VP08R from Infectious Spleen and Kidney Necrosis Virus Is a Novel Component of the Virus-Mock Basement Membrane

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ABSTRACT

Infectious spleen and kidney necrosis virus (ISKNV), the type species of the genus Megalocytivirus, family Iridoviridae, brings great harm to fish farming. In infected tissues, ISKNV infection is characterized by a unique phenomenon, in that the infected cells are attached by lymphatic endothelial cells (LECs), which are speculated to wall off the infected cells from host immune attack. A viral membrane protein, VP23R, binds and recruits the host nidogen-1 protein to construct a basement membrane (BM)-like structure, termed virus-mock basement membrane (VMBM), on the surface of infected cells to provide attaching sites for LECs. VMBMs do not contain collagen IV protein, which is essential for maintenance of BM integrity and functions. In this study, we identified the VP08R protein encoded by ISKNV. VP08R was predicted to be a secreted protein with a signal peptide but without a transmembrane domain. However, immunofluorescence assays demonstrated that VP08R is located on the plasma membrane of infected cells and shows an expression profile similar to that of VP23R. Coimmunoprecipitation showed that VP08R interacts with both VP23R and nidogen-1, indicating that VP08R is a component of VMBM and is present on the cell membrane by binding to VP23R. Through formation of intermolecular disulfide bonds, VP08R molecules self-organized into a multimer, which may play a role in the maintenance of VMBM integrity and stability. Moreover, the VP08R multimer was easily degraded when the ISKNV-infected cells were lysed, which may be a mechanism for VMBM disassembly when necessary to free LECs and release the mature virions.

IMPORTANCE

Infectious spleen and kidney necrosis virus (ISKNV; genus Megalocytivirus, family Iridoviridae) is most harmful to cultured fishes. In tissues, the ISKNV-infected cells are attached by lymphatic endothelial cells (LECs), which are speculated to segregate the host immune system. A viral membrane protein, VP23R, binds and recruits the host nidogen-1 protein to construct virus-mock basement membranes (VMBMs) on the surface of infected cells to provide attaching sites for LECs. Although VMBMs lack the collagen IV network, which is an essential structural part of true BMS, VMBMs still show an intact structure. An ISKNV-encoded VP08R protein can self-assemble into a multimer and bind both VP23R and nidogen-1 to maintain the integrity and stability of VMBMs. On the basis of these facts, we redrew the putative schematic illustration of the VMBM structure. Our study suggests that the virus adopts a strategy to remodel the cellular matrix and may provide an important reference to elucidate BM functions and the mechanisms of lymphangiogenesis.

Iridoviruses, a group of icosahedral cytoplasmic DNA viruses with circularly permuted and terminally redundant DNA genomes, infect invertebrates and poikilothermic vertebrates, including insects, fish, amphibians, and reptiles (1–6). The family Iridoviridae has been subdivided into five genera: Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus, and Megalocytivirus (7). Megalocytiviruses, named after their ability to cause swelling of infected cells, are a group of viruses most harmful to cultured fishes (7). Infectious spleen and kidney necrosis virus (ISKNV), which causes high mortality rates in farmed mandarin fish (Siniperca chuatsi), is the type species of the Megalocytivirus genus and has been detected in more than 60 marine and freshwater fishes (8–12). The ISKNV genome (~110 kb) has been sequenced, and the 125 putative open reading frames (ORFs) have been analyzed (GenBank accession no. AF371960) (13).

In tissues, ISKNV infection is characterized by a unique phenomenon, in that the infected cells are attached by a layer of lymphatic endothelial cells (LECs), which are speculated to wall off the infected cells from attack by the host immune system (14). VP23R, a plasma membrane-localized viral protein encoded by ORF023R of ISKNV, plays a central role in this pathological phenomenon. Residues 292 to 576 of VP23R are homologous to the laminin γ1 chain III2-6 fragment and can bind to nidogen-1 (14), also called entactin-1, an important component of the basement membrane (BM) (15–17). By recruiting nidogen-1, VP23R mediates the formation of virus-mock basement membrane (VMBM), a low-electron-density BM-like structure of 40 to 50 nm in thickness, on the surface of ISKNV-infected cells to provide attaching sites for lymphatic endothelial cells (14).

BM is a dense and sheet-like specialized form of the extracellular matrix (ECM) which mediates tissue compartmentalization, provides structural support for the endothelium, epithelium, fat cells, muscle, peripheral cells, and nerve axons, as well as acts as a...
functional foundation of the vasculature (18–20). The fundamental structure of BMs consists of a laminin layer, a type IV collagen layer, and the nidogen protein, which bridges the two layers (21–25). In contrast, VMBM has a unique structure consisting of VP23R and nidogen-1, as well as the infected cell’s plasma membrane, which plays the role of the laminin polymer layer of BMs (14). In BMs, both laminin and collagen IV form complex networks through inter- and intramolecular self-interactions (22, 25, 26), while no molecular network structure has been found in VMBM. Besides the fundamental structure, BMs also contain more than 50 known components which interact with the laminin network or the collagen IV network to organize a functional BM on the basolateral aspect of the cells (19), while only 2 protein components, VP23R and nidogen-1, have been identified in VMBM, until now.

The processes of VMBM formation and LEC attachment may involve migration and proliferation of LECs and interactions between LECs and VMBM components and could be similar to those that occur during lymphangiogenesis (27–29). Studies on the structure of VMBM and the behavior of the attached LECs may help elucidate the functions of BM components and the mechanisms of lymphangiogenesis. Unlike true BMs, VMBMs do not contain collagen IV molecules, which are cross-linked into a layer of a network that constructs part of the fundamental structures of BMs (26). Although collagen IV is not implicated in the association of BMs with cellular receptors, it plays an important role in the maintenance of BM integrity and functions (30). The absence of collagen IV α1 and α2 chain genes causes structural deficiencies in BMs and failure of the integrity of Reichert’s membrane, resulting in the death of mouse embryos at embryonic days 10.5 to 11.5 (30). Mutants of collagen IV can cause Alport syndrome and thin basement membrane nephropathy (31, 32). However, it has been shown that the absence of collagen IV does not affect the integrity and function of VMBMs (14), indicating that there may be an unknown mechanism that maintains the integrity of VMBM. In the present study, we identified a novel component of VMBM, the VP08R protein encoded by ORF008R of ISKNV, which could interact with both VP23R and nidogen-1. VP08R also interacted with itself and could therefore form a multimer structure through intermolecular disulfide bonds, which may play a role in the maintenance of VMBM integrity.

MATERIALS AND METHODS

Ethics statement. The animal use protocol listed below has been reviewed and approved by the Animal Ethical and Welfare Committee (AEWC) of Sun Yat-sen University with the permit number IACUC-2012-0406. All animal experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People’s Republic of China.

Fish and virus. Healthy mandarin fish weighing about 200 g each were from a local fish farm in Guangzhou City, Guangdong Province, China, and kept in separate tanks at 28°C. Tank water was filtered through a sand and carbon layer and aerated before use. ISKNV was purified from diseased mandarin fish identified in our laboratory, propagated in a cultured mandarin fish fry cell line (MFF-1), and stored at −80°C (12, 33). At 6 days postinfection (dpi) of ISKNV, the supernatants of the cultured cells were collected, and the virus titer was determined using the 50% tissue culture infective dose (TCID50) method, as described previously (33). The titer of ISKNV was calculated to be 107.4 TCID50 ml−1. The virus stock was diluted 1:10 in sterile phosphate-buffered saline (PBS; pH 7.4), and 0.5 ml/fish of the dilution was intraperitoneally injected into the healthy mandarin fish.

5’ RACE. Total RNA was isolated from the spleens of the ISKNV-infected mandarin fish. The 5’ ends of the ORF008R transcript were mapped by use of a GeneRacer kit (Invitrogen) according to the manufacturer’s instructions. 5’ rapid amplification of cDNA ends (RACE)-PCR amplification was performed with GeneRacer 5’ RACE primer and an ORF008R-specific reverse primer (5’-GCCACCCGTCCAAGGTGTCT CG-3’). Nested PCR was subsequently performed with GeneRacer 5’ RACE nested primer and an ORF008R-specific nested primer (5’-GGTC GGGCCACATCAGTGCAG-3’) using the first-round PCR product as the template. The PCR products were subcloned into the PMD19-T TA vector (Takara, China) and sequenced.

Antibody preparation. The full-length mature ORF008R without the signal peptide-encoding sequence was amplified and cloned into a glutathione 3-transferase (GST)-fusion protein cloning vector, pGEX-4T-1 (Pharmacia). The recombinant vector was transformed into Escherichia coli strain BL21(DE3) (Invitrogen) to express the GST-VP08R fusion protein. The protein was purified using glutathione-Sepharose 4B (GE Healthcare Life Sciences) and separated by electrophoresis in 12% SDS-polyacrylamide gels. The gel slice containing the GST-VP008R band was cut out and ground with Freund’s adjuvant to immunize BALB/c mice to produce anti-VP008R polyclonal antibody.

Plasmid construction. Full-length VP08R, VP23R, and Tetraodon nigroviridis nidogen-1 were cloned into pEGFP-N3 (Clontech) and pcDNA 3.1/V5-His A (Invitrogen) to express secreted GFP-tagged proteins. As shown in Fig. 3F, respectively, which express green fluorescent protein (GFP)- and V5-tagged protein, respectively. A Flag tag (DYKDDDDK)-encoding sequence with a stop codon was linked to the N terminus of ORF008R and cloned into pcDNA 3.1/V5-His A to generate the PC-08F vector to express Flag-tagged VP08R. As shown in Fig. 3B, residues 1 to 818 (the extracellular region), 1 to 602, and 1 to 291 of VP23R were cloned into pcEGFP-N3 and pcDNA 3.1/V5-His A to generate PC-23N602/PC-23N291 and PC-23N818/PC-23N602/PC-23N291, respectively, which express GFP- and V5-tagged truncation mutants of VP23R, respectively. Residues 292 to 602 and 603 to 818 of VP23R were also linked with the signal peptide (residues 1 to 19) of VP23R and cloned into pEGFP-N3 to generate PC-23N292-602 and PC-23N603-818, respectively, which express secreted GFP-tagged proteins. As shown in Fig. 3F, ORF008R was divided into 5 fragments, each of which was linked to the signal peptide of VP08R and cloned into pcEGFP-N3 to generate PC-08N-1/PC-08N-2/PC-08N-3/PC-08N-4/PC-08N-5. The G1 (residues 32 to 305), G2 (residues 306 to 602), and G3 (residues 888 to 1216) domains of T. nigroviridis nidogen-1 were linked to the nidogen-1 signal peptide (residues 1 to 31) and cloned into pEGFP-N3 and pcDNA 3.1/V5-His A to generate the vectors PN-NiG1/PN-NiG2/PN-NiG3 and PC-NiG1/PC-NiG2/PC-NiG3, respectively.

Immunofluorescence analyses. The spleens of ISKNV-infected mandarin fish and mandarin fish mock infected with PBS (as controls) were collected at 1, 2, 3, 4, 5, and 6 dpi, fixed with 4% paraformaldehyde, paraffin embedded, and sectioned to a 4-μm thickness (14). The sections were deparaffinized in xylene and rehydrated through a gradient of ethanol solutions. Endogenous peroxidase activity was blocked in a 3% hydrogen peroxide solution, and staining of sections was improved by heating for 10 min in citrate buffer (pH 6.0) using a microwave oven. Nonspecific binding was blocked by incubation in 10% normal goat serum. Sections were then incubated with rabbit anti-major capsid protein (anti-MCP) polyclonal antibody in a humidified chamber at 37°C for 1 h. After washing in PBS, sections were incubated with Alexa Fluor 633-conjugated secondary goat anti-rabbit antibody (Invitrogen) for 30 min. After washing, fluorescein isothiocyanate (FITC)- and Alexa Fluor 488-conjugated secondary goat anti-mouse antibody (Invitrogen). Double-stained sections were observed using a Leica LSM 410 confocal microscope (Germany) at 633 nm for Alexa Fluor 633 and 486 nm for Alexa Fluor 488. At 2 and 3 dpi, sections were observed with a Leica DM5000 B microscope (Germany) at 400×.
also stained with 2 μg/ml Hoechst 33258 (Sigma-Aldrich) to visualize the nuclei.

Immunofluorescence was also performed on cultured cells. Briefly, at 48 h posttransfection, zebrafish 2F-4 cells cotransfected with PC-08 and PC-23 or transfected with PC-08 alone (as a control) were fixed with 4% paraformaldehyde, incubated with mouse anti-VP08R polyclonal antibody, and detected with Alexa Fluor 546-conjugated goat antimouse secondary antibody (Invitrogen). VP23R was then detected with mouse anti-VP23R polyclonal antibody, which was directly labeled with Alexa Fluor 488 using a Zenon mouse IgG labeling kit (Invitrogen). The cell nuclei were also stained with Hoechst 33258 (Invitrogen). The stained cells were observed with a Nikon TE2000 microscope (Japan).

Co-IP. Immunoprecipitation (co-IP) and reciprocal co-IP assays were performed on plasmid-cotransfected fathead minnow (FHM) cells using anti-V5 antibody. To detect the interactions between VP08R and VP23R, PC-08 was cotransfected with PN-23N818, PN-23N602, PN-23N291, PN-23N292-602, PN-23N603-818, and pEGFP-N3 (as control) for co-IP experiments, and PC-23N818, PC-23N602, and PC-23N291 were cotransfected with PN-08 and pEGFP-N3 (as a control) for reciprocal co-IP. To detect the interactions between VP08R and nidogen-1, PC-08 was cotransfected with PN-Ni, PN-NiG1, PN-NiG2, PN-NiG3, and pEGFP-N3 (as a control) for co-IP, and PC-NiG1, PC-NiG2, and PC-NiG3 were cotransfected with PN-08 and pEGFP-N3 (as control) for reciprocal co-IP. To determine the binding sites of VP23R or nidogen-1 to VP08R, PC-23 and PC-Ni were cotransfected with PN-08-1, PN-08-2, PN-08-3, PN-08-4, and PN-08-5 for co-IP. To detect the self-interactions of VP08R, PC-08 was cotransfected with PN-08 for co-IP. Co-IP and reciprocal co-IP were performed using an anti-V5 agarose affinity gel (Sigma-Aldrich), GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich). GFP-tagged proteins in co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody (Sigma-Aldrich).

To analyze the interaction of VP08R with VP23R and nidogen-1 in ISKNV-infected tissues, at 5 dpi spleens from six ISKNV-infected mandarin fish were homogenized and lysed with cell lysis buffer (150 mM NaCl, 50 mM Tris at pH 8.0, 1% Triton X-100, 10 mM EDTA) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and 0.5 mM glutathione disulfide (GSSG; Roche, Switzerland). Co-IP was performed using 50 μl of a suspension of protein A/G-Sepharose (Santa Cruz) and 5 μl of mouse anti-VP08R polyclonal antibody or unimmunized mouse serum (as a control). The precipitates were detected by Western blotting using rabbit anti-nidogen-1 and mouse anti-VP08R polyclonal antibodies (14). Reciprocal co-IP was performed using 5 μl anti-nidogen-1 and anti-VP23R antibodies, and Western blotting was then performed using anti-VP08R antibody.

Western blotting. To further detect the self-interactions of VP08R, PC-08F was cotransfected with PN-08-1, PN-08-2, PN-08-3, PN-08-4, PN-08-5, and pEGFP-N3 (as a control) into FHM cells. At 72 h posttransfection, cells were sampled and each sample was treated with SDS-loading buffer with or without β-mercaptoethanol. Western blotting assays were then performed using rabbit anti-GFP antibody (Sigma-Aldrich). Western blotting was also used to detect the virus-expressed VP08R protein. Briefly, MFF-1 cells were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum at 27°C. After reaching 90% confluence, cells were infected with ISKNV at a multiplicity of infection (MOI) of 3, and 60 h later, the infected cells were collected and lysed using cell lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich). After storing at 4°C for 0, 0.5, 1, and 2 h, the cell lysates were treated with SDS sample buffer and subjected to Western blotting analysis for VP08R using mouse anti-VP08R antibody. MFF-1 cells transfected with PC-08F were used as a control. For the GSSG treatment assay, ISKNV-infected MFF-1 cells were lysed with cell lysis buffer containing 0.5 mM GSSG and subjected to Western blotting analysis using anti-VP08R antibody after storing at 4°C.

RESULTS

Bioinformatics analyses of ORF08R. The previous prediction showed that ORF008R of ISKNV covered nucleotides 6669 to 8246 of the virus genome (13). However, 5’ RACE analysis showed that the transcription start site of ORF008R is located at ISKNV genomic coordinate 6694, which is 35 bp downstream of the previously predicted initiation codon. Based on this result and the comparison of the sequence with the sequences of orthologous ORFs from other megalocytiviruses, we realigned the ORF008R sequence by using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and predicted that ORF008R actually begins at nucleotide 6702 and terminates at nucleotide 8246; encodes a putative protein of 514 amino acids, designated VP08R, which contains 34 cysteine residues; and has an apparent molecular mass of 56.8 kDa and an isoelectric point of 5.62 (Fig. 1). VP08R shows 89%, 89%, 88%, and 85% identities to its orthologous proteins from rock bream iridovirus (GenBank accession no. AAT71824), orange-spotted grouper iridovirus (GenBank accession no. AAX82318), red sea bream iridovirus (GenBank accession no. BAK14275), and turbot reddish body iridovirus (GenBank accession no. ADE34353), respectively (data not shown). There is no significant homology between VP08R and other known proteins outside the Megalocytivirus genus, and no known conserved domain in the VP08R sequence can be predicted. Failure to amplify the 3’ end of the ORF008R transcript using the poly(A) tail method may indicate that the transcript probably has no poly(A) tails.

Relocation of the ORF008R start codon makes the prediction of the signal peptide more reasonable. There is a 19-residue N-terminal signal peptide predicted by the SignalP (version 4.0) program (http://www.cbs.dtu.dk/services/SignalP/), but no transmembrane domain was predicted by the TMHMM (version 2.0; http://www.cbs.dtu.dk/services/TMHMM-2.0/) and SOSUI programs, and no nuclear localization signal sequence in the VP08R sequence was predicted by the PredictNLS program, indicating that VP08R is a secretory protein.

VP08R is localized on the plasma membrane of infected cells. To identify the cellular localization of the VP08R protein, immunofluorescence assays using mouse anti-VP08R polyclonal antibody were performed on a series of sections of spleens from ISKNV-infected mandarin fish at 1, 2, 3, 4, 5, and 6 dpi (Fig. 2A). As megalocytiviruses are typical cytoplasmic DNA viruses and their MCPs are expressed in the cytoplasm (7), the MCP of ISKNV was detected using rabbit anti-MCP polyclonal antibody to provide cytoplasmic counterstaining for VP08R localization. The VP08R signals showed a circular green fluorescence around the areas of red fluorescence of the MCP signals, suggesting that VP08R is localized on the plasma membrane of the infected cells. VP08R began to appear on the membrane of the infected cells at 1 dpi, and MCP began to be expressed inside the infected cells at 3 dpi. At 5 dpi, MCP began to be released from the infected cells. At 6 dpi, a large number of MCP signals were found outside the infected cells, while the VP08R protein remained on the membrane even after all the virions were released from the infected cells. As VP08R was predicted to contain a signal peptide without a transmembrane domain or a membrane anchor domain, there may be an unknown mechanism that mediates the localization of VP08R on the membrane of the ISKNV-infected cells.

To further confirm the localization of VP08R, at 2 and 3 dpi,
sections were treated with Hoechst 33258 for nuclear staining (Fig. 2B). The results demonstrated that the VP08R signals surrounded the plasma area but not the nucleus, confirming that VP08R locates on the plasma membrane. At 3 dpi, the replicated virus DNA had been released into the plasma and could also be stained by Hoechst 33258. We also observed at 3 dpi that the nuclei of many infected cells had been degenerated and disappeared, which accords with the findings of previous studies of Megalocytivirus infection (34).

Interaction between VP08R and VP23R. Since the cellular localization, expression profile, and behavior of VP08R are very similar to those of VP23R (14), a key component of VMBM, we speculated that there may be a relationship between the two viral proteins. The possible interaction between VP08R and VP23R was then analyzed by immunoprecipitation assays (Fig. 3). As shown in Fig. 3A, VP23R could be coprecipitated with the V5-tagged VP08R, while the GFP control was not coprecipitated with VP08R. In order to confirm the interaction between VP08R and VP23R and identify the binding site of VP23R for VP08R, co-IP and reciprocal co-IP assays were performed with VP08R plus a series of truncated VP23R proteins (Fig. 3B to D). The results demonstrated that all the truncated VP23R proteins could be co-precipitated with VP08R, suggesting that the binding site of VP23R for VP08R may be located on the shortest truncated segment, residues 19 to 276 of VP23R. Co-IP assays also showed that VP08R could not bind with the secreted forms of residues 292 to 576 and 576 to 856 of VP23R (Fig. 3E), confirming that VP08R binds to residues 19 to 276 of VP23R. To identify the binding site of VP08R for VP23R, the sequence of the VP08R protein was divided into five fragments, and the signal peptide of VP08R was linked to the N terminus of each fragment to obtain the secreted and fully modified forms (Fig. 3F). Co-IP assays were performed using cells expressing V5-tagged VP23R together with each of the five GFP-tagged fragments of VP08R, and the results showed that the fourth fragment could be coprecipitated with VP23R, suggesting that the binding site of VP08R for VP23R is located between residues 271 and 417 of VP08R (Fig. 3G).

FIG 1 Nucleotide sequence of the VP08R transcript and deduced amino acid sequence of the VP08R protein. The amino acid sequence is represented in one-letter code above the nucleotide sequence, and cysteine residues are marked with bold letters. The transcription start site is marked with a curved arrow, and the previously predicted start codon is shaded. The putative signal peptide is boxed, and the VP23R-binding region is underlined.
n nidogen-1, suggesting that the interaction between VP08R and nidogen-1 could be complicated by multiple binding sites. Furthermore, the binding sites of VP08R for nidogen-1 were determined by co-IP using cells expressing V5-tagged nidogen-1 together with five GFP-tagged fragments of VP08R. All VP08R fragments except the first one were coprecipitated with nidogen-1, suggesting that the binding site of VP08R for nidogen-1 is located between residues 99 and 514 of VP08R (Fig. 4D).

**Self-interaction of VP08R.** Co-IP showed that GFP-tagged VP08R, but not the control GFP, could be coprecipitated with V5-tagged VP08R, suggesting that VP08R may interact with itself (Fig. 5A). Without β-mercaptoethanol in the SDS-loading buffer, the precipitated GFP-tagged VP08R showed a high molecular mass of more than 250 kDa, indicating that VP08R can multimerize through the formation of intermolecular disulfide bonds. We further analyzed the multimerization properties of VP08R in PN-08-transfected FHM cells by SDS-PAGE and Western blotting using SDS-loading buffer with or without β-mercaptoethanol (Fig. 5B). We observed that the GFP-tagged VP08R, but not the control GFP, could form multimers in cells, and β-mercaptoethanol treatment could dissociate the multimerization of VP08R. We further detected the expression of the VP08R protein in ISKNV-infected mandarin fish spleen tissues. The infected tissues were lysed and treated with SDS-loading buffer with or without β-mercaptoethanol and subjected to Western blotting analysis using anti-VP08R antibody. The result demonstrated that the virus-expressed VP08R could also form a multimer which could be disassembled by β-mercaptoethanol treatment (Fig. 5C), confirming that the virus-expressed VP08R protein forms a multimer through intermolecular disulfide bonds in vivo.

To identify the binding sites for VP08R self-interaction, five GFP-tagged VP08R fragments were cotransfected with full-length VP08R into FHM cells and detected by Western blotting using SDS-loading buffer with or without β-mercaptoethanol (Fig. 5D). The results demonstrated that without β-mercaptoethanol treatment, each of the VP08R fragments was involved in the multimers formed by the full-length VP08R protein with a molecular mass of more than 250 kDa, and the interactions could be dissociated by breaking the disulfide bonds with β-mercaptoethanol, suggesting that the binding sites between VP08R molecules are multiple and the intermolecular disulfide bonds can be formed on each fragment. Since a disulfide bond can be formed only between two cysteine residues, these results indicate that VP08R molecules may interact with themselves through multiple intermolecular disulfide bonds between cysteine residues in different regions of VP08R and then several VP08R molecules are cross-linked into a multimer.

To further investigate the properties of the VP08R multimer, ISKNV-infected mandarin fish MFF-1 cells were lysed in cell lysis buffer containing protease inhibitor cocktail and stored at 4°C for 0, 0.5, 1, and 2 h, followed by treatment with SDS sample buffer without β-mercaptoethanol. Western blotting was then performed to detect the virus-expressed VP08R protein (Fig. 5E, left). At 0 h after cell lysis, the virus-expressed VP08R protein also showed a high-molecular-mass band, confirming that VP08R can form multimers. Interestingly, we found that VP08R multimers could be quite easily degraded after the infected cells were lysed. In the infected cell lysates, although the control β-actin protein could not be degraded, the virus-expressed VP08R multimer was disassociated into monomers within 0.5 h after storage at 4°C and the VP08R monomer protein was further degraded even in the

forms of the GFP-tagged G1, G2, and G3 domains of nidogen-1 were analyzed by co-IP assays. All the domains of nidogen-1 exhibited affinity for VP08R and could be coprecipitated with V5-tagged VP08R (Fig. 4B). To further confirm the interactions, reciprocal co-IP (Fig. 4C) experiments were performed using cells expressing GFP-tagged VP08R together with the V5-tagged G1, G2, and G3 domains of nidogen-1, and the results showed that VP08R could be coprecipitated with all the globular domains of

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**FIG 2** Immunofluorescence analysis of spleens from ISKNV-infected mandarin fish. (A) Mandarin fish spleens were sampled at 1, 2, 3, 4, 5, and 6 dpi, embedded in paraffin, cut into sections, and analyzed by immunofluorescence analysis using rabbit anti-MCP antibody and mouse anti-VP08R antibody. The infected cells were labeled with Alexa Fluor 488 for VP08R detection (green fluorescence), and the ISKNV particles were labeled with Alexa Fluor 633 for MCP detection (red fluorescence). White arrows, a large number of released virions. Bars, 20 μm. (B) At 2 and 3 dpi, sections were treated with Hoechst 33258 for nuclear staining. White arrows, nuclei of infected cells; white arrowheads, replicated virus DNA that was stained with Hoechst 33258. Bars, 10 μm.
presence of protease inhibitor cocktail, suggesting that the VP08R multimer structure is unstable when the ISKNV-infected cells are lysed. In contrast, the recombinant VP08R multimer in the plasmid-transfected MFF-1 cell lysates demonstrated good stability and could not be easily disassociated and degraded (Fig. 5E, right). During the degradation of virus-expressed VP08R, the multimer was initially disassembled, indicating that the break of the intermolecular disulfide bonds and disassembly of the multimer could be the first step of VP08R degradation. Therefore, we added 0.5 mM GSSG, a disulfide derived from two short peptides (with each peptide consisting of γ-Glu–Cys–Gly) (36), into the lysis buffer to protect the intermolecular disulfide bonds of the VP08R multimer. The cell lysates stored at 4°C for 0, 1, 2, and 4 h were detected by Western blotting using anti-VP08R antibody. We observed that GSSG could stabilize the ISKNV-expressed VP08R multimer in lysed cells (Fig. 5F).

**FIG 3 Interaction between VP08R and VP23R.** Co-IP was performed on plasmid-cotransfected FHM cells using anti-V5 antibody. GFP-tagged proteins in the co-IP samples and 1/30 of the input cell lysates were detected by Western blotting using rabbit anti-GFP antibody. (A) GFP-tagged VP23R (200 kDa; black arrow) can be coprecipitated with V5-tagged VP08R, whereas the control GFP (29 kDa; black arrow) cannot be coprecipitated. (B) The GFP-tagged VP23R truncation mutants consisting of residues 1 to 818 (200 kDa; black arrow), 1 to 602 (120 kDa; black arrow), and 1 to 291 (60 kDa; black arrow) of VP23R can be coprecipitated with V5-tagged VP08R. Black arrowhead, a possible degradation product of GFP-tagged VP23R. (C) The VP08R fragments. (D) Reciprocal co-IP shows that the GFP-tagged VP08R (110 kDa, consistent with a potential posttranslational modification) can be coprecipitated with VP23R truncation mutants 1 to 818, 1 to 602, and 1 to 291 of VP23R. Black arrow, the control GFP (29 kDa).
DISCUSSION
Like other members of the Megalocytivirus genus, ISKNV-infected cells in tissues are characterized by marked hypertrophy and are much larger than healthy cells (12). As a place for virus assembly and replication, the enlarged cells could be a sitting target for immune attack from the host, and megalocytiviruses could be urged to take corresponding measures to avoid attacks from immune cells, such as killer T cells, natural killer cells, and macrophages. The infected cells are attached and enclosed by LECs, which may segregate the infected cells from the host immune system, and this unique phenomenon has been speculated to be a unique immune evasion strategy adopted by megalocytiviruses to effectively shield the infected cells from immune attacks before virions mature (14). To provide attaching sites on the membrane of the infected cells for LECs, megalocytiviruses developed the VMBM structure, which mimics the functions of true BMs.

Besides the fundamental structure consisting of a layer of laminin polymer, a layer of the collagen IV network, and the nidogen-1 protein, BMs also contain more than 50 other known components that form a complex structure (19). In contrast, the structure of VMBM formed by megalocytiviruses is simple, and its organization and formation processes may follow the laws of parsimony, simplicity, and minimum work. For example, in BMs, laminin-1 is a large (~800-kDa) cross-shaped molecule consisting of three different chains (α1, β1, and γ1) which interact with each other to form a laminin network on the cell surface by binding to the cell receptors (e.g., integrins and dystroglycans) (37–40). The short arm of the γ1 chain extends out of the laminin...
network layer to bind nidogen-1 protein, which also links the collagen IV network to form the basic structure of BMs (41–43). In VMBM, the structure is simple: ISKNV encodes the membrane protein VP23R, which contains a region homologous to the laminin γ1 chain II2-6 that mimics the γ1 short arm that extends out of the laminin polymer layer to bind nidogen-1, and the plasma membrane of the infected cells is actually utilized to play the role of the laminin network layer and its cell receptors (14, 41). As an important component of the BM, nidogen-1 is also present in VMBM and could provide attaching sites for LECs (14). VMBM does not contain a collagen IV network, which is essential to maintenance of BM integrity and functions (30). As collagen IV is a large molecule and the collagen IV network is much more complicated in structure, it could be difficult for megalocytivirus to recruit collagen IV molecules to form a network in VMBMs. However, the question is, how can VMBM keep its integrity and stability without the collagen IV network? In the present study, we identified the VP08R protein and proved that VP08R can interact with both VP23R and nidogen-1. VP08R contains a signal peptide and the nidogen-1-binding region homologous to laminin γ1 chain II2-6 (14). So, VP08R, VP23R, and nidogen-1 could form a stable trimer structure, which may contribute to the stability of the VMBM structure. Moreover, VP08R could self-assemble to a multimer through the formation of intermolecular disulfide bonds between different regions of the VP08R sequence. There are 34 cysteine residues in the VP08R sequence, suggesting that a number of inter- or intramolecular disulfide bonds could be formed. As a covalent bond, the disulfide bond has a force much stronger than that of noncovalent interactions between proteins, such as antibodies with antigens and ligands with receptors. So, the VP08R multimer could be a very stable structure which may play a role in the maintenance of VMBM integrity and stability and thus may replace some of the functions of the complex collagen IV network.

We have observed that in ISKNV-infected mandarin fish tissues, when the virions are mature, the infected cells that were enclosed by LECs could be uncovered, leading to the release of virions from the cells (14) (Fig. 5G and H). These processes could involve the disassembly of VMBMs (at least partially) and the departure of LECs. Interestingly, in this study, we observed that the VP08R multimer was easily degraded in the lysed ISKNV-infected cells. The underlying mechanism is worthy of in-depth study. In the virus life cycle, the ease of degradation of the VP08R multimer may have its own biological significance to disassemble the VMBM structure on the plasma membrane of the infected cell when the virions are mature and it is necessary to free LECs and release the virions. Since the presence of the VP08R multimer could make VMBM a stable structure, the degradation of VP08R multimers could be important for VMBM disassembly. Interestingly, although the VP08R multimer was easily degraded in the lysed ISKNV-infected cells, it was stable in the plasmid-expressed cells. We also observed that during the degradation process, the virus-expressed VP08R multimer was initially disassembled and GSSG could protect the multimer from disassembly, indicating that the break of the intermolecular disulfide bonds of the multimer could be essential for VP08R degradation. Based on these phenomena, we speculate that ISKNV could produce unknown factors that can disassociate the VP08R multimer and degrade the VP08R protein. When the virions are mature or the infected cells are lysed, these factors could be released and act on the VP08R multimer. Thus, the degradation of the VP08R multimer as well as the disassembly of the VMBM structure could be a mechanism for virus release. Further studies should be performed to confirm this speculation.

The interactions between VP23R, nidogen-1, and the VP08R network make VMBM a stable structure. Based on the findings of this study, we have redrawn the putative schematic illustration of the VMBM structure (Fig. 7). By encoding VP08R, the virus could develop a mechanism to maintain the integrity and stability of VMBM, and the function of VP08R may be to replace some of the functions of the complex collagen IV network in true BMs. It may be a smart, effective, and economical strategy for megalocytiviruses to perfect the structure of VMBM and the mechanism of LEC attachment on infected cells. The precise structure of the

![Image](http://jvi.asm.org)
FIG 7 Putative schematic of VMBM and the possible structure of VP08R multimers. Purple symbols, nidogen-1 proteins; red squares and lines, VP08R proteins; hexagons, virions; EGF Lam, the laminin-type epidermal growth factor-like domain in the VP23R protein; green lines, each with five EGF Lam domains, VP23R proteins.

VP08R multimer and its interactions with VP23R, nidogen-1, and lymphatic endothelial cells need further study.

The structural proteins of purified ISKNV have been systematically and comprehensively identified and analyzed (44). Neither VP23R nor the novel identified VP08R was present in purified virions, suggesting that they could be nonstructural viral proteins and the VMBM structure may not be involved in virion assembly. Electron microscopy also demonstrated that the virions are not present in the attached LECs (14), indicating that there may be a mechanism that prevents the virus in the infected cells from entering the attached LECs through cell-to-cell connections, which needs further evidence. As the target cells of *Megalocytivirus* infection have not been identified, the interactions between infected cells, LECs, and VMBMs during virus infection are worthy of further investigation. It could also facilitate studies on the pathogenic mechanisms of megalocytiviruses, BM functions, and mechanisms of lymphangiogenesis.

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