VP23R of infectious spleen and kidney necrosis virus mediates formation of virus-mock basement membrane to provide attaching sites for lymphatic endothelial cells

Running title: Functions of ISKNV VP23R Gene.

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ABSTRACT: Putative open reading frames (ORFs) encoding laminin-like proteins are found in all members of the genus *Megalocytivirus*, family Iridoviridae. This is the first study that identified the VP23R protein encoded by ORF23R of the infectious spleen and kidney necrosis virus (ISKNV), a member of these genes of megalocytiviruses. The VP23R mRNA covering the ISKNV genomic coordinate 19547–22273, was transcribed ahead of major capsid protein (MCP). Immunofluorescence analysis demonstrated that VP23R was expressed on the plasma membrane of the ISKNV-infected cells and could not be a viral envelope protein. Residues 292–576 of VP23R are homologous to the laminin γ1III2-6 fragment, which covers the nidogen-binding site. Immunoprecipitation assay showed that VP23R could interact with nidogen-1, and immunohistochemistry showed that nidogen-1 was localized on the outer membrane of the infected cells. Electron microscopy showed that a virus-mock basement membrane (VMBM) was formed on the surface of the infected cells and a layer of endothelial cells (ECs) was attached to VMBM. VMBM contained VP23R and nidogen-1 but not collagen IV. The attached ECs were identified as lymphatic endothelial cells (LECs), which have unique feature of overlapping intercellular junctions and can be stained by immunohistochemistry using an antibody against a specific lymphatic marker, Prox-1. Such infection signs have never been described in viruses. Elucidating the functions of LECs attached to the surface of the infected cells may be useful for studies on the pathogenic mechanisms of megalocytiviruses, and may also be important for studies on lymphangiogenesis and basement membrane functions.

KEYWORDS: ISKNV; VP23R; laminin; nidogen; Virus-mock basement membrane; lymphatic endothelial cell
INTRODUCTION

Basement membrane (BM), a dense and sheet-like structure that is always associated with cells, is a very important specialized form of extracellular matrix (ECM) (31, 67). BMs mediate tissue compartmentalization and provide structural support to the epithelium, endothelium, peripheral nerve axons, fat cells, and muscle cells, as well as structural and functional foundations of the vasculature (25, 31, 52). BM is also an important regulator of cell behaviors, such as adhesion, migration, proliferation, and differentiation. BMs are highly cross-linked and insoluble materials. They are high complex and are made up of more than 50 known components (31, 54). Although the molecular composition of BMs is unique in each tissue, their basic structures are similar. Even if many more isoforms exist in different species, the major BM proteins and their receptors are conserved from Caenorhabditis elegans to mammals. BM consists of a layer of laminin polymer, a layer of type IV collagen network, and the nidogen protein, which acts as a cross-linker of these two networks. Other BM components, such as perlecan and fibulin, interact with the laminin polymer and the type IV collagen network to organize a functional BM on the basolateral aspect of the cells (31, 52).

The components of BM are able to self-assemble and form a sheet-like structure, and laminin is the key molecule in this process (50). Laminin protein consists of three different chains (α, β, and γ), which comprise a cross-shaped molecular structure with three short amino-terminal arms and a long carboxyl-terminal triple-helical arm (58, 68). The three short arms of this cross-shaped structure can interact with each other in the presence of calcium. Through the binding of globular G domain at the carboxyl-terminal end of the α chain to the cell receptors (e.g., integrins and dystroglycans), laminin self-assembles into polygonal
lattices on cell surfaces. This process initiates BM self-assembly (15, 21, 25, 38, 65, 66). To date, 17 laminin isoforms have been observed in different tissues (51). Among them, laminin-1, the crux of early embryonic BM assembly, has been well-studied. Laminin-1 consists of α1, β1, and γ1 chains and can interact with nidogen-1 with high affinity through a laminin-type epidermal growth factor-like (LE) module, γ1III4, within the domain III of the γ1 chain (1, 42). The heptapeptide “NIDPNAV” within the γ1III4 motif of laminin-1 is essential for the interaction between laminin-1 and nidogen-1 (41, 46). Blocking the interactions between laminin-1 and nidogen-1 leads to the disruption of BMs. This indicates that the formation of laminin/nidogen complex is essential for BM assembly and stability (30, 61). Nidogen-1, also called entactin-1, is a dumbbell-shaped sulfated 150 kDa glycoprotein consisted of three domains (G1, G2, and G3) (12). By interacting with collagen IV through its G2 domain and binding with laminin γ1 chain through its G3 domain, nidogen-1 bridges the layers of the laminin network and the collagen IV network to construct the fundamental structure of BMs (48). Collagen IV is a triple-helical trimer composed of three α chains. Through the hexamer formation of the carboxyl-terminal globular non-collagenous (NC)-1 domain of each α chain, two collagen IV proteins assemble into a dimer. Dimers of collagen IV connect with each other via their amino-terminal 7S domains and self-assemble into a network (27, 31, 32). Six kinds of α chains of collagen IV have been identified in mammals. Among them, α1 and α2 chains are most abundant forms of collagen IV found in all basement membranes (BM) (19, 23). They commonly form a collagen IV molecule with a α1 and α2 ratio of 2:1 (31, 35).

Iridoviruses infect invertebrates and poikilothermic vertebrates, including insects, fish,
amphibians, and reptiles. They are a group of icosahedral cytoplasmic DNA viruses with circularly permuted and terminally redundant DNA genomes (8, 9, 10, 57, 62). The family Iridoviridae has been subdivided into five genera: Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus, and Megalocystivirus (7). The genus Megalocystivirus, characterized by the ability to cause swelling of the infected cells, is one group of the most harmful viruses to cultured fish (7, 26, 29). Infectious spleen and kidney necrosis virus (ISKNV), the causative agent of a disease that causes high mortality rates in farmed mandarin fish, Siniperca chuatsi, and large-mouth bass, Micropterus salmoides, is regarded as the type species of Megalocystivirus (7). Similar to infection caused by other members of Megalocystivirus, fish ISKNV infection is characterized by cell hypertrophy in the spleen, kidney, cranial connective tissue, and endocardium (16, 17). Aside from mandarin fish and large-mouth bass, ISKNV-like virus can also be detected in the tissues of more than 60 marine and freshwater fishes (14, 28, 59, 64). The entire genome of ISKNV has been sequenced, and the organization of open reading frames (ORFs) of ISKNV was analyzed by using DNASTAR Omiga 2.0 and Genescan (18). The ISKNV genome is about 110 kbp and contains 125 putative ORFs (GenBank accession no. AF371960).

Putative ORFs, encoding viral proteins containing a fragment homologous to laminin and a putative transmembrane fragment, were found in all the sequenced genomes of the members of Megalocystivirus. These ORFs include ORF23R of ISKNV (GenBank accession no. AAL98747), laminin-like protein gene of olive flounder iridovirus (OFIV) (GenBank accession no. AAT76907), ORF-2 of sea perch iridovirus (SPIV) (GenBank accession no. AAV51313), predicted laminin-type epidermal growth factor-like protein of large yellow
croaker iridovirus (LYCIV) (GenBank accession no. ABI32391), unknown gene of red sea bream iridovirus (RSIV) (GenBank accession no. AAQ07956), ORF-2 of rock bream iridovirus (RBIV) (GenBank accession no. AAN86692), and laminin-type epidermal growth factor-like protein of orange-spotted grouper iridovirus (OSGIV) (GenBank accession no. AAX82335). These putative proteins are highly homologous to each other in amino acid sequence (65%-99% identity). However, functions of these proteins have never been identified. This is the first study to identify that the VP23R protein encoded by ORF23R of ISKNV is a plasma membrane-localized viral protein. In addition, we discovered a new function of VP23R in a unique pathological phenomenon of virus infection – the attachment of lymphatic endothelial cells (LECs) to the infected cells. Nidogen-1 assisted VP23R in the construction of a BM-like structure, providing an attachment site for LECs. This unique pathological phenomenon has never been found in viruses, and is an attractive direction for studies of pathogenic mechanisms of megalocystiviruses. Moreover, studies on the unique profiles of the virus-mock BM can help us learn more about the functions of BM components and the mechanisms of lymphangiogenesis.

MATERIALS AND METHODS

**Fish and virus.** Mandarin fish were from a local fish farm in Guangzhou City, Guangdong Province, China, and were kept in separate tanks at 28°C. Tank water was filtrated through a sand and carbon layer and aerated before use. ISKNV was purified from diseased mandarin fish identified in our laboratory (16) and propagated in cultured mandarin fish fry cell line (MFF-1) (11). The supernatants of the cultured cells at 6 days post infection (dpi) of ISKNV
were collected, and the virus titer was determined using the 50% tissue culture infective dose (TCID$_{50}$) method as described previously (11).

5'- and 3'- RACE. Total RNA was isolated from spleens of ISKNV-infected mandarin fish. The 5' and 3' ends of ORF23R transcript were mapped by using the GeneRacer kit (Invitrogen, USA) according to the manufacturer’s instructions. 5'-Rapid amplification of cDNA ends (RACE)-PCR amplification was performed with GeneRacer 5'-RACE primer and ORF23R specific reverse primer (5'-GCAGTTGCCGCTCAAACACTCTGG-3'). Nested PCR was subsequently performed with GeneRacer 5'-RACE nested primer and ORF23R nested primer (5'-CACTCCATGTTCAGACTTGCTGC-3') by using the first-round PCR product as template. 3'-RACE-PCR was performed by using GeneRacer 3'-RACE primer together with an ORF23R-specific forward primer (5'-GTCATTGGGTCTTGCATGGTTGCCGC-3'). The PCR products were subcloned into the PMD19-T TA vector (TaKaRa, China) and sequenced.

cDNA of mandarin fish BM components. A series of partial cDNA of mandarin fish BM components were cloned, sequenced and submitted to GenBank. Briefly, RNA was extracted from kidneys of healthy mandarin fish by using the TRIzol method, and the first-strand cDNA was synthesized by SuperScript III RT (Invitrogen, USA). The specific forward primer (5'-CAGCTGTAAGCCAGGAGTGA-3') and reverse primer (5'-ACCTTGTCTCTGACCAGGCTGTA-3') were designed based on the known laminin $\gamma_1$-chain coding sequences of green-spotted pufferfish (Tetraodon nigroviridis) and zebrafish (Danio rerio) and used to amplify the laminin $\gamma_1$ fragment of mandarin fish (GenBank accession no. HM153806) by RT-PCR. Primers MNi1
(5’-TACCAGTGGCGTCAGACCATCACCTTCC-3’) and MNi2 (5’-
GCGTCGTTGGGACATCTGCAGG-3’) were designed based on the known human,
zebrafish, and green-spotted pufferfish nidogen-1 G2/G3 domains coding sequences for
amplification of mandarin fish nidogen-1 (GenBank accession no. HM138201). A collagen IV
α1 fragment from mandarin fish (GenBank accession no. HM138202), which is orthologous
to residues 1408–1607 of human collagen IV α1 (GenBank Accession No. CAM14222) was
amplified by using A1-1 (5’-CCGGGCCCTCCTCAATG-3’) and A1-2
(5’-GGTAGCAAGCCAGAAGCTGT-3’) primers. A collagen IV α2 fragment from mandarin
fish (GenBank accession no. HM138203), which is orthologous to residues 1488–1667 of
human collagen IV α2 (NCBI Accession No. NP_001837) was amplified by using A2-1
(5’-ATGCCGGGCCGCAGCGTCAGC-3’) and A2-2
(5’-CAGAGCTGAGCCAGAAGCTGTGCTTGTT-3’) primers. All cDNA fragments were
cloned into PMD-19T vector and sequenced.

**Antibody preparation.** A DNA fragment corresponding to amino acid residues 18–169 of
VP23R was amplified and cloned into the PQE30 vector (Qiagen, USA) (referred to as
PQE30-XF23 vector). The recombinant vector was transformed into the M15 *Escherichia coli*
strain (Qiagen, USA) to express 6xHis-XF23 fusion protein. The protein was purified with
Ni-NTA and separated by electrophoresis in 15% SDS-polyacrylamide gels. The gel slice
containing 6xHis-XF23 band was cut out and ground with adjuvant to immunize BALB/c
mice. Full length ISKNV major capsid protein (MCP) gene (ORF006L) was cloned into the
PRSET-A vector (Invitrogen, USA). Recombinant MCP was expressed in BL21 (DE3) *E. coli*
strain (Novagen, USA), purified, and used as an antigen to immunize rabbits. Mandarin fish
nidogen-1 G3 domain was expressed in PMAL-C2X vector (NEB, UK) in *E. coli* Origami strain (Novagen, USA). The MBP tag was cut out by Factor Xa (NEB, UK), and nidogen-1 G3 fragment was separated by SDS-PAGE. Then, nidogen-1 G3 bands were cut out to immunize BALB/c mice. The collagen IV α1 and α2 fragments were cloned into the PRSET-A vector and expressed in BL21 (DE3), and recombinant proteins were used to immunize BALB/c mice. Rabbit anti-prox-1 polyclonal antibody was purchased from Abcam (UK).

**Immunohistochemistry and immunofluorescence analyses.** Spleens of ISKNV- and PBS mock-infected (as controls) mandarin fish were collected, fixed with 4% paraformaldehyde, paraffin-embedded, and sectioned. The 4 µm sections of tissue samples 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. Non-specific binding was blocked by incubation in 10% normal goat serum.

Sections for immunofluorescence were incubated with rabbit 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 goat anti-rabbit secondary antibody (Invitrogen, USA) for 30 min. VP23R was detected using mouse anti-VP23R polyclonal antibody, followed by Alexa Fluor 488-conjugated secondary goat anti-mouse antibodies (Invitrogen, USA). Double-stained sections were observed using a Leica LSM 410 confocal microscope (Germany) at 633 nm for Alexa Fluor 633 and 488 nm for Alexa Fluor 488.

Nidogen-1, prox-1, collagen IV α1 and α2 chains were detected with their specific
antibodies by immunohistochemistry. Sections were pretreated using the antigen retrieval-pepsin method. After incubation with specific antibodies at 37°C for 1 h, Horseradish Peroxidase (HRP)-conjugated secondary antibodies (Sigma, USA) were added to the sections, incubated with 3-amino-9-ethylcarbazole (AEC) and counter-stained with hematoxylin for microscopic examination. Sections were observed using a Zeiss Axioskop 40 microscope (Germany).

**Co-immunoprecipitation.** As a negative control, the heptapeptide “NIDDNPV” deletion mutant of VP23R was constructed by overlapping PCR, generating VPΔ23 mutant. As a positive control, the laminin γ1-homologous sequence of VP23R was displaced with the mandarin fish laminin γ1III2-6 fragment, generating a mutant termed VP23LN. The 1-1086 nucleotide sequences of VP23R, VPΔ23, and VP23LN were cloned into PEGFP-N3 (Clontech, USA) and PCDNA 3.1/V5-His A (Invitrogen, USA), generating PN-23/PN-Δ23/PC-23LN and PC-23/PC-Δ23/PC-23LN, which express GFP- and V5-tagged proteins, respectively. The full length *T. nigroviridis* nidogen-1 (GenBank accession no. HM138204) was cloned into PEGFP-N3 and PCDNA 3.1/V5-His A, generating PN-Ni and PC-Ni that express GFP- and V5-tagged nidogen-1, respectively. For co-immunoprecipitation, PC-Ni was co-transfected with PN-23, PN-Δ23, or PN-23LN into fathead minnow (FHM) fish cells. For reciprocal co-immunoprecipitation, PC-23, PC-Δ23, or PC-23LN was co-transfected with PN-Ni into FHM cells. After 72 h, cells were collected and lysed. Co-immunoprecipitation and reciprocal co-immunoprecipitation were performed using anti-V5 Agarose Affinity Gel (Sigma, USA). Western blotting was performed using rabbit anti-GFP antibody (Sigma, USA) and alkaline phosphatase (AP)-conjugated goat anti-rabbit
secondary antibodies (Sigma, USA).

Co-immunoprecipitation assays were also performed with the ISKNV-infected spleens of mandarin fish. Briefly, spleens from the ISKNV-infected mandarin fish at 5 dpi were homogenized and lysed. co-immunoprecipitation was performed using 5 µl anti-VP23R mouse polyclonal antibodies and 50 µl of a suspension of protein A/G-Sepharose (Santa Cruz, USA). Western blotting was performed using anti-nidogen-1 rabbit polyclonal antibodies.

Electron microscopy analysis. Spleens of the ISKNV-infected mandarin fish were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Specimens were then rinsed with 0.1 M phosphate buffer 4 times, post-fixed in 0.1 M phosphate buffer containing 2.0% osmium tetroxide for 1 h at 4°C, and embedded in Epon's 812 after dehydration. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Then, they were examined on a Philips CM10 electron microscope.

RESULTS

Structural and bioinformatics analyses of ORF23R. 5'-RACE showed that the transcription starting site of ORF23R was at the ISKNV genomic coordinate 19547, which extended only 14 bp upstream of ORF23R. 3'-RACE showed that the ORF23R mRNA contained a typical poly(A) tail and the transcription ended at 22273, extended 140 bp downstream of ORF23R. A computer-assisted analysis of the ISKNV genome indicated that ORF23R began at nucleotide 19562 and terminated at nucleotide 22132, which encoded a putative protein of 856 amino acids with an apparent molecular mass of 90.7 kDa and an isoelectric point of 4.8. The protein, designated as VP23R, was predicted to have a 17-residue signal peptide and a potential transmembrane domain localized between residues 818–840.
Eight N-glycosylation and six O-glycosylation sites were predicted in the entire protein sequence. Therefore, VP23R protein may retain characteristics of a membrane protein, presenting a large N-terminal ectodomain, a C-terminal hydrophobic anchor, and a short C-terminal cytoplasmic tail with only 16 residues (Fig. 1A).

An initial BLAST search showed that residues 292–576 of VP23R is homologous to the γ1III2-6 LE motifs of laminin γ1 chain. We have cloned the mandarin fish laminin γ1 chain fragment, including the γ1III2-6 motifs. Sequence comparison shows that there is 44.6% identity in amino acid sequence between mandarin fish laminin γ1III2-6 fragment and VP23R residues 292-576. This region (residues 292-576) of VP23R also shows 43.5%, 41.3%, 43.8% and 44.2% identity to the laminin γ1III2-6 fragments of *T. nigroviridis* (GenBank accession no. CAD27803), *Danio rerio* (GenBank accession no. CAK05288), *Homo sapiens* (GenBank accession no. AAA59492), and *Mus musculus* (GenBank accession no. AAA39405), respectively (Fig. 1B). The γ1III4 motif of laminin γ1 chain is the binding site of nidogen-1. The heptapeptide NIDPNAV within γ1III4 is essential for the interactions between laminin and nidogen-1 (41, 46). In VP23R, a homologous heptapeptide NIDDNPV was also found, and only two residues were different.

**VP23R is localized on plasma membrane.** In ISKNV-infected cultured MFF-1 cells, VP23R mRNA began to transcribe at 8 hours post infection (hpi), prior to transcription of MCP, which began at 36 hpi (data not shown). To identify the cellular location of VP23R protein, a series of mandarin fish spleen sections were prepared at 1, 2, 3, 4, 5, and 6 dpi (Fig.2). Mice anti-VP23R polyclonal antibody, together with rabbit anti-MCP polyclonal antibody, was used to perform immunofluorescence assays on these sections. As ISKNV is a typical cytoplasmic
DNA virus and MCP of ISKNV is expressed in the cytoplasm (7). MCP staining can provide a cytoplasmic counter-staining for VP23R localization. The MCP signals (red fluorescence) were surrounded by the green fluorescence of VP23R signals (Fig. 2 C, D), suggesting that VP23R is localized on the plasma membrane of the infected cells. VP23R began to appear on the membrane of the infected cells at 1 dpi (Fig. 2 A), and MCP began to express inside the infected cells at 3 dpi (Fig. 2 C). At 5 dpi, MCP began to release out of the infected cells (Fig. 2E, white arrow). At 6 dpi, almost all MCP was found outside of the infected cells (Fig. 2F, white arrows). On the contrary, VP23R protein appeared on the membrane during the whole infection process.

**Interaction between VP23R and nidogen-1.** Immunoprecipitation assays showed that VP23R and its laminin-replaced mutant exhibited affinity to nidogen-1 and could be co-precipitated by the V5-tagged nidogen-1, while the “NIDDNPV” deletion mutant of VP23R was not co-precipitated with nidogen-1 (Fig. 3A). Reciprocal co-immunoprecipitation showed that GFP-tagged nidogen-1 was also co-precipitated with V5-tagged VP23R and its laminin-replaced mutant but not with its “NIDDNPV” deletion mutant (Fig. 3B). To further confirm the interaction between VP23R and nidogen-1, immunoprecipitation was also performed in the spleen of the virus-infected cells (Fig. 3C). The result showed that nidogen-1 in the spleen of ISKNV-infected mandarin fish was co-precipitated with anti-VP23R polyclonal antibody, indicating that VP23R can interact with nidogen-1 in vivo.

Immunohistochemistry of the virus-infected spleens showed that nidogen-1 was present on the outer-membrane of the ISKNV-infected cells (Fig. 4A), which were characterized by marked hypertrophy (16, 17). VP23R was expressed on the plasma membrane of the infected
cells. This result confirmed that nidogen-1 interacts with VP23R in the ISKNV-infected
tissues. Nidogen-1 proteins on the surface of infected cells may be recruited by VP23R.
Nidogen-1 is also a ligand for collagen IV. However, no collagen IV was detected on the
membrane of ISKNV-infected cells by anti-collagen IV α1 and α2 chain antibodies (Fig.4C,
E).

Interaction of lymphatic endothelial cells with virus infected cells. Under the Electron
microscope, almost all the ISKNV-infected cells were found to be attached with layers of flat
cells (Fig. 5). The flat cells were identified by electron microscopy as endothelial cells (ECs).
A low-electron-dense structure of 40-50nm thickness existed between the plasma-membrane
of the infected cells and the surrounding of ECs (Fig. 5F, black arrowheads), which are
always attached to the BMs. This result showed that VP23R and nidogen-1 may provide an
attaching site for ECs. The 40–50 nm thick structures outside of the infected cells did not
show an electron-dense zone, which is present in true BMs and consists of collagen IV
network (20). This confirmed that collagen IV is not present outside the membrane of the
infected cells. These 40–50 nm thick structures, composed of VP23R and nidogen-1, did not
contain collagen IV α1 and α2 chains, and worked in the same manner as true BMs to provide
attaching sites for ECs, were termed virus-mock basement membranes (VMBM). Fig. 5G and
5H showed an infected cell releasing endocytes and mature virions.

The attached ECs showed overlapping intercellular junctions (Fig. 5A-C, black arrows),
which were regarded as the unique profile of LECs (39, 43, 63). Thus the attached ECs can be
identified as LECs. The result was confirmed by immunohistochemical assay using antibody
against Prox-1 protein, a specific marker for LECs (13, 44). As a LEC-specific transcription
DISCUSSION

The predicted ORF23R, located in 19562-22132 of the ISKNV genome, encodes an 856-residue protein, termed VP23R. The putative transmembrane region near the carboxyl-terminus and the putative signal peptide at the amino-terminal end make VP23R a cell plasma membrane-localized protein. The putative extracellular region of VP23R is homologous to the γ1III2-6 fragment of laminin-1 that contains the high affinity nidogen-binding site of laminin, indicating that VP23R may have functions related to BM. This is the first study to identify VP23R protein and analyze its functions in mediating formation of BM-like structures on the surface of ISKNV-infected cells, providing a site for LEC adhesion.

The double-stain immunofluorescence assays showed that VP23R protein was expressed earlier than MCP in the spleens of infected mandarin fish. VP23R was localized on the plasma membrane of the infected cells. The location of VP23R did not coincide with MCP, which represents virus particles. During the process of ISKNV infection, VP23R was constantly localized on the plasma membrane as it was detected on the plasma membrane even after viral particles were released outside the cells. Based on these results, we infer that VP23R is a plasma membrane-localized viral protein, but it cannot be an envelope protein of ISKNV. This result also disproved previous assumption that accumulation of viral particles results in megalocytosis of the infected cells (7), because the infected cells enlarged without appearance of MCP.

Residues 292–576 of VP23R are homologous to the γ1 chain III2-6 motifs of mandarin factor, Prox-1 was specifically stained in the attached LECs (Fig. 4G).
fish laminin with 43% identity, which contain the high affinity nidogen-binding site. By co-immunoprecipitation, we verified that VP23R could interact with nidogen-1. The heptapeptide “NIDPNAV” within the γ1 chain III4 motif of laminin is crucial for interaction of laminin γ1 with nidogen-1 (41, 46). In VP23R, the heptapeptide “NIDPNAV” was substituted by “NIDDNPV”, in which only two amino acids were changed, and this alteration did not affect the interaction between VP23R and nidogen-1. Deletion of the heptapeptide “NIDDNPV” resulted in a loss of VP23R binding ability to nidogen-1. This result indicates that the heptapeptide is essential for the interaction between VP23R and nidogen-1.

Immunohistochemistry assay showed that nidogen-1 was present on cell membrane of ISKNV-infected cells. Electron microscopy showed that VP23R along with nidogen-1 formed a low electron-density BM-like structure (VMBM), to which a layer of LECs were attached. Fig. 6 shows the schematic illustration of VMBM. In VMBM, the plasma membrane of the infected cells take the roles of the laminin polymer layer of true BMs, which always binds to cell receptors and initiates BM self-assembly. The laminin γ1 chain III2-6 homologous region of VP23R mimics the γ1 short arms that extend out of the laminin polymer layer to bind nidogen-1 (Fig 6). In true BMs, nidogen-1 bridges the laminin polymer and type IV collagen network. The collagen IV network showed an electron-dense layer in true BM (20). However, under the electron microscope, no electron-dense zone appeared in VMBM. In true BMs, collagen IV α1 and α2 chains are most abundant forms of collagen IV and expressed in all BMs (19, 23). Immunohistochemical analysis showed that collagen IV protein was absent in the VMBM. Because of the absence of collagen IV, VMBM is only 40–50 nm thick, about half of that of true BMs.
Collagen IV is essential to the maintenance of BM integrity and functions. Collagen IV-absent BMs are found in small amounts in newly formed endothelial cells sprouts during the early steps of angiogenesis (49). 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 death of mouse embryo at the stages E10.5–E11.5 (47). Mutants of collagen IV in the BMs cause the Goltz syndrome (3, 37). However, absence of collagen IV network does not affect BMs self-assembly (2), and in true BMs, collagen IV networks have not been implicated in association with cellular receptors (50). Thus, absence of collagen IV beneath the attached LECs may not affect the VMBM function to provide attaching sites for LECs. Nidogen-1 plays a role in cell attachment in true BMs (5, 34, 53). In VMBM, nidogen-1 protein recruited by VP23R can provide the binding site for LECs. As other components in VMBM will be identified in further studies, more attaching sites will also be discovered.

Based on the overlapping intercellular junctions (39, 43, 63), we identified the attached ECs as LECs. This result was confirmed by immunohistochemistry using specific antibody against Prox-1 (13, 44). The formation of VP23R-mimicked BMs and the attachment of LECs on the infected cells are unique phenomena that have never been found in viruses. Attached LECs enclose the infected cells like a bag. The functions of these infection signs need further studies. Under an electron microscope, an infected cell was observed to release endocytes and mature virions (Fig. 5 G and H). One of the LECs uncovered the enclosed bag and allowed virions and endocytes to be released. The immunofluorescence assay at 6 dpi also demonstrated that the plasma membrane of the infected cells was still in intact, even after all the virions were released. The signals of VP23R also remained strong. Based on these results,
we speculate that attachment of LECs can segregate the infected cells from the host immune system. By encoding a laminin-like membrane protein VP23R, ISKNV generates a BM-like structure on the infected cells to house LECs, building a “camouflaged bunker” against the attack of host immune cells. This phenomenon is a unique strategy of virus to effectively shield from immune attacks. Further studies should be performed to confirm this hypothesis.

The process of LECs attaching to the infected cells may be similar to that of lymphangiogenesis, especially in pathological conditions, such as tumor metastasis, inflammation, and transplant rejection that involve migration and proliferation of ECs from preexisting vessels and recruitment and differentiation of bone marrow endothelial progenitor cells (33, 40, 56, 69). Although the precise molecular mechanisms that regulate lymphangiogenesis remain largely unknown, it is clear that some members of the vascular endothelial growth factor (VEGF)/platelet-derived growth factor (PDGF) family, such as VEGF-A, PDGF-BB, and particularly VEGF-C and VEGF-D, play important roles in this process (4, 22, 55, 56). Interestingly, it has been reported that the ISKNV genome contains an ORF48R gene, which belongs to the VEGF/PDGF family and functions through the VEGF receptor, Flk-1 (60). Association of ORF48R with the origination of the attached LECs is worth of further studies.

Studies on VP23R functions elucidate the unique infection signs of megalocystiviruses. Functions of VP23R in attaching LECs to the surface of infected cells through VMBM can help us learn more about the pathogenetic mechanism of megalocystiviruses. This is essential for *Megalocystivirus* prevention and cure. Furthermore, in view of the unique profile of VMBM and unique behaviors of the attached LECs, many questions remain to be answered:
Are there any other components in VMBM and if so, how do they function? Why did LECs, but not vascular ECs, appear on the surface of the infected cells? Where do the attached LECs come from? Further studies are necessary in order to answer these questions. The results are also important for studies of BM functions and lymphangiogenesis mechanisms.

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REFERENCES


FIGURE LEGENDS:

FIG. 1. (A) Nucleotide sequence of VP23R transcript and deduced amino acid sequence of VP23R protein. Amino acid sequence is represented with one-letter codes above the nucleotide sequence. The laminin-γ1-homologous domain is framed with red lines. The putative signal peptide and transmembrane fragment are framed with green and blue lines, respectively. (B) Sequence alignment of VP23R with the laminin γ1 III2-6 fragments of mandarin fish, *Siniperca chuatsi* (GenBank accession no. HM153806), *T. nigroviridis* (GenBank accession no. CAD27803), *Danio rerio* (GenBank accession no. CAK05288), *Homo sapiens* (GenBank accession no. AAA59492), and *Mus musculus* (GenBank accession no. AAA39405). Identical residues in at least two sequences are shaded and the comparison of the heptapeptide “NIDDNPV” in VP23R and “NIDPNAV” in laminin γ1 are framed.

FIG. 2. Immunofluorescence analysis of spleens from ISKNV-infected mandarin fish. The infected cells are labeled with Alexa Fluor 488 for VP23R (green fluorescence) and the ISKNV particles are labeled with Alexa Fluor 633 for MCP (red fluorescence, white arrows). Spleens were from different times post-infection: (A) 1 dpi; (B) 2dpi; (C) 3dpi; (D) 4dpi; (E) 5dpi; (F) 6dpi. White arrows: the released virions.

FIG. 3. (A) Co-immunoprecipitation assays in FHM cells. GFP-tagged VP23R (VP23R-GFP) (Lane 1) and its laminin-replaced mutant (VP23LN-GFP) (Lane 2) could be co-precipitated by the V5-tagged nidogen-1 (nidogen-V5), whereas the GFP-tagged “NIDDNPV” deletion mutant of VP23R (VPA23-GFP) could not be co-precipitated (Lane 3). The GFP-fusion proteins in the lysates of FHM cells transfected with PC-Ni along with PN-∆23 (Lane 4), PN-23 (Lane 5) or PN-23LN (Lane 6) were detected by using anti-GFP antibody, whereas the
lysates of the untransfected FHM cells showed no positive signals (Lane 7). (B) Reciprocal co-immunoprecipitation showed that GFP-tagged nidogen-1 (nidogen-GFP) was co-precipitated by the V5-tagged VP23R (VP23R-V5) (Lane 1) and its laminin-replaced mutant (VP23LN-V5) (Lane 2), but was not co-precipitated by the V5-tagged “NIDDNPV” deletion mutant of VP23R (VPΔ23-V5) (Lane 3). The nidogen-GFP fusion protein in the lysates of FHM cells transfected with PN-Ni along with PC-23 (Lane 4), PC-23LN (Lane 5), or PC-Δ23 (Lane 6) could be detected by using anti-GFP antibody, whereas the lysates of the untransfected FHM cells showed no positive signals (Lane 7). (C) Immunoprecipitation assays performed in the ISKNV infected mandarin fish spleens. Nidogen-1 (~150kDa) was co-precipitated by anti-VP23R antibodies (Lane 2, black arrow) but not by control sera (Lane 1).

**FIG. 4.** Immunohistochemical assays of ISKNV- and mock-infected (as controls) mandarin fish spleens (magnification×400). In ISKNV-infected spleens (A, C, E, G), the infected cells showed signs of hypertrophy (red arrows). No enlarged infected cells were observed in mock-infected spleens (B, D, F, H). Nidogen-1 was detected on the plasma-membrane of the enlarged ISKNV-infected cells (A and Inset, red arrows) and on true BMs (A, B, black arrows). The α1 and α2 chains of collagen IV were detected in true BMs (C, D, E, F, black arrows), but not on the plasma-membrane of the enlarged ISKNV-infected cells (C and E, red arrows). (G) Attached ECs on the plasma-membrane of the enlarged ISKNV-infected cells showed prox-1 positive (red arrows). (H) Lymphatic endothelial cells (LECs) in mock-infected tissues showed prox-1 positive (blue arrows).

**FIG. 5.** Electron microscope assays of the ISKNV-infected cells in spleens of mandarin fish.
The ISKNV-infected cells (IC) were attached by lymphatic endothelial cells (LECs). Black arrows: the overlapping intercellular junctions of the attached LECs; black arrowheads: VMBM, a low-electron-density layer. (A) magnification×6000, the area of the overlapping intercellular junctions of the attached LECs was framed with black lines and was enlarged in (B); (C) magnification×6000; (D) magnification×4000; (E) magnification×8000; (F) magnification×15000. (G) magnification×2000, attached LECs uncovered their surroundings, and infected cell released endocytes and mature virions. The location of the crack of the LECs “bags” is framed in black box and enlarged in (H). As controls, no infected cells were observed in spleens of mock-infected mandarin fish (I, magnification×1650).

**FIG. 6.** Schematic illustration of VMBM. The plasma membrane of the infected cells took the roles of the laminin polymer layers of true BMs, and the laminin γ1 chain III2-6 homologous region of VP23R mimicked the γ1 arms that extend out of the laminin polymer layer to bind nidogen-1. VMBMs are free of collagen IV. Nidogen-1 may provide attaching sites for LECs.
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**Note:** The table contains nucleotide sequences. Each row represents a sequence, and the columns are likely positions within the sequence.
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