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Rana grylio virus (RGV) envelope protein 2L: subcellular localization and essential roles in virus infectivity revealed by conditional lethal mutant

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Rana grylio virus (RGV) is a pathogenic iridovirus that has resulted in high mortality in cultured frog. Here, an envelope protein gene, *2L*, was identified from RGV and its possible role in virus infection was investigated. Database searches found that RGV *2L* had homologues in all sequenced iridoviruses and is a core gene of iridoviruses. Western blotting detection of purified RGV virions confirmed that 2L protein was associated with virion membrane. Fluorescence localization revealed that 2L protein co-localized with viral factories in RGV infected cells. In co-transfected cells, 2L protein co-localized with two other viral envelope proteins, 22R and 53R. However, 2L protein did not co-localize with the major capsid protein of RGV in co-transfected cells. Meanwhile, fluorescence observation showed that 2L protein co-localized with endoplasmic reticulum, but did not co-localize with mitochondria and Golgi apparatus. Moreover, a conditional lethal mutant virus containing the *lac* repressor/operator system was constructed to investigate the role of RGV 2L in virus infection. The ability to form plaques and the virus titres were strongly reduced when expression of 2L was repressed. Therefore, the current data showed that 2L protein is essential for virus infection. Our study is the first report, to our knowledge, of co-localization between envelope proteins in iridovirus and provides new insights into the understanding of envelope proteins in iridovirus.

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INTRODUCTION

Iridoviruses are large, enveloped DNA viruses which contain circularly permutated and terminally redundant double-stranded DNA genomes (Chinchar *et al.*, 2011; Williams *et al.*, 2005). Based on the ninth report of the International Committee on Taxonomy of Virus (ICTV), the family *Iridoviridae* could be classified into five genera: *Ranavirus*, *Megalocytivirus*, *Lymphocystivirus*, *Iridovirus* and *Chloriridovirus* (Jancovich *et al.*, 2012). Similarly to other aquatic viruses (such as aquareoviruses, turtle herpesviruses and novirhabdoviruses), iridoviruses could also infect a variety of invertebrates and ectothermic vertebrates, leading to heavy economic losses in the aquaculture industry and resulting in great threat to wildlife conservation (Leong & Kurath, 2011; Ke *et al.*, 2011; Whittington *et al.*, 2010; Work *et al.*, 2009).

Although many iridovirus genomes have been completely sequenced (Chen *et al.*, 2013; Gui & Zhu, 2012; Huang *et al.*, 2009; Jancovich *et al.*, 2010; Lei *et al.*, 2012a; Mavian *et al.*, 2012; Wong *et al.*, 2011; Zhang *et al.*, 2004), a number of viral-encoded proteins, including the viral envelope proteins, have not been identified and their functions remain unclear. Viral envelope proteins have been reported to play important roles in virus infection and assembly (Zhao *et al.*, 2008; Zhou *et al.*, 2011). Recently, several envelope or membrane proteins have been identified in some iridoviruses (Ao & Chen, 2006; Ince *et al.*, 2010, 2013; Shuang *et al.*, 2013; Whitley *et al.*, 2010; Wong *et al.*, 2011; Zhao *et al.*, 2008; Zhou *et al.*, 2011). However, the exact role of envelope proteins in virus infection as well as the relationship between envelope proteins and other proteins are still unclear.

Rana grylio virus (RGV), a member of *Ranavirus* belonging to the family *Iridoviridae*, has been reported to induce high mortality in cultured pig frog (*Rana grylio*) (Zhang *et al.*, 1999, 2001, 2006). Previous studies showed that at least 16 structural proteins were detected in RGV. Some RGV-encoded proteins involved in DNA replication, gene transcription, viral infection and assembly have been investigated and analysed (Huang *et al.*, 2007; Ke *et al.*, 2009; Kim *et al.*, 2010; Lei *et al.*, 2012b; Sun *et al.*, 2006; Zhao *et al.*, 2007, 2008, 2009; Zhang *et al.*, 2001). Moreover, it was demonstrated that RGV could be used as a viral vector for foreign gene expression in fish cells (He *et al.*, 2012). A conditional lethal recombinant RGV containing the *lac* repressor/operator system was proven to be a powerful tool

The sequence of RGV *2L* is extracted from the complete genome sequence of RGV (GenBank/EMBL/DDBJ accession number: JQ654586).

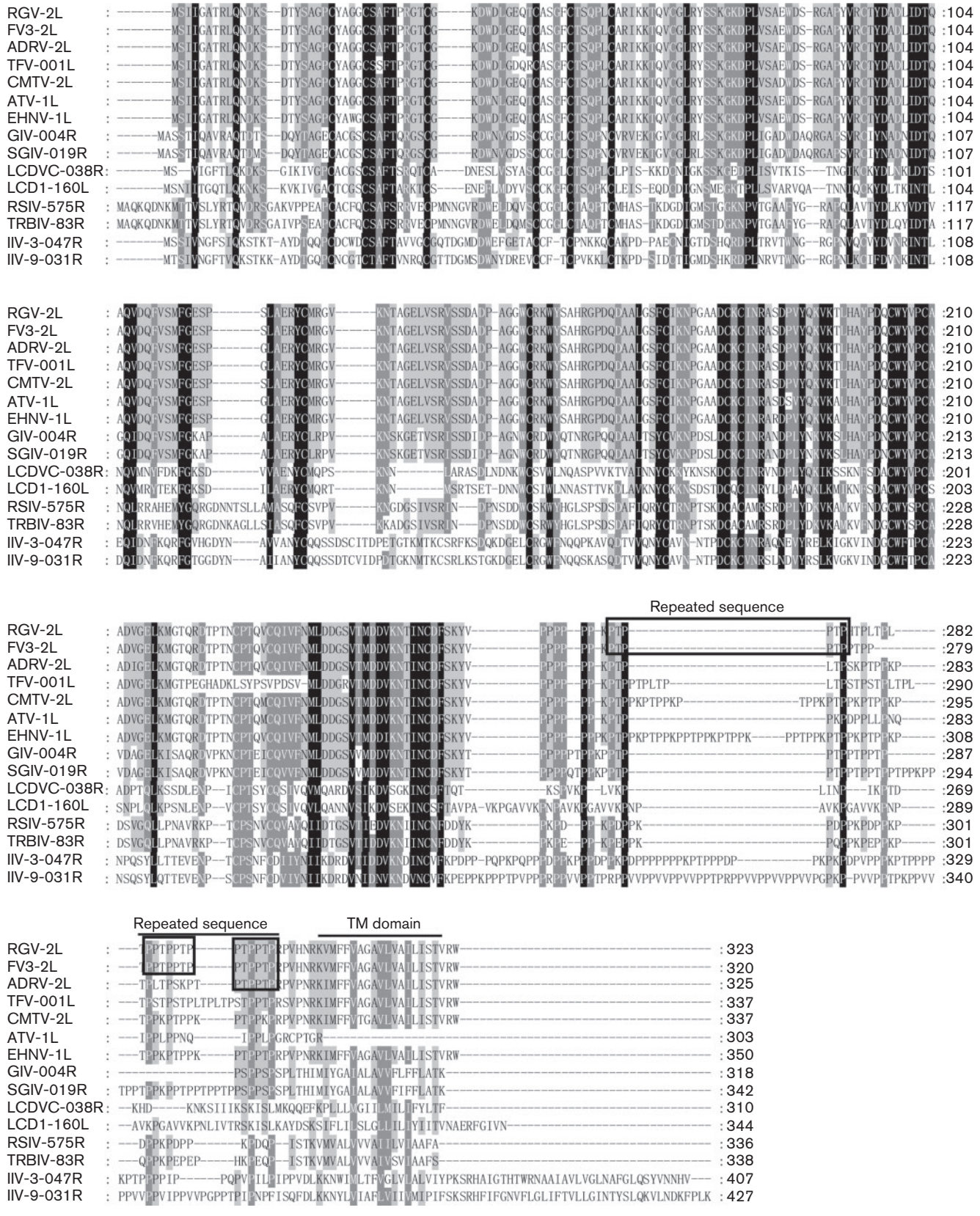

Fig. 1. Multiple sequence alignment of 2L homologues in iridoviruses. RGV, *Rana grylio* virus; FV3, *Frog virus 3*; ADRV, *Andrias davidianus* ranavirus; TFV, tiger frog virus; CMTV, common midwife toad ranavirus; ATV, *Ambystoma tigrinum virus*; EHNV, *Epizootic haematopoietic necrosis virus*; GIV, grouper iridovirus; SGIV, Singapore grouper iridovirus; LCDV1, *Lymphocystis*

Fig. 1. (cont.) *disease virus 1*; LCDVC, lymphocystis disease virus isolate China; RSIV, red sea bream iridovirus; TRBIV, turbot reddish body iridovirus; IIV-3, Invertebrate iridescent virus 3; IIV-9, *Invertebrate iridescent virus 9*. RGV, FV3, ADRV, TFV, CMTV, ATV, EHNV, GIV and SGIV belong to the genus *Ranavirus*. LCDV1 and LCDVC belong to the genus *Lymphocystivirus*. RSIV and TRBIV belong to the genus *Megalocytivirus*. IIV-3 belongs to the genus *Chloriridovirus*. IIV-6 belongs to the genus *Iridovirus*. The black shaded regions indicate completely conserved residues, whilst the grey shaded regions are partially conserved residues with greater than 80 % identity. The repeated sequences are boxed and the predicted TM domain is indicated.

for the analysis of gene function (He *et al*., 2013). Recently, the complete genome of RGV has been sequenced and analysed. It was found that the RGV genome is closely related to that of frog virus 3 (FV3), the type species of the genus *Ranavirus* (Lei *et al*., 2012a).

Analysis of the RGV genome showed that RGV contained 106 open reading frames (ORFs). Similarly to ORF 53R, ORF 2L was predicted to encode a myristylated membrane protein and to be one of the 26 core genes that are conserved in family *Iridoviridae* (Eaton *et al*., 2007; Lei *et al*., 2012a; Zhao *et al*., 2008). Although the *2L* gene and its homologues have been proved to be late genes in family *Iridoviridae* (Majji *et al*., 2009; Xu *et al*., 2007), the function of 2L protein in virus infection is still unknown, and the relationship between 2L and other proteins remain unclear. In this study, we cloned and characterized the *2L* gene from RGV, analysed the relationship between 2L protein and other viral-encoded proteins, and revealed its possible role in virus infection.

RESULTS

Sequence characteristics of RGV 2L

The nucleotide sequence of RGV ORF *2L* has a length of 972 bp and encodes a peptide of 323 amino acids (aa) with a predicted molecular mass of 35 kDa. Sequence analysis showed that the *2L* gene had homologues in all sequenced iridoviruses and was more conserved in *Ranavirus*. A transmembrane (TM) domain was predicted to exist between aa positions 304 and 321, and a continuous tri-repeated sequence (PTPPTP) was present at the C-terminal (Fig. 1). Additionally, the N-terminal region was more conserved than the C-terminal region.

RGV 2L is a viral envelope protein

Plasmid pET-32a/2L was transformed into *Escherichia coli* BL21(DE3) and the expression of 2L fusion protein was induced by IPTG. As shown in Fig. 2(a), the fusion protein was approximately 50 kDa. The fusion protein was purified and used for anti-RGV 2L serum preparation in mice. Fig. 2(b) shows that an approximate 35 kDa protein band was detected in the lysates of cells that were infected with RGV and harvested at 24 and 48 h post-infection (p.i.) by using the anti-RGV 2L serum. However, no band was detected in mock infected cells. Moreover, when 2L protein was repressed in a conditional lethal mutant, the protein band was hard to detect (see Fig. 6d below). These results suggested that anti-RGV 2L serum is specific to 2L protein. The predicted TM domain region indicated that RGV 2L protein might be associated with the viral envelope fractions. Using Triton X-100 treatment, the envelope and capsid protein fractions were separated from purified RGV virions. These fractions were subjected to SDS-PAGE and Western blotting analysis by anti-RGV 2L serum. As shown in Fig. 2(c), most of the 2L protein was present in the supernatant, whereas a small portion of 2L protein was observed in the pellet. As a control, envelope protein 53R was also concentrated in the supernatant and only a small portion of 53R protein was detected in the pellet (Fig. 2d). In addition, no components of major capsid protein (MCP) were detected in the supernatant (Fig. 2e). These results suggested that 2L was an envelope protein of RGV.

Subcellular localization and distribution of RGV 2L

Immunofluorescence assay was carried out to reveal the intracellular localizations of RGV 2L in infected cells. As shown in Fig. 3, when epithelioma papulosum cyprini (EPC) cells were infected with RGV for 24 h, fluorescence signals mainly distributed in the cytoplasm of infected cells. At 36 h p.i., fluorescence signals were stronger than at 24 h p.i. and viral factories were observed near the nucleus. At 48 h p.i., viral factories were more obvious and almost all the fluorescence co-localized with viral factories. As a negative control, no fluorescence signals were observed in mock infected cells.

2L protein localized to the endoplasmic reticulum

To investigate the precise localization of 2L protein and the relationship between 2L protein and organelles such as endoplasmic reticulum (ER), mitochondria (MT) and Golgi apparatus, plasmid pEGFP-2L was co-transfected with plasmid pDsRed2-ER or pDsRed2-MT or pDsRed-Monomer-Golgi and cells were subjected to fluorescence observation at 48 h post-transfection (p.t.). As shown in Fig. 4(a), 2L-GFP co-localized with specific regions in the ER. However, 2L-GFP did not co-localize with mitochondria and Golgi apparatus (Fig. 4b, c). The results suggested that 2L protein localized to the ER in EPC cells. Moreover, the results also showed that the globular inclusions formed by 2L protein are not aggresomes for degradation because they localized to ER.

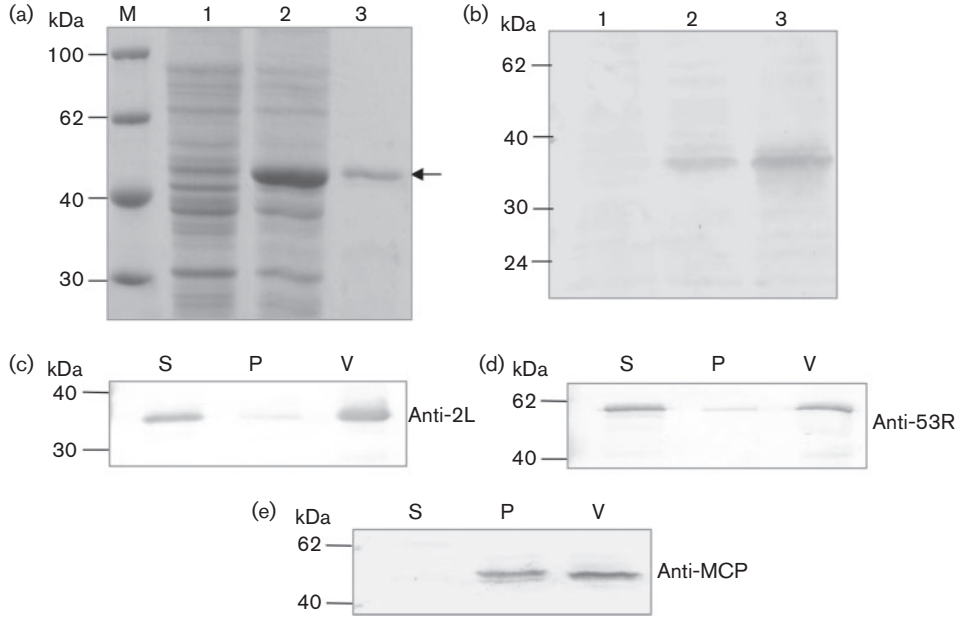

Fig. 2. Prokaryotic expression and Western blotting analysis of RGV 2L. (a) Prokaryotic expression of RGV 2L. M, protein molecular mass marker; lane 1, lysate of bacteria containing pET-32a/2L without IPTG induction; lane 2, lysate of bacteria containing pET-32a/2L with IPTG induction; lane 3, purified 2L fusion protein. The arrow indicates the purified 2L protein. (b) Confirmation of the specificity of anti-RGV 2L serum. Lane 1, lysate of mock infected cells; lane 2, lysate of RGV infected cells harvested at 24 h; lane 3, lysate of RGV infected cells harvested at 48 h. (c) Detergent extraction and Western blotting detection of RGV 2L in purified RGV virions. S, supernatant; P, pellet; V, purified RGV virions. (d) Detergent extraction and Western blotting detection of 53R protein in purified RGV virions. (e) Detergent extraction and Western blotting detection of MCP protein in purified RGV virions.

Co-localization of 2L and other viral-encoded proteins

Although RGV 2L co-localized with viral factories in infected cells and presented as globular inclusions in transfected cells, the relationship between 2L and other viral-encoded proteins is unclear. Thus, plasmid pEGFP-2L was co-transfected with plasmid pDsRed2-22R or pDsRed2-53R or pDsRed2-MCP in order to find out the relationship between 2L protein and other viral-encoded proteins. 22R protein, whose homologue in tiger frog virus (TFV) has been proved to be a membrane protein (Wang *et al.*, 2008), was predicted to contain TM domains. As shown in Fig. 5(a), no co-localization was observed in the plasmid pEGFP-2L and pDsRed2-C1 co-transfected cells, which were used as a negative control. When plasmid pEGFP-2L was co-transfected with pDsRed2-22R or pDsRed2-53R, green fluorescence overlapped with red fluorescence in transfected cells (Fig. 5b, c), suggesting that 2L co-localized with 22R and 53R. However, when plasmid pEGFP-2L was co-transfected with plasmid pDsRed2-MCP, green and red fluorescence did not overlap (Fig. 5d), suggesting that 2L did not co-localize with MCP. In addition, when plasmid pDsRed2-22R was co-transfected with plasmid pEGFP-53R, green and red fluorescence also overlapped (Fig. 5e), suggesting that 22R co-localized with 53R.

Investigating the role of 2L using a conditional lethal mutant virus

Construction of a conditional lethal mutant virus in which 2L was inducibly expressed. A conditional lethal mutant RGV in which 2L protein was inducibly expressed was constructed to investigate the role of 2L protein in virus infection. After eight successive rounds of plaque isolation via red fluorescence protein (RFP) selection in the presence of IPTG, a conditional lethal mutant virus, i2L-RGV-lacIO, was purified. i2L-RGV-lacIO contained *lacI* and *lacO* elements of the *lac* repressor/operator system. The predicted structure of i2L-RGV-lacIO is shown in Fig. 6(a). The *lacI* gene and EGFP gene were inserted into the TK locus. A hybrid promoter p50-lacO-8 (He *et al.*, 2013), which contained the *lacO* sequence 8 bp downstream of the TATA-like box of RGV p50 promoter, was placed in front of the *2L* gene. After being infected with i2L-RGV-lacIO, cells showed not only plaques, but also green and red fluorescence. Moreover, the plaques and the fluorescent areas overlapped completely. As a control, no fluorescence was observed in RGV infected cells (Fig. 6b).

PCR was carried out to confirm whether the hybrid promoter p50-lacO-8 and chimeric gene p50-RFP were inserted in front of the *2L* gene. Using specific primers for foreign genes (p50-lacO-8 and p50-RFP), no DNA band

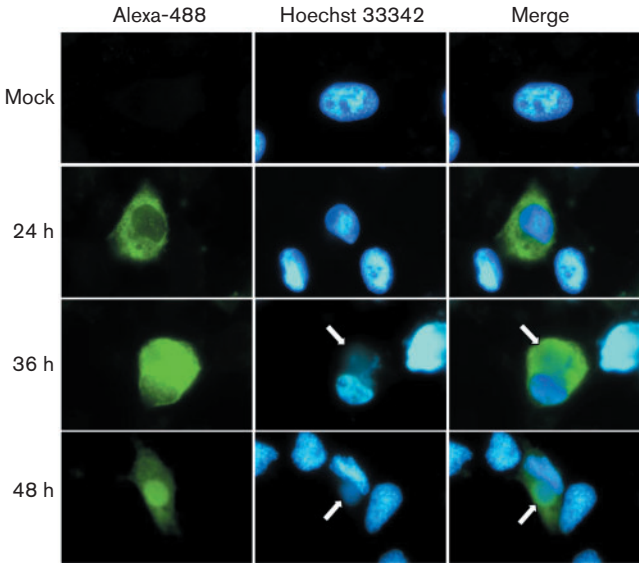

Fig. 3. Immunofluorescence localization of RGV 2L in infected cells. EPC cells were infected with RGV for 24, 36 and 48 h and the cells were fixed, permeabilized and stained with anti-RGV 2L serum and Alexa-488-conjugated goat anti mouse IgG, followed by Hoechst 33342. Mockinfected cells were used as a negative control. Green fluorescence shows the distribution of 2L; blue fluorescence shows the nucleus. The arrows indicate virus factories stained by Hoechst 33342. Magnification ×100 (oil-immersion objective).

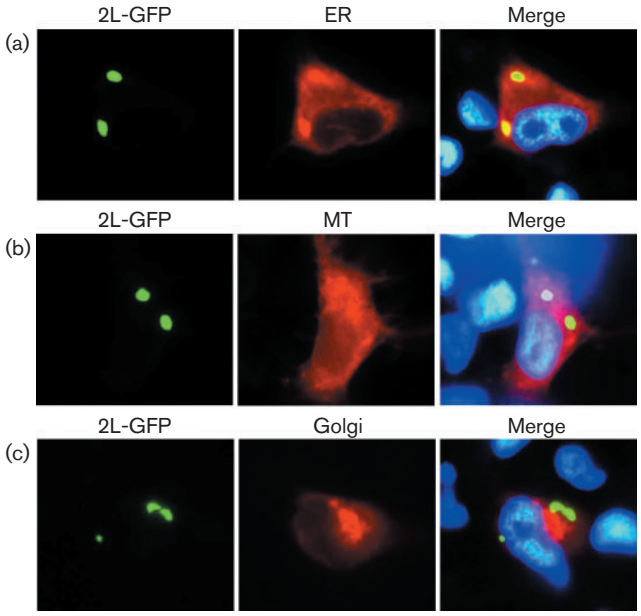

Fig. 4. The relationship between 2L protein and cell organelles. Cells were transfected with plasmids and subjected to fluorescence observation at 48 h p.t. Green fluorescence shows the distribution of fusion protein containing GFP; red fluorescence shows the distribution of cell organelles (ER, MT and Golgi apparatus); blue fluorescence shows the nucleus. (a) Distribution of 2L-GFP fusion protein and ER in co-transfected cells. (b) Distribution of 2L-GFP fusion protein and MT in co-transfected cells. (c) Distribution of 2L-GFP fusion protein and Golgi apparatus in co-transfected cells. Magnification ×100 (oil-immersion objective).

was obtained from mock infected or RGV infected cells. In contrast, an approximately 1.2 kb DNA band was obtained from i2L-RGV-lacIO infected cells (Fig. 6c). This DNA band corresponded to the length of foreign genes p50-lacO-8 and p50-RFP, implying that they had been inserted. The insertion of foreign genes was further confirmed by sequencing of the PCR product (data not shown).

Western blotting was employed to study the effect of IPTG on the expression of 2L protein in RGV or i2L-RGV-lacIO infected cells. As shown in Fig. 6(d), it can be seen that IPTG had no effect on the expression level of 2L protein in RGV infected cells. However, in i2L-RGV-lacIO infected cells, the expression of 2L protein was highly dependent on IPTG, being significantly reduced in its absence. This result revealed that the expression of 2L protein in i2L-RGV-lacIO infected cells was induced by IPTG.

Replication efficiency of i2L-RGV-lacIO was strongly decreased when 2L was repressed. Plaque assay and one-step growth curves were performed to study the role of 2L protein in virus infection. As shown in Fig. 7(a), a large number of plaques were observed in RGV infected cells regardless of whether IPTG was present. Although the number of plaques obtained in i2L-RGV-lacIO infected cells with IPTG was slightly lower than that of RGV, the number of plaques obtained in i2L-RGV-lacIO infected cells without IPTG was about 10 % of that obtained in RGV infected cells (Fig. 7a). One-step growth curves showed results similar to the plaque assay. The titres of i2L-RGV-lacIO in the absence of IPTG were significantly decreased. The titres of RGV were about 50-fold more than that of i2L-RGV-lacIO at 48 h p.i. without IPTG (Fig. 7b). These results suggested that the replication efficiency of i2L-RGV-lacIO was strongly reduced when the expression of 2L protein was repressed.

i2L-RGV-lacIO induced weak cytopathic effect when 2L was repressed. The ability of i2L-RGV-lacIO to induce cytopathic effect (CPE) in the presence or absence of IPTG was investigated and compared with that of RGV. As shown in Fig. 7(c), obvious CPE was observed in RGV infected cells regardless of whether IPTG was present, implying that IPTG has no effect on the ability of RGV to induce CPE. The CPE induced by i2L-RGV-lacIO in the presence of IPTG was slightly lower than that induced by RGV. However, i2L-RGV-lacIO induced weak CPE when IPTG was absent. These data revealed that 2L protein played an important role in i2L-RGV-lacIO infection.

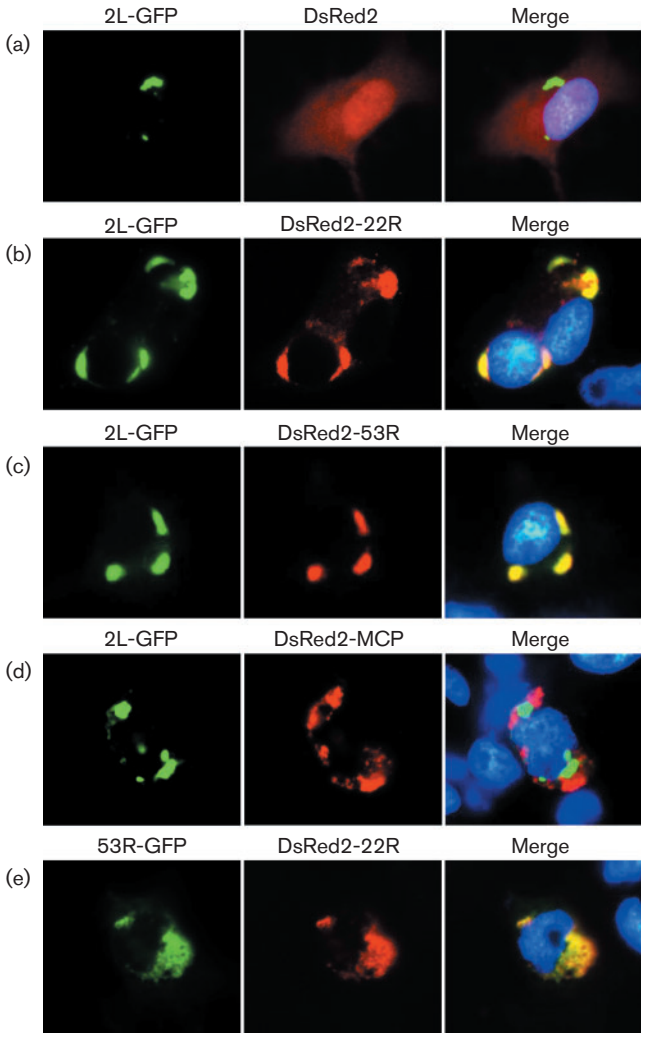

Fig. 5. Analysis of the co-localization between 2L and 22R or 53R or MCP. Cells were transfected with plasmids and subjected to fluorescence observation at 48 h p.t. Green fluorescence shows the distribution of fusion protein containing GFP; red fluorescence shows the distribution of fusion protein containing DsRed2; blue fluorescence shows the nucleus. (a) Distribution of 2L-GFP fusion protein and DsRed2 in co-transfected cells. (b) Distribution of 2L-GFP fusion protein and DsRed2-22R fusion protein in co-transfected cells. (c) Distribution of 2L-GFP fusion protein and DsRed2-53R fusion protein in co-transfected cells. (d) Distribution of 2L-GFP fusion protein and DsRed2-MCP fusion protein in co-transfected cells. (e) Distribution of 53R-GFP fusion protein and DsRed2-22R fusion protein in co-transfected cells. Magnification ×100 (oil-immersion objective).

DISCUSSION

In this study, we cloned and characterized the *2L* gene from RGV. Database searches found that the *2L* gene had homologues in all sequenced iridoviruses, whereas no homologues or orthologues were found among non-iridoviruses. Moreover, no conserved domain or motif was found from either the *2L* gene or its homologues. These data suggested that the *2L* gene may be an iridovirus-specific gene with unknown function. Therefore, the RGV *2L* gene was selected for further study in order to understand its possible role in virus infection. A TM domain was found at the C-terminal of 2L, implying that 2L protein may be associated with the viral envelope. Viral envelope proteins are considered to play important roles in virus infection (Zhao *et al.*, 2008; Zhou *et al.*, 2011). By detergent extraction and Western blotting analysis, 2L was identified as an envelope protein of RGV.

Immunofluorescence assay showed that 2L protein co-localized with viral factories in the later stages of RGV infection. Interestingly, the localization of 2L protein in co-transfected cells presented as globular inclusions near the nucleus at the later stage of transfection. The globular inclusions were similar to viral factories formed by iridovirus (Netherton *et al.*, 2007) or inclusion structures formed by reovirus μNS (Becker *et al.*, 2003; Broering *et al.*, 2005). The capacity of RGV 2L protein to form globular inclusions implies that a new method could be used to study the relationship between 2L and other viral-encoded proteins by co-transfecting 2L protein with other proteins. This method was widely used to identify protein–protein associations in reovirus and was proven to give results similar to co-immunoprecipitation (Brandariz-Nuñez *et al.*, 2010; Miller *et al.*, 2007). Thus, 2L was co-transfected with 22R or 53R or MCP for fluorescence observation. The results showed that 2L, 22R and 53R co-localized, and these three proteins were identified or predicted as envelope proteins of iridovirus (Wang *et al.*, 2008; Zhao *et al.*, 2008); as they co-localized with each other they may interact. Both envelope proteins and MCP are the components of viral factories (Huang *et al.*, 2011), but 2L did not co-localize with MCP in the co-transfected cells, suggesting that the globular inclusions formed by 2L protein may not be bona fide viral factories. This interesting phenomenon that 2L did not co-localize with MCP needs further research. Envelope proteins of *White spot syndrome virus* (WSSV) could interact with each other, some of which could bind to form a complex (Chang *et al.*, 2008; Zhou *et al.*, 2009). WSSV is the only member of family *Nimaviridae* and is phylogenetically near *Herpesvirus*, *Poxvirus* and *Iridovirus* (Lo *et al.*, 2012). Moreover, both the iridovirus and WSSV are large double-stranded DNA viruses. They may have similar strategies in the interactions of envelope proteins.

ER is one of the organelles that many viruses exploit during infection (Shibata *et al.*, 2009). Previous studies have revealed that several membranous materials were observed in the viromatrix of RGV (Huang *et al.*, 2006; Zhang & Gui, 2012). This membranous material may contain components of ER. Membranes of ER were also shown to participate in vaccinia virus assembly (Risco *et al.*, 2002). Vaccinia virus A11 protein was associated with viral crescent membranes which co-localized in cytoplasmic factories with ER, and was considered to be involved in recruitment of ER for virus assembly (Maruri-Avidal *et al.*, 2013). Our observation

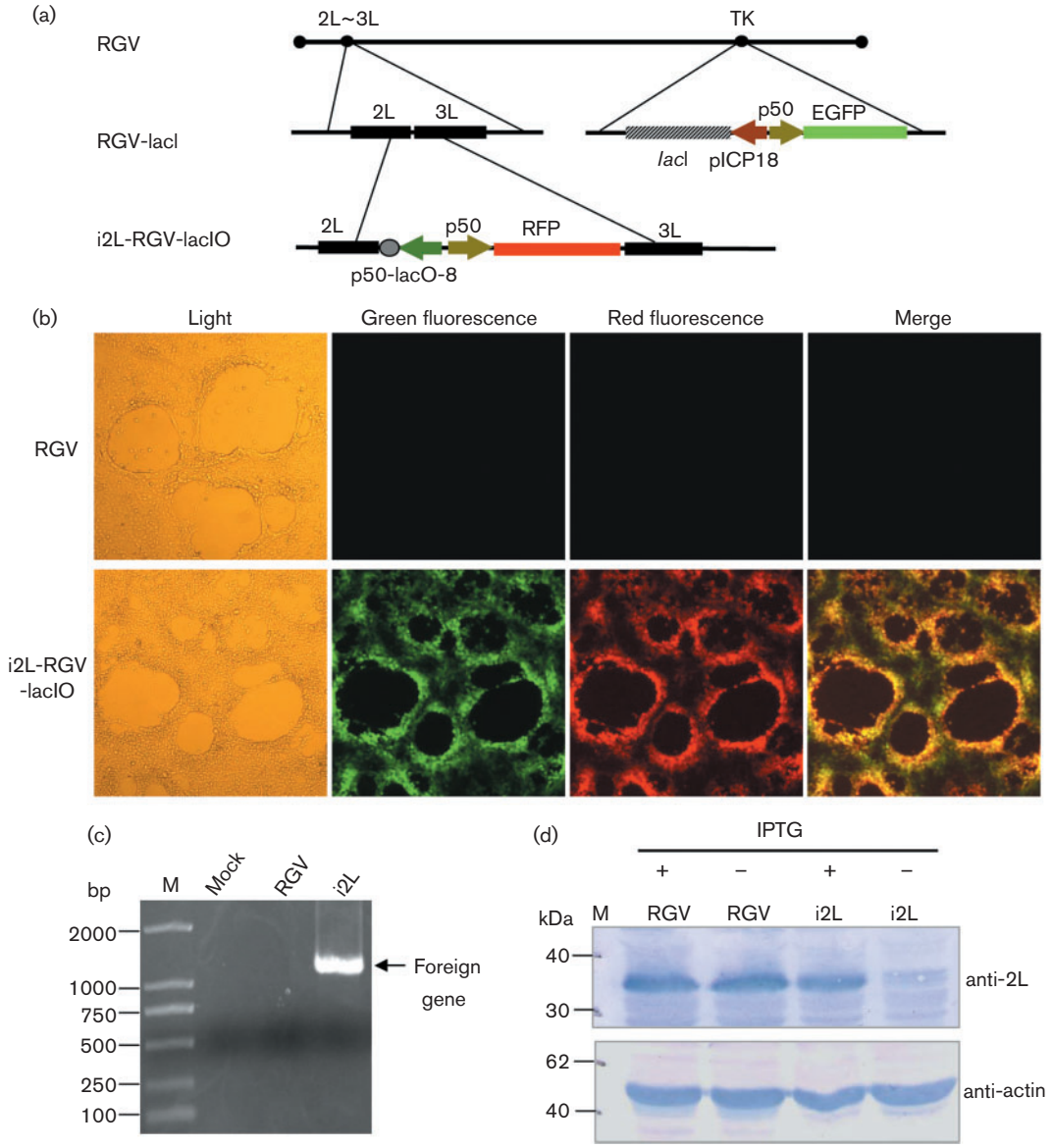

Fig. 6. Analysis of the conditional lethal mutant virus i2L-RGV-lacIO. (a) Schematic diagram of i2L-RGV-lacIO structure. i2L-RGV-lacIO contained hybrid promoter p50-lacO-8 and chimeric gene p50-RFP in the gene spacer between *2L* and *3L*. (b) Light and fluorescence micrographs of RGV and i2L-RGV-lacIO infected cells. Cells were infected with RGV or i2L-RGV-lacIO at an m.o.i. of 1. At 48 h p.i. the plaques formed and cells were subjected to fluorescence observation. Column 4 (merge) is the overlap of columns 2 (green fluorescence) and 3 (red fluorescence). Magnification ×5. (c) PCR analysis by primers for foreign genes (p50-lacO-8 and p50-RFP). Cells were mock infected or infected with RGV or i2L-RGV-lacIO and total DNA was extracted for PCR analysis. M, DNA marker. (d) Western blotting analysis of the expression of 2L induced by IPTG. Cells were infected with RGV or i2L-RGV-lacIO at an m.o.i. of 1 in the presence or absence of IPTG. The cells were harvested at 24 h p.i. and subjected to Western blotting using anti-2L serum. Actin was detected under the same conditions as an internal control. M, protein molecular mass marker.

showed that 2L protein co-localized with the specific regions of ER, but did not co-localize with mitochondria and Golgi apparatus. Thus, the observation that 2L co-localized with ER suggested that 2L may recruit some components of ER to participate in the formation of viral membrane or virus assembly. FV3 ORF 97R localized to ER, and the C-terminal TM domain of 97R was essential for this localization (Ring *et al.*, 2013). 2L also had a TM domain at its C-terminal. Whether this domain was required for localization needs further confirmation.

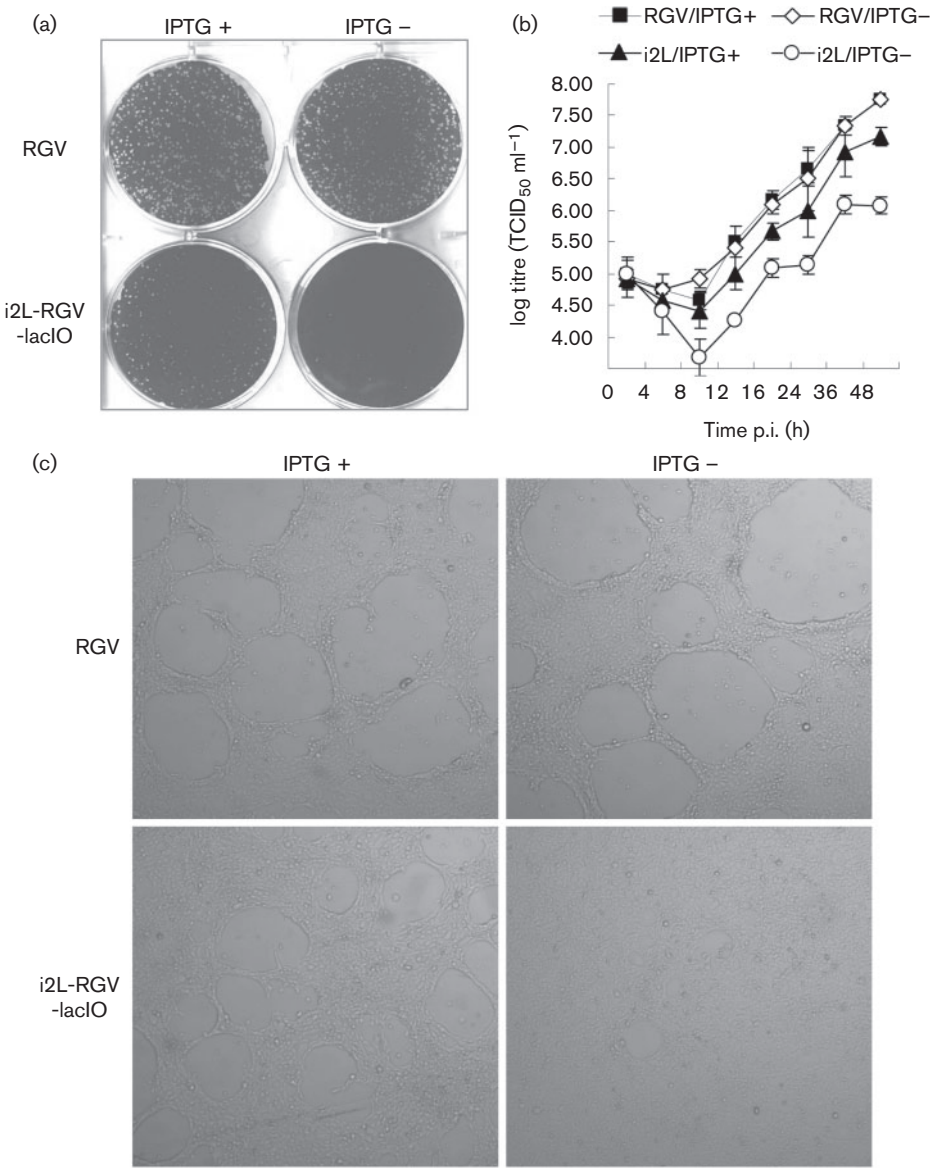

Fig. 7. Comparison between i2L-RGV-lacIO and wild-type RGV. (a) Plaque assay of i2L-RGV-lacIO and RGV. Cells were infected with RGV or i2L-RGV-lacIO at an m.o.i. of 0.01 in the presence or absence of IPTG for plaque assay. (b) One-step growth curves of i2L-RGV-lacIO and RGV in EPC cells. Cells were infected with RGV or i2L-RGV-lacIO at an m.o.i. of 1 and then harvested at different times (0, 4, 8, 12, 16, 24, 36 and 48 h p.i.) for titration. The data show the average titres of three independent experiments as log $TCID_{50}$ ± SD. (c) CPE induced by RGV or i2L-RGV-lacIO. Cells were infected with RGV or i2L-RGV-lacIO at an m.o.i. of 1 in the presence or absence of IPTG. At 48 h p.i. CPE was evident and cells were observed under a light microscope. Magnification ×5.

The *lac* repressor/operator system has been thoroughly studied and is the best understood system that regulates gene transcription by protein–nucleic acid interactions (Jacob & Monod, 1961). Recently, we have constructed a conditional lethal mutant RGV containing the *lac* repressor/operator system, which was proven to be a powerful tool for the analysis of gene function (He *et al.*, 2013). Based on the system, we constructed another conditional lethal mutant virus named i2L-RGV-lacIO. Western blotting showed that the expression of 2L protein in recombinant virus i2L-RGV-lacIO was dependent on IPTG. When 2L protein was repressed, plaque formation ability, virus titres and CPE were strongly reduced compared with wild-type RGV. All the data suggested that 2L protein was essential for virus infection. These data also suggested that conditional lethal mutant RGV containing the *lac* repressor/operator system is a powerful tool for gene function analysis.

Table 1. Primers for plasmid construction (enzyme cleavage site is underlined)

Primers	Primer sequences (5→3′)	Constructed plasmid or fragment
32a-2L-F	GCTGAATTCATGTCCATCATCG (*Eco*RI)	pET-32a/2L
32a-2L-R	TATAAGCTTTTACCATCTCACTGTAGAG (*Hin*dIII)	
2L-F	GGCGAATTCAATGTCCATCATC (*Eco*RI)	pEGFP-2L
2L-R	TATGGTACCATACCATCTCACTGTAGAG (*Kp*nI)	
22R-F	CACGAATTCGATGTTGCAGAATTAC (*Eco*RI)	pDsRed2-22R
22R-R	GCAGGTACCGTATGAGCTCCCGT (*Kp*nI)	
53R-F	TACAGATCTATGTAGGGAAAATGGGAG (*Bgl*II)	pEGFP-53R and pDsRed2-53R
53R-R	CCTGAATTCCCATAACCCCTGTG (*Eco*RI)	
MCP-F	TAAGAATTCAATGTCTTCTGTAACTGGT (*Eco*RI)	pDsRed2-MCP
MCP-R	AAAGGTACCATACAAGATTGGGAAT (*Kp*nI)	
p50-lacO-8-F	CTCGGATCCAACCTCTGAGAAAGC (*Bam*HI)	p50-lacO-8
p50-lacO-8-R	GTCGCTCCGATGATGGACATACCAGTTACAGAAGACATTT	
2L-L-F	AAATGTCTTCTGTAACTGGTATGTCCATCATCGGAGCGAC	2L-L
2L-L-R	AAGAAGCTTAGGGCGGCGTCCTGGT (*Hin*dIII)	
p50-RFP-F	TATGGATCCTTACAGGAACAGGTGG (*Bam*HI)	p50-RFP
p50-RFP-R	GAGAGAAAAAGGCTATTAAACTCCGCAAAAACCTCTGAGA	
2L-R-F	TCTCAGAGGTTTTTGCGGAGTTTAATAGCCTTTTTCTCTC	2L-R
2L-R-R	CAGGAATTCGGACAGAGAGTTCCAC (*Eco*RI)	

In conclusion, 2L was identified as an envelope protein of RGV. 2L protein co-localized with viral factories in infected cells and presented as globular inclusions in transfected cells. Moreover, 2L protein co-localized with ER, envelope protein 22R, and envelope protein 53R in co-transfected cells. The conditional lethal mutant revealed that 2L protein was essential for virus infection. The results obtained in the present study will provide important information for better understanding of envelope proteins in iridovirus and their cooperation between each other.

METHODS

Virus and cell line. *Rana grylio* virus (RGV) was used in the study. RGV propagation and viral titre determination were performed as described previously (Zhang *et al.*, 1999, 2006). EPC cells used in the study were maintained in TC199 medium (Hyclone) supplemented with 10 % FBS at 25 °C.

Plasmid construction and protein sequence analysis. To construct plasmids for prokaryotic expression of 2L protein, the complete sequence of ORF 2L was amplified from RGV genomic DNA by specific primers (Table 1). The obtained fragments were digested with corresponding restriction enzymes and inserted into pET-32a vector (Novagen) which had been treated with the same enzymes to give plasmid pET-32a/2L.

To analyse the subcellular location of 2L protein and its co-localization with other viral-encoded proteins, DNA fragments that contained ORFs 2L, 22R and 53R, and MCP of RGV were amplified from RGV DNA. Each fragment was cut with corresponding restriction enzymes and inserted into either pEGFP-N3 (Clontech) or pDsRed2-C1 (Clontech) vector which had been treated with the same enzymes to give plasmids pEGFP-2L, pDsRed2-22R, pEGFP-53R, pDsRed2-53R and pDsRed2-MCP, respectively.

To generate plasmids for the construction of conditional lethal mutant virus, two DNA fragments, 2L-L and 2L-R, that contained a 5′ end or partial coding sequence of the RGV *2L* gene (2L-L: +1 to +509 relative to the ATG of RGV *2L* gene; 2L-R: −509 to −1 relative to the ATG of RGV *2L* gene), respectively, were obtained, from RGV DNA via PCR. A hybrid promoter p50-lacO-8, which contained the *lacO* sequence 8 bp downstream of the TATA-like box of the RGV p50 promoter (He *et al.*, 2012, 2013), was amplified and fused with DNA fragment 2L-L by overlapped PCR. The obtained fragment was digested with *Bam*HI/*Hin*dIII and inserted into pUC19 vector (TaKaRa) to generate plasmid p19-lacO-2L-L. A DNA fragment, p50-RFP, which contained the complete ORF of the red fluorescence protein (RFP) gene which was promoted by the p50 promoter, was amplified from plasmid pRFP-lacO-53R (He *et al.*, 2013) and fused with DNA fragment 2L-R. The fused DNA fragment was cut with *Bam*HI/*Eco*RI and inserted into plasmid p19-lacO-2L-L, generating plasmid p19-lacO-2L. Plasmid p19-lacO-2L was used for the construction of conditional lethal mutant virus. All constructed plasmids mentioned above were confirmed by restriction enzymes and DNA sequencing.

The sequence data for ORF 2L 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. Multiple sequence alignments were conducted using CLUSTAL_X v2.0 and edited using GeneDoc.

Prokaryotic expression, protein purification and antibody preparation. Plasmid pET-32a/2L was transformed into *Escherichia coli* BL21(DE3) and the bacterium was induced for 6 h with 1 mM IPTG at 37 °C to express fusion protein. The fusion protein was purified using the HisBind purification kit (Novagen), mixed with an equal volume of Freund's adjuvant (Sigma) and then used to immunize mice by hypodermal injection once every 7 days. Anti-RGV 2L serum was collected after the fifth immunization. Mouse anti-RGV 53R polyclonal antibody has been described previously (Zhao *et al.*, 2008). Mouse anti-RGV MCP polyclonal antibody was produced in our laboratory (data not shown).

Animal experimental procedures were conducted under the institutional guidelines of Hubei province. The protocol was approved by

the Committee of Wuhan University Center for Animal Experiment (permit number SCXK 2008-0004). All surgery was performed under the anaesthetic sodium pentobarbital, and all efforts were made to minimize animal suffering.

Immunofluorescence microscopy observation. EPC cells that grown on coverslips in six-well plates were mock infected or infected with RGV at an m.o.i. of 0.5 and harvested at 24, 36 and 48 h p.i. The cells were rinsed with PBS and fixed with 4 % paraformaldehyde for 30 min at room temperature. Fixed cells were permeabilized with 0.2 % Triton X-100 and then blocked in 10 % normal goat serum at room temperature for 1 h. The cells were incubated with anti-RGV 2L serum diluted in 1 % normal goat serum for 2 h, rinsed three times for 10 min each with PBS containing 1 % normal goat serum and then incubated with Alexa-488-conjugated goat anti mouse IgG (Invitrogen) followed by staining of nucleus and viral factories by Hoechst 33342 (Sigma). Finally, the cells were rinsed with PBS, mounted with 50 % glycerol and visualized under a fluorescence microscope (Leica). The images were processed with Adobe Photoshop (Adobe Systems).

Transfection and subcellular localization. EPC cells grown on coverslips in six-well plates were transfected with plasmid pEGFP-2L and fixed at 12, 24 and 48 h p.t. The fixed cells were permeabilized with 0.2 % Triton X-100, stained by Hoechst 33342 and observed by fluorescence microscopy. To investigate the co-localization of 2L protein with other viral-encoded proteins, plasmid pEGFP-2L was co-transfected with plasmid pDsRed2-22R or pDsRed2-53R or pDsRed2-MCP, and then fixed at 48 h p.t. The fixed cells were stained as above and observed by fluorescence microscopy. At the same time, plasmid pDsRed2-22R was co-transfected with plasmid pEGFP-53R to investigate the co-localization between 22R and 53R. To find out the relationship between 2L protein and organelles such as ER, MT and Golgi apparatus, three organelle-specific markers (plasmid pDsRed2-ER (marker for ER; Clontech), pDsRed2-MT (marker for MT; Clontech) and pDsRed2-Monomer-Golgi (marker for Golgi apparatus; Clontech)) were used. Plasmid pEGFP-2L was co-transfected with these plasmids and subjected to fluorescence observation at 48 h p.t.

Detergent extraction and phase separation of purified virions. The extraction of envelope proteins of RGV was performed as described previously (Zhou *et al.*, 2011). In brief, purified RGV virions 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 at 15 000 ***g*** for 1 h at 4 °C. Proteins from the pellet and supernatant were analysed by 12 % SDS-PAGE and transferred to PVDF membranes for Western blotting analysis.

Western blotting. The proteins from the pellet and supernatant phase were analysed by Western blotting as described previously (He *et al.*, 2012). Anti-RGV 2L serum was used as the primary antibody at a 1 : 1000 dilution, followed by alkaline phosphatase-conjugated goat anti mouse IgG (H + L) antibody at a 1 : 1000 dilution (Vector Laboratories) as the secondary antibody. To analyse expression of 2L protein in RGV or i2L-RGV-lacIO, cells were infected with RGV or i2L-RGV-lacIO at an m.o.i. of 1 in the presence or absence of IPTG and harvested at 24 h p.i. The harvested cells were subjected to Western blotting as above.

Generation of conditional lethal mutant virus. Plasmid p19-lacO-2L was used for the construction of conditional lethal mutant virus in which 2L protein was inducibly expressed. EPC cells were transfected with plasmid p19-lacO-2L and then infected with RGV-lacI, an RGV derived recombinant virus expressing the LacI repressor of the *lac* repressor/operator system (Fig. 6a) (He *et al.*, 2013). Cells were harvested at 48 h p.i. and were diluted to infect EPC cells in the presence of 1 mM IPTG. The infected cells were covered with 0.7 % melted soft agar containing 1 mM IPTG and observed under a fluorescence microscope. The plaques emitted green and red fluorescence; they were marked and selected to infect fresh cells in the presence of 1 mM IPTG. The infected cells were cultured and purified as above. In this way, the RGV-lacI derived recombinant virus was purified by eight successive rounds of plaque isolation. This virus, which contains chimeric gene pICP18-lacI and p50-EGFP in the TK locus, a hybrid promoter p50-lacO-8 and chimeric gene p50-RFP in front of the *2L* gene, was named i2L-RGV-lacIO (Fig. 6a).

Plaque assay. EPC cells seeded in six-well plates were infected with RGV or i2L-RGV-lacIO at an m.o.i. of 0.01 in the presence or absence of 1 mM IPTG. After 1 h absorption, unbound virus was removed and then cells were overlaid with medium containing 0.7 % melted soft agar in the presence or absence of 1 mM IPTG. Four days later, the plaques formed and the medium was removed, and the cells fixed with 20 % formaldehyde and stained with 1 % crystal violet.

One-step virus growth curves. Cells were infected with RGV or i2L-RGV-lacIO at an m.o.i. of 1 in the presence or absence of 1 mM IPTG. The cells were harvested at various intervals (0, 4, 8, 12, 16, 24, 36 and 48 h) and titrated on duplicate monolayers of EPC cells in the presence of 1 mM IPTG.

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