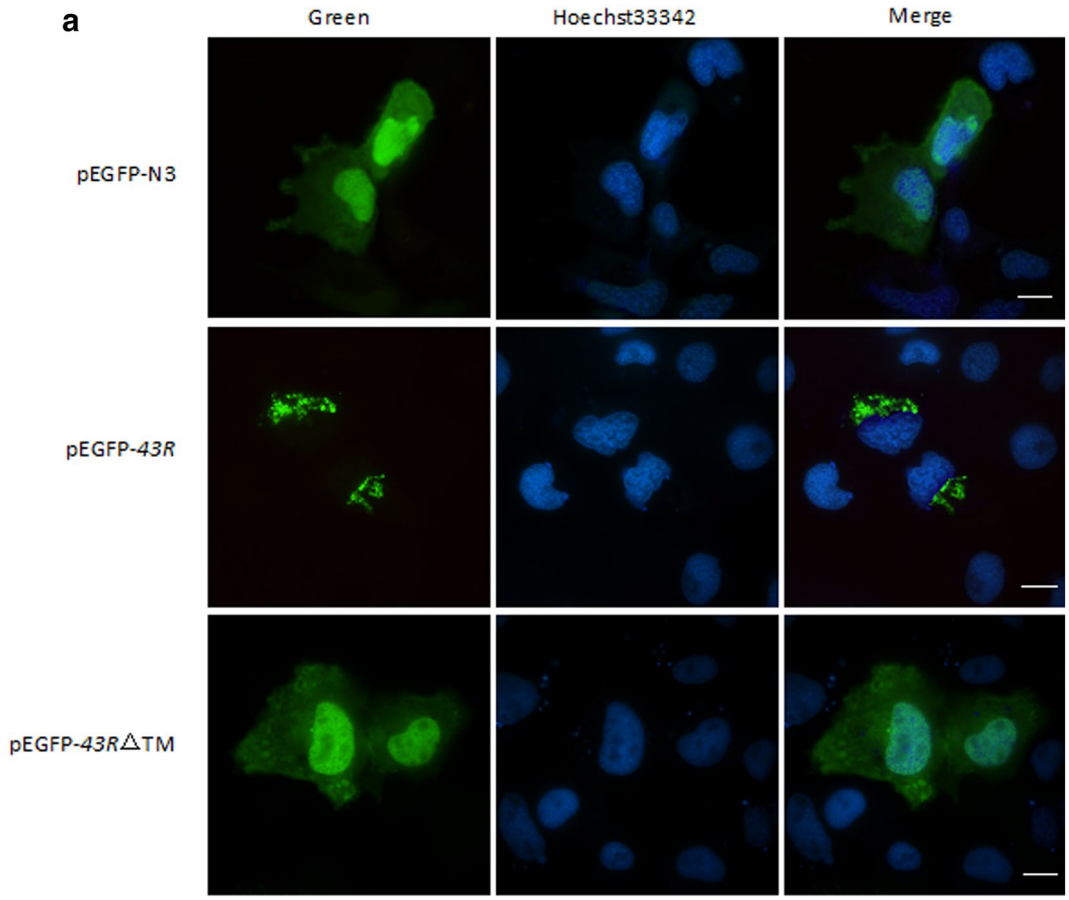

Fig. 3 Subcellular localization of 43R detected by 43R-EGFP fusion protein. **a** EPC cells transfected with plasmid pEGFP-N3, pEGFP-*43R* or pEGFP-*43R*ΔTM were observed at 24 hpt. 43R-EGFP fusion protein distributed in the cytoplasm while 43RΔTM-EGFP fusion protein distributed in the cytoplasm and nucleus. Bar = 10 μm. **b** EPC cells transfected with plasmid pMD-p43R-*EGFP* or pMD-*43R-EGFP* for 24 h were mock infected or infected with RGV for 16, 24, 36, and 48 h, then the cells were observed. 43R-EGFP fusion protein colocalized with viral factories. Bar = 10 μm

48 h, EGFP distributed in both cytoplasm and the nucleus. As negative control, no green fluorescence signals were observed in the cells transfected with the plasmid pMD-*43R-EGFP* or pMD-p43R-*EGFP*.

Confirmation of recombinant RGV deleting *43R* (Δ43R-RGV)

A recombinant virus Δ43R-RGV in which *43R* was deleted was constructed by homologous recombination to investigate its role in virus infection. In Δ43R-RGV, the nucleotide positions from + 245 to + 504 relative to the translation initiation codon of *43R* were replaced by p50-EGFP (Fig. 4a).

Cells infected with Δ43R-RGV second filial generation (F2) showed plaques and green fluorescence, but the plaques and the fluorescent areas overlapped incompletely. Cells infected with Δ43R-RGV tenth filial generation (F10) showed plaques and green fluorescence, and the plaques and the fluorescent areas overlapped completely (Fig. 4b). These data showed that Δ43R-RGV was purified through ten generations of plaque isolation via EGFP selection.

43R deletion was further verified by PCR analysis and western blot analysis, using the anti-43R. Using RGV or Δ43R-RGV as templates, predicted products were amplified by PCR analysis (data not shown). A specific band of about 38 kDa was detected in RGV-infected cells, but it was not present in cells infected with Δ43R-RGV (Fig. 4c), indicating that the expression of 43R was completely interrupted in the cells infected with Δ43R-RGV.

43R deletion resulted in reducing cytopathic effects and virus titers

The effect of *43R* deletion on cytopathic effect (CPE) were examined by light microscopy of EPC cells separately infected with RGV or Δ43R-RGV at a MOI of 0.1. The cells were viewed at 12, 24, and 36 hpi. As shown in Fig. 5a, no CPE was observed in cells infected with each virus at

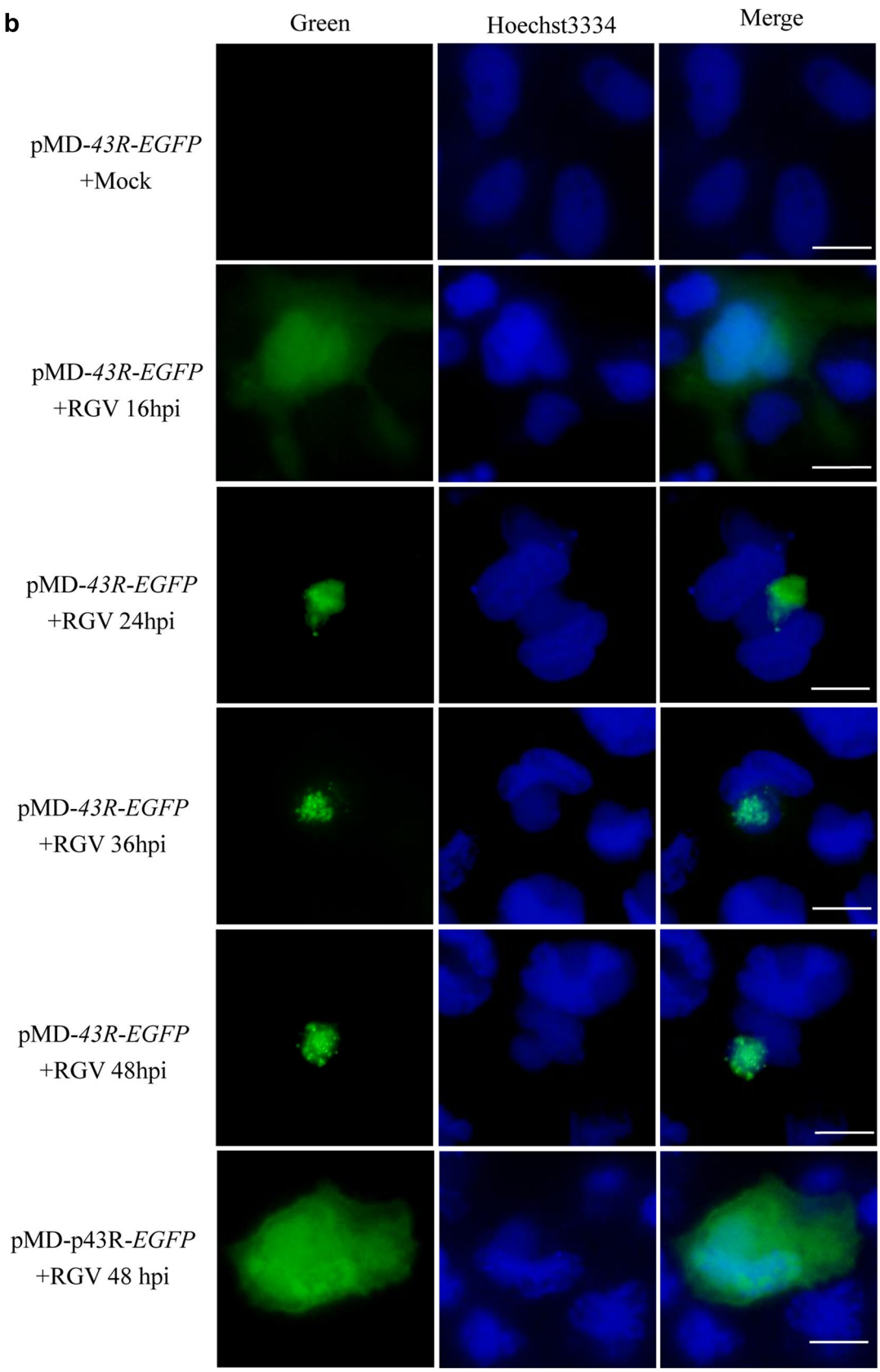

Fig. 3 (continued)

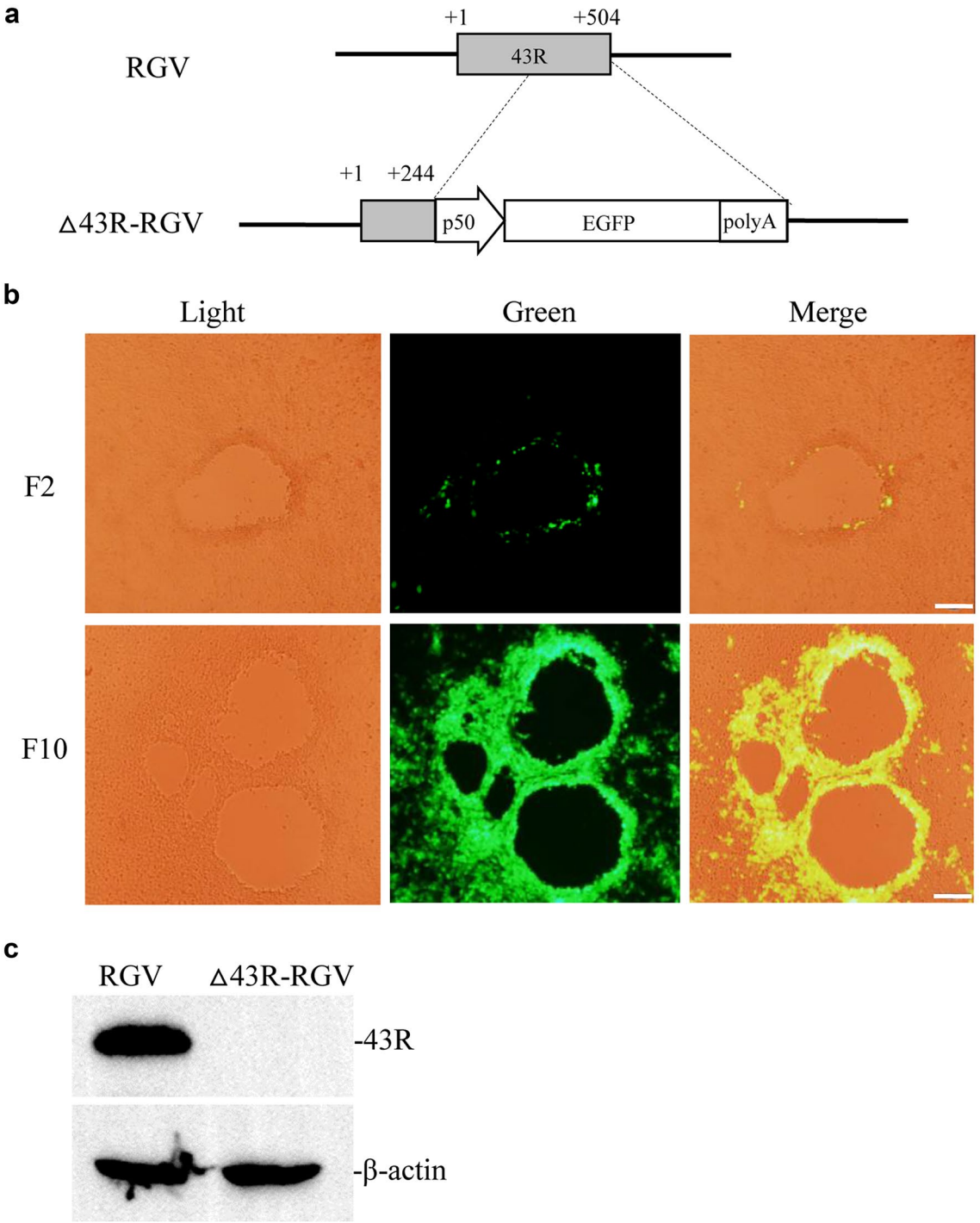

Fig. 4 Construction of recombinant virus Δ43R-RGV. **a** Schematic diagram of Δ43R-RGV structure. The nucleotide positions from +245 to +504 relative to the translation initiation codon of *43R* were replaced by p50-EGFP. **b** Light and green fluorescence micrographs of cells infected with Δ43R-RGV. The column 3 (Merge) is the overlap of the column 1 (Light) and column 2 (Green). The plaques and the fluorescent areas overlapped incompletely after EPC cells infected with Δ43R-RGV second filial generation (F2) for 72 h. The plaques and the fluorescent areas overlapped completely after cells infected with Δ43R-RGV tenth filial generation (F10) for 76 h. Bar = 100 μm. **c** Western blot analysis for presence or absence of 43R protein in the cells infected by RGV or Δ43R-RGV at 36 hpi

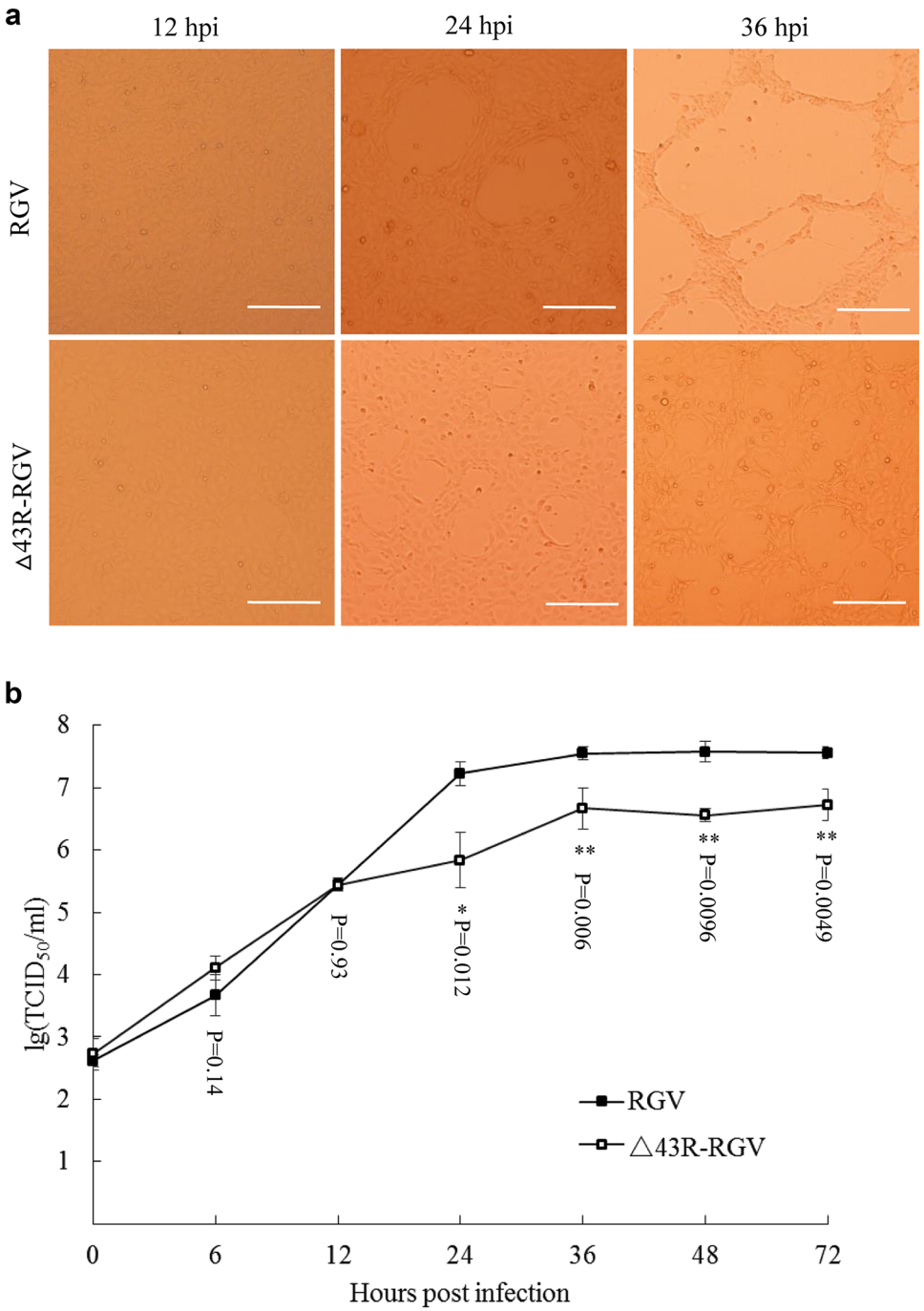

Fig. 5 Comparison between Δ43R-RGV and RGV in the ability to induce CPE and virus titer. **a** Light microscopic micrographs of cells infected with RGV or Δ43R-RGV at a MOI of 0.1. At 24 and 36 hpi, the CPEs in cells infected with RGV were more obvious than these of Δ43R-RGV. Scale bar, 100 μm. **b** Virus growth curves of RGV or Δ43R-RGV in EPC cells. The titers of Δ43R-RGV from 24 hpi to 72 hpi were significantly lower than these of RGV. Each data point represents the average titer of three independent infections. Error bars indicate standard deviations. * and ** represent $P < 0.05$ and $P < 0.01$, respectively

12 hpi. The CPEs were observed in cells infected with RGV or Δ43R-RGV at 24 and 36 hpi, and the CPEs in RGV-infected cells were more obvious than these in cells infected with Δ43R-RGV. These indicating that compared with RGV, the ability of Δ43R-RGV to induce the cytopathic effect was reduced.

The effects of *43R* deletion on the virus titers were evaluated by virus growth curve analysis. As shown in Fig. 5b, the titers of Δ43R-RGV and RGV at 0 hpi were not significantly different ($P = 0.453$). The titers of Δ43R-RGV from 24 hpi to 72 hpi were significantly lower than these of RGV. These indicated that *43R* deletion significantly reduced the virus titers.

43R deletion affected virus entry but not viral DNA replication

Virus particles number in virus supernatant which was used in CPE analysis was analyzed by qPCR. The average virus particles number per $TCID_{50}$ of Δ43R-RGV is 216, while

the number of RGV is 103 virus particles/$TCID_{50}$. Approximately 2.1 times of virus particles are needed for Δ43R-RGV to reach a similar percentage of CPE to those of RGV, demonstrating that *43R* deletion reduced RGV infectivity.

Then the virus genome copies in infected cells were analyzed, but not in virus supernatant. The DNA levels of RGV and Δ43R-RGV in the infected cells at the early stage of virus infection were measured to examine the effect that *43R* deletion have on virus entry and viral DNA replication. The results are shown in Fig. 6. At 0 hpi, the viral genome copy number of Δ43R-RGV was significantly lower than that of RGV ($P = 0.0003$), indicating that *43R* deletion affects Δ43R-RGV entry. The viral genome copy number increased steadily from 2 hpi to 8 hpi upon Δ43R-RGV infection, which suggests continuous replication of the viral genome. An identical pattern was also obtained with RGV, with values at the four time points examined being higher than Δ43R-RGV by approximately 3.5-fold. The slope of regression line remains unaltered by *43R* deletion. Thus, *43R* deletion primarily affects viral entry but not viral DNA replication.

Anti-43R serum has neutralization effect at late stages of infection

RGV neutralization was carried out to further determine whether 43R is an envelope protein and is important in virus entry. The results are shown in Fig. 7. When RGV was neutralized by incubation with anti-43R serum prior to inoculation, the titers slightly reduced from 36 hpi to 72 hpi, and

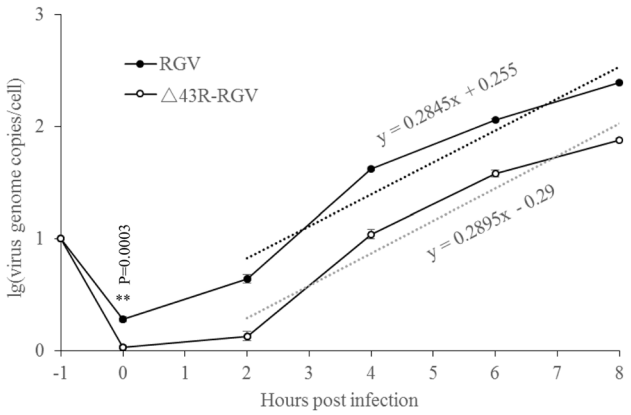

Fig. 6 qPCR analysis of viral DNA levels in EPC cells infected with RGV and Δ43R-RGV. After infection with 10 viral particles per EPC cell in 6-well plates, virus genome DNA from infected cells at indicated hpi was extracted and quantified by qPCR. At 0 hpi, the viral genome copy number of Δ43R-RGV was significantly lower than that of RGV. The viral genome copy number increased steadily from 2 hpi to 8 hpi upon RGV or Δ43R-RGV infection. Dotted lines represent the regression lines of the values from 2 hpi to 8 hpi. Linear regression equations were also shown

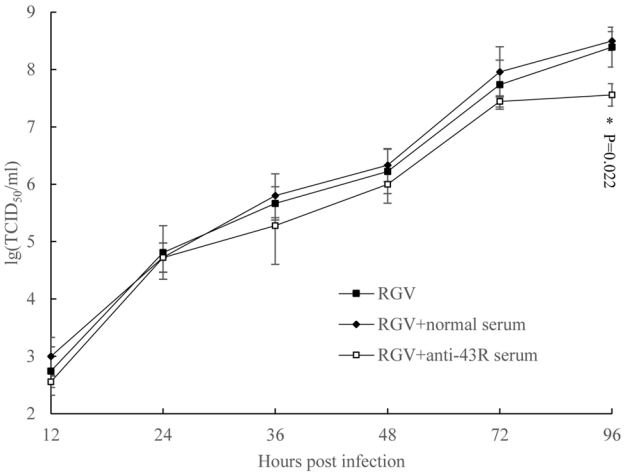

Fig. 7 Virus growth curves of RGV neutralized with anti-43R serum in EPC cells. RGV neutralization with anti-43R serum slightly reduced the virus titer from 36 hpi to 72 hpi and significantly reduced the virus titer at 96 hpi. Each data point represents the average titer of three independent infections. Error bars indicate standard deviations. * represent $P < 0.05$

significantly reduced at 96 hpi. As control, pre-incubation with normal serum had no effect on virus titer. These results further confirmed that 43R is on the surface and is involved in virus entry.

Discussion

RGV 43R was predicted to contain a TM domain and a RGD motif. Some envelope proteins of ranaviruses were also reported to contain TM domain and RGD motif playing an important role in virus attachment and entry [22, 23]. These strongly implied that 43R might be an envelope protein. To determine whether 43R is an envelope protein, the envelope fractions and nucleocapsid fractions of purified RGV were separated using detergent Triton X-100 treatment, and detected by western blot analysis. Surprisingly, a little of RGV 2L was detected in the pellet. It is reported that the detergent Triton X-100 did not completely separate the membrane from the viral particles [10, 24] Moreover, a little of MCP was detected in the supernatant. It is possible that little viral particles or capsid proteins dissolved from viral particles are in the supernatant. It is reported that the detergent might dissolved ranavirus particles [24]. However, RGV 43R was detected in the viral particles and supernatant but not in the pellet, suggesting that 43R is a viral envelope protein.

The temporal expression profile of RGV 43R was analyzed, showing that the transcript encoding 43R and the protein itself was detected late during RGV infection, and its expression was blocked by viral DNA replication

inhibitor, indicating that *43R* belongs to the late expression class of genes. Its homologue in frog virus 3 (FV3) was also reported as late gene [25]. In general, late genes of large DNA virus encode mainly structural proteins, involving in virus entry, assembly, trafficking and budding [26, 27].

TM domains of some viral envelope proteins were reported as the localization signal [28]. Here, the role of TM domain in the subcellular distribution of 43R was investigated, showing that 43RΔTM-EGFP diffusely distributed in cytoplasm and nucleus while 43R-EGFP distributed in cytoplasm. These indicated that TM domain is very important for the subcellular distribution of 43R. Subcellular localization of 43R during RGV infection showed that 43R-EGFP fusion protein diffusely distributed in both cytoplasm and the nucleus at 16 hpi and ultimately localizes to viral factory. Similar phenomena have been observed in other envelope proteins of ranaviruses [10, 29, 30]. Viral factory of ranavirus is the place for virus assembly [31]. These implied that 43R might participate in virus assembly.

To further elucidate the role of envelope protein RGV 43R in virus infection, a recombinant virus Δ43R-RGV was constructed and analyzed in comparison with wild type RGV. Compared with RGV, the ability of Δ43R-RGV to induce CPEs and its virus titers from 24 to 72 hpi were significantly reduced. To explore the mechanism behind the phenotypic variations resulting from *43R* deletion, the viral entry, DNA replication and morphogenesis were evaluated. By measuring and comparing the DNA levels of RGV and Δ43R-RGV in the infected cells at the early stage of infection, it is revealed that *43R* deletion inhibited Δ43R-RGV entry but did not affect viral DNA replication. RGV neutralization with anti-43R serum slightly reduced the virus titer from 36 hpi to 72 hpi and significantly reduced the virus titer at 96 hpi, further confirming that 43R is on the surface and is important in virus entry. The neutralization effect at early stage of infection is not obvious. It could be resulted from two reasons. One, the anti-43R serum is prepared based on a truncated 43R, which may not cover all sites of the protein. Two, some other RGV-encoded proteins may also affect the neutralization results. Electron microscopy showed typical characteristics of ranavirus infection in EPC cells infected with RGV or Δ43R-RGV at 24 hpi or 36 hpi, such as viral factory, intracytoplasmic paracrystalline arrays, chromatin condensation, vesiculation, and budding viral particles from the plasma membrane (data not shown), indicating that *43R* deletion did not significantly influence virus morphogenesis. Therefore, these data suggested that *43R* deletion reduced virus infectivity through inhibiting viral entry. Enveloped ranavirus is thought to enter cells by receptor-mediated endocytosis, followed by release of non-enveloped virions into the cytoplasm [32]. Furthermore, iridovirid entry may also involve interaction between virions and caveolae [33, 34]. However, whether RGV 43R interacts with host receptor to affect virus entry needs more evidences.

In conclusion, RGV *43R* was identified as a late gene encoding an envelope protein. 43R-EGFP fusion protein distributed in the cytoplasm and TM domain is essential for its subcellular distribution in cytoplasm. 43R-EGFP fusion protein colocalizes with the viral factory in RGV-infected cells. *43R* deletion reduced the ability of Δ43R-RGV to induce CPE and virus titers. It is further revealed that *43R* deletion significantly inhibited viral entry. This study extends the knowledge about the envelope protein of ranavirus in virus infection.

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Author contributions QYZ conceived and designed the study. XCG and XTZ performed experiments. QYZ and XTZ analyzed the data and wrote the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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