Mandarin Fish Caveolin 1 Interaction with Major Capsid Protein of Infectious Spleen and Kidney Necrosis Virus and Its Role in Early Stages of Infection

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Infectious spleen and kidney necrosis virus (ISKNV) is the type species of the genus Megalocytivirus from the family Iridoviridae. ISKNV is one of the major agents that cause mortality and economic losses to the freshwater fish culture industry in Asian countries, particularly for mandarin fish (Siniperca chuatsi). In the present study, we report that the interaction of mandarin fish caveolin 1 (mCav-1) with the ISKNV major capsid protein (MCP) was detected by using a virus overlay assay and confirmed by pulldown assay and coimmunoprecipitation. This interaction was independent of the classic caveolin 1 scaffolding domain (CSD), which is responsible for interacting with several signaling proteins and receptors. Confocal immunofluorescence microscopy showed that ISKNV MCP colocalized with mCav-1 in the perinuclear region of virus-infected mandarin fish fry (MFF-1) cells, which appeared as soon as 4 h postinfection. Subcellular fractionation analysis showed that ISKNV MCP was associated with caveolae in the early stages of viral infection. RNA interference silencing of mCav-1 did not change virus-cell binding but efficiently inhibited the entry of virions into the cell. Taken together, these results suggested that mCav-1 plays an important role in the early stages of ISKNV infection.

Iridoviruses, with large, icosahedral, and double-stranded DNA (dsDNA), can infect both invertebrates (particularly insects), and poikilothermic vertebrates (fish, amphibians, and reptiles), leading to systematic diseases (1). To date, more than 100 iridovirus strains have been isolated, and the entire genomes of 20 strains have been completely sequenced. The Iridoviridae family is composed of five genera, namely, Iridovirus, Chloriridovirus, Ranavirus, Lymphocystivirus, and Megalocytivirus, according to viral particle size, host range, DNA cross-hybridization, the presence of a methyltransferase, and the major capsid protein (MCP) sequence (1, 2). Infectious spleen and kidney necrosis virus (ISKNV) is the type species of the genus Megalocytivirus (2, 3). The ISKNV genome is 111,362 bp in length, consisting of 124 potential open reading frames (ORFs) with encoding capacities ranging from 40 to 1,208 amino acids (aa) (3). Proteomic analysis of ISKNV shows that 38 viral structural proteins are present in ISKNV virions (4). Fifteen highly abundant ISKNV structural proteins were identified. ISKNV MCP is the main structural component of the virus particle, comprising 40% to 45% of the total particle polypeptide (4, 5). ISKNV is one of the fatally infectious agents rendering tremendous economic losses in Chinese mandarin fish cultures (3). Control of ISKNV infection is desperately needed. A better understanding of the ISKNV infection process is the first step toward viral control and prevention.

An increasing number of studies have shown that lipid rafts, liquid-ordered, plasma membrane microdomains, also known as detergent-resistant membranes (DRMs) or caveolae, are involved in virus internalization (6), in addition to signal transduction (7, 8). These viruses include Ebola and Marburg viruses (9), measles virus (10, 11), rotavirus NSP4 (12), immunodeficiency virus (13–15), simian virus 40 (SV40) (16), influenza viruses (17), and respiratory syncytial virus (RSV) (18). Caveolae are 50-nm- to 80-nm-diameter flask-shaped plasma membrane invaginations that are marked by the presence of a caveolin protein family member (19) as well as by polymerase I and the transcript release factor/cavin (20–22). Caveolin 1 (Cav-1), a 21- to 24-kDa scaffolding protein, is the principal structural component of caveolar membranes and is essential for caveola formation during endocytosis (23, 24).

We previously reported that ISKNV may internalize into mandarin fish fry (MFF-1) cells through a caveola-dependent endocytic pathway (25, 26). We postulated that certain ISKNV proteins may interact with mandarin fish Cav-1 (mCav-1). Here, we show that the interaction of ISKNV MCP with mCav-1 and mCav-1 are essential in the early stages of ISKNV infection.

MATERIALS AND METHODS

Cells and reagents. Homo sapiens HeLa (ATCC CCL-2) and HEK293T (ATCC CRL-11554) cells were cultured as a monolayer at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco) under a humidified atmosphere of air containing 5% CO2. MFF-1 cells were maintained in DMEM supplemented with 10% FBS and passed every 3 to 4 days via trypsinization in monolayer cultures at 27°C under a humidified atmosphere of 5% CO2 (27). Rabbit polyclonal anti-MCP, anti-ORF101L, and anti-mCav-1 and mouse polyclonal anti-mCav-1 antisera were previously...
generated in our laboratory (26). Mouse monoclonal antibodies (Abs) raised against α-actin, the Flag tag, the green fluorescent protein (GFP) tag, the glutathione S-transferase (GST) tag, the maltose-binding protein (MBP) tag, β-tubulin, and the V5 tag were obtained from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488-labeled goat anti-mouse IgG and Alexa Fluor 594-labeled goat anti-rabbit IgG secondary antibodies, Hoechst 33342, enhanced chemiluminescent reagent, phenylmethylsulfonyl fluoride (PMSF), and a cocktail of protease inhibitors were purchased from Invitrogen Corporation (Carlsbad, CA). Sucrose and Triton X-100 were obtained from Sigma-Aldrich.

**Plasmid construction and transient transfection.** A DNA fragment of the ISKNV mcp gene was amplified by PCR using the corresponding primers. The PCR fragment, digested with EcoRI and Xhol, was subcloned into the plasmid vectors pcDNA3.1/V5-His (Invitrogen, Carlsbad, CA), pGEX-4T-1 (Amersham Biosciences, Sweden), and pCMV-Myc (Clontech/Takara Bio, CA) to generate recombinant plasmids pcDNA3.1-MCP, pGEX-4T-MCP, and pCMV-myc-MCP, respectively. Gene fragments encoding the functional domains of mCav-1 at aa 1 to 104 [mCav-1(1–104)] and mCav-1(1–139) were amplified by PCR using pCMV-myc-mCav-1 as the template, together with primer-1/primer-2 (primer-1, 5′-CCGAAATTCGGACAGAAGGTAGCAAGACGG-3′; primer-2, 5′-CCGCTCGAGGAAAGGAATCCCCGGATCCATGC-3′) and primer-3/primer-4 (primer-3, 5′-CCGAAATTCGGACAGAAGGTAGCAAGACGG-3′; primer-4, 5′-CCGCTCGAGGAAAGGAATCCCCGGATCCATGC-3′), respectively, for the expression of the Myc-tagged mCav-1(1–104) [Myc–mCav-1(1–104)] protein, covering the mCav-1 sequence from aa 1 to 104, and the Myc–mCav-1(1–139) protein, covering the mCav-1 sequence from aa 139 to 181. The PCR fragments were digested with EcoRI and Xhol and then subcloned into the pcDNA-myc vector to generate pCMV-myc-mCav-1(aa 1–104) and pCMV-myc-mCav-1(aa 139–181). A new fragment, Myc–mCav-1ΔCSD (aa 1 to 85 and aa 105 to 181), with a deleted Cav-1 scaffolding domain (CSD) (28), was generated from mCav-1 (CSD) (aa 1 to 85 and aa 105 to 181) by PCR using primers. The PCR fragments were digested with EcoRI and XhoI and then subcloned into the pCMV-Myc vector to generate pCMV-myc-mCav-1, pCMV-myc-mCav-1ΔCSD, and pCMV-myc-mCav-1ΔCSD (Myc–mCav-1 (1–104)). The orientations of all plasmid constructions were confirmed by DNA sequencing. The transient transfection of recombinant DNA plasmids into HEK293T cells and HeLa cells was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the instructions of the manufacturer.

**Viral infection and purification.** The ISKNV used in this study was originally isolated from disease-infected mandarin fish and preserved in our laboratory (3). MEF cells for infection were cultured overnight in 25-cm² flasks at 5 × 10⁵ cells prior to further treatment. Each flask was inoculated with the virus suspension (multiplicity of infection [MOI] = 5). The cells were harvested at different times according to the experimental design. Viral titers were calculated according to the Reed-Muench and Spearman-Karber methods (29,30). Virus purification by sedimentation through sucrose density gradients was performed as described previously (26).

**Western blot analysis.** The protein concentrations of the purified viruses were determined by using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL). Thirty micrograms of the sample was boiled in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and then separated via SDS-PAGE using a 12% polyacrylamide Tris-HCl gel. The gel-separated protein bands were blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA), which were then blocked in 5% nonfat dried milk in Tris-buffered saline (TBS) with the detergent Tween 20 (TBS) (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.05% Tween 20) at room temperature (RT) for 1 h. Thereafter, the membranes were incubated overnight with specific antibodies at 4°C under constant rotation. The membranes were then washed three times with TBS at RT for 5 min under agitation. Immunoreactive bands were visualized via incubation with the appropriate horseradish peroxidase (HRP)-coupled secondary antibodies and ECL Western blotting detection reagent (Invitrogen).

**Virus overlay assay (far-Western blotting).** Far-Western blotting was performed as described previously by Kikkert et al. (31), with some modifications. Briefly, purified ISKNV (100 μg) was denatured in water at 90°C for 5 min. The denatured product was resolved in triplicates on three 12% SDS-PAGE gels. After electrophoresis, the viral structural proteins in two gels were transferred onto a PVDF membrane via the Bio-Rad blotting procedure (50 V for 2 h), whereas another gel was stained with Coomassie brilliant blue G-250 for a parallel experiment. The membrane was washed twice in TBS for 5 min each. The blots were then blocked overnight in renaturing buffer (20 mM Tris-HCl, 1 mM EDTA, 0.5 mol/liter NaCl, 0.05% Tween 20, 1 M guanidine hydrochloride, and 5% nonfat dried milk powder) at 4°C and then incubated with 10 μg/ml MBP or MBP–mCav-1 protein for 2 h at RT. The membrane was incubated with anti-MBP antibody (1:2,000 dilution) in TBS for 2 h at RT after washing three times in TBS for 10 min each. The membrane was washed as described above, and the antigen-antibody complexes were then detected by using HRP-conjugated goat anti-mouse secondary antibody (1:5,000 dilution) in TBS for 1 h. The immobilized conjugates on the membrane were subsequently visualized by using an HRP substrate solution after another series of washes.

**Coimmunoprecipitation (co-IP).** HEK293T cells were cotransfected with the appropriate combination of the following constructs, according to the recommendations of the manufacturer: pCMV-myc-MCP and pFlag-CMV4-mCav-1, pCMV-myc and pFlag-CMV4-mCav-1, pCMV-myc-MCP and pFlag-CMV4, or pCMV-myc and pFlag-CMV4. The cells were lysed on ice with lysis buffer (10 mM Tris-HCl [pH 7.5], 0.4 M NaCl, 1% NP-40, 0.4% Triton X-100, 0.2% sodium deoxycholate, 1 mM EDTA, protease inhibitors [Calbiochem]) for 30 min at 48 h after transfection. The cell lysates were then centrifuged at 12,000 × g for 10 min. The supernatants were preabsorbed into protein A/G plus agarose (Calbiochem) in a rotation wheel at 4°C for 1 h. After centrifugation at 4,000 × g for 5 min, 1 μg mouse anti-Flag antibody was added to the supernatants for mCav-1 immunoprecipitation. After incubation at 4°C for 2 h, 30 μl protein A/G plus beads was added to the lysates with anti-Flag antibody and then incubated for another 2 h. The beads were washed five times with 1 ml of wash buffer (10 mM Tris-HCl [pH 7.5], 0.2 M NaCl, 0.5% NP-40, 0.2% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, protease inhibitors, 1 mM PMSF) for 3 min each time, followed by centrifugation at 2,000 × g at 4°C for 3 min. The pellets were resuspended in 2× SDS loading buffer and then analyzed via SDS-PAGE. The interaction domains in mCav-1 and ISKNV MCP were mapped by preparing lysates from HEK293T cells that had been cotransfected with Myc-tagged mCav-1ΔCSD (Myc–mCav-1ΔCSD) and pcDNA-V5-MCP (V5-MCP), Myc–mCav-1ΔCSD and the pcDNA3.1/V5-His empty vector, V5-MCP and the pCMV-Myc empty vector, or the pcDNA3.1 empty vector and the pCMV-Myc empty vector, using Lipofectamine 2000 (Invitrogen). The protein lysates were immunoprecipitated by using rabbit anti-Myc and mouse anti-V5 antibodies, as described above. Bead-bound proteins were extracted by using electrophoresis sample buffer and then analyzed by immunoblotting with anti-V5 or anti-Myc antibodies.

**GST fusion protein expression and pulldown assays.** GST-MCP fusion proteins were expressed in Escherichia coli BL21 by incubation in lysogenic broth (LB) medium supplemented with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and ampicillin (100 μg/ml) at 37°C for 3 h. The cells were lysed in lysis buffer (1× phosphate-buffered saline [PBS] [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors).
hibitor mixture) via sonication on ice. The lysate was partially purified by centrifugation at 15,000 × g at 4°C for 20 min. The resulting supernatant was used as the GST-MCP probe in a pulldown assay. GST alone was also prepared and served as a negative control in the assay. The GST-MCP probe was incubated with glutathione-Sepharose beads (Invitrogen) in lysis buffer at 4°C for 2.5 h. GST-MCP-glutathione-agarose beads were washed three times with lysis buffer to remove unbound GST-MCP and were used in pulldown assays for binding to Myc-tagged Cav-1 (see below).

HEK293T cells were transiently transfected with pCMV-Myc-mCav-1. After 48 h, the cells were washed three times with ice-cold PBS and then resuspended in 500 μl lysis buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM MgCl2, 1% polyoxyethylene nonylphenol, 1 mM dithiothreitol, 5% glycerol, and protease inhibitors). The lysates were briefly vortexed, incubated on ice for 40 min, and then centrifuged at 12,000 × g for 10 min at 4°C. The resulting supernatants were incubated with beads coupled to the GST-MCP probe (described above) at 4°C for 2 h with rotation. After incubation, the beads were washed three times with lysis buffer, resuspended in 2× SDS-PAGE sample loading buffer, and boiled for 10 min. The samples were analyzed via SDS-PAGE and immunoblotting using rabbit anti-Myc antibody to detect Myc-mCav-1 proteins.

The lysates of HEK293T cells transfected with pCMV-Myc-MCP were incubated with immobilized MBP or MBP-mCav-1 and then processed for the MBP–mCav-1 binding assays, as described above. The samples were subjected to SDS-PAGE, and Myc-tagged MCP proteins were detected with an anti-Myc antibody.

**Immunofluorescence labeling and confocal microscopy.** MFF-1 cells were seeded onto 12-mm circular glass coverslips and prechilled to investigate the colocalization of mCav-1 with MCP in ISKNV-infected cells. The cells were then incubated with ISKNV at an MOI of 5 at 4°C. The cells were rinsed with PBS at 1 h postinfection (p.i.) and then replensed with fresh DMEM containing 5% FBS. The cells were incubated at 27°C for the specified times, subsequently washed with PBS buffer, and then fixed with prechilled methanol. The cells were washed three times with 1× PBS and permeabilized with 1% Triton X-100 in 1× PBS for 10 min. The cells were rinsed three times with 1× PBS, and nonspecific binding was reduced by blocking with 5% normal goat serum for 30 min at RT. The cells were incubated with anti-rabbit MCP and anti-mouse mCav-1 antibodies in PBS (PBS, 0.05% Tween 20) containing 5% normal goat serum for 60 min at RT. The cells were then rinsed three times with PBS for 10 min and incubated with Alexa Fluor 488 anti-mouse secondary antibody and Alexa Fluor 594 anti-rabbit secondary antibody (1:1,000 dilution) for 1 h. The coverslips were then washed several times with PBS and incubated with Hoechst 33342. The samples were viewed and evaluated under a confocal microscope (LSM510; Zeiss, Germany) equipped with 555-nm/488-nm argon-krypton and 543-nm helium-neon lasers.

**Isolation of caveola-enriched membrane fractions from MFF-1 cells.** MFF-1 cells were infected with ISKNV to investigate the association of ISKNV with cholesterol-rich microdomains. At the specified times, caveola-enriched membrane fractions were prepared by sucrose density gradient sedimentation, as described previously (26). Each sucrose density gradient fraction was resolved by SDS-PAGE and then analyzed via immunoblotting.

**Knockdown of mCav-1 by siRNA.** Three target sequences were selected, and the following small interfering RNAs (siRNAs) against mCav-1 mRNA were synthesized (Invitrogen): siRNA 07 (5′-TGGACAA CGACACGCTGCAAGCAG-3′), siRNA 09 (5′-CACACAAAAGAGAT CGACTTGCTCA-3′), and siRNA 11 (5′-GGTCAAGACCTACTGAT CGAGAT-3′). A control siRNA (NC) (Guangzhou Ribobio Co., Ltd.) that has no homology with mCav-1 mRNA was used as a control. MFF-1 cells were transfected with siRNA by using Lipofectamine 2000 according to the instructions of the manufacturer. The transfected cells were collected and analyzed via Western blotting after 72 h. The MFF-1 cells were bound with ISKNV on ice for 1 h and harvested or incubated for another 4 h at 27°C at 48 h after siRNA transfection. The cells were analyzed via Western blotting, and the effect of mCav-1 knockdown on ISKNV ORF101L protein expression was determined.

**Quantitative real-time PCR.** MFF-1 cells were infected with ISKNV at an MOI of 5 for 1 or 4 h at 48 h after transfection with siRNA. The cells were harvested, and DNAs were extracted for quantitative real-time PCR (qPCR) analysis by using an EZNA tissue DNA kit (Omega) according to the instructions of the manufacturer. The ISKNV genome equivalent (GE) level was determined by qPCR using a LightCycler 480 instrument (Roche, Germany). Briefly, reactions were performed in a 10-μl volume containing 2 μl of total DNA, 5 μl of 2× SYBR Premix Ex Taq (TaKaRa, China), 200 nM forward (F) and reverse (R) primers (MCP-specific forward primer 5′-CAATGTGACCCAGACTGACC-3′, MCP-specific reverse primer 5′-ACCTCAGCTCCTGCTTGTC-3′, β-actin sense primer 5′-CCCTCTGACCCCAAAAGCCA-3′, and β-actin antisense primer 5′-CAGGCTGTAGGCAAGTGATCA-3′), and 3.6 μl double-distilled water (ddH2O). The cycling parameters were as follows: 1 cycle at 95°C for 30 s and 40 cycles at 95°C for 5 s, 60°C for 20 s, and 72°C for 20 s, followed by 1 cycle at 95°C for a calefactor velocity, to generate the melting curve. Fluorescence measurements were conducted at 70°C for 0.1 s during each cycle. qPCR was carried out in three replicates per sample. The β-actin gene was used as an endogenous reference to normalize the cell number variations in each sample. Data were analyzed by using Q-gene statistics, followed by an unpaired-sample t test (32), to determine the statistical significance between controls and the experimental groups. Statistical significance was accepted at a P value of <0.05. The data are expressed as means ± standard deviations (SD).

**RNA extraction and quantitative reverse-transcription PCR (qRT-PCR) analysis.** MFF-1 cells were infected with ISKNV at an MOI of 5 for 1 to 48 h at 48 h after siRNA transfection. Total RNAs of MFF-1 cells were isolated at 1 to 48 h p.i. All total RNA samples were extracted by using the RNeasy minikit (Qiagen, Germany) and subsequently reverse transcribed to cDNA by using a PrimeScript RT Reagent kit (TaKaRa) according to the instructions of the manufacturer. qPCR assays were carried out with a Roche LightCycler480 thermal cycler (Roche Applied Science, Germany). The primers for the mCav-1, mcp, and ISKNV ORF001L genes were as follows: sense primer 5′-ATGGCAACAGAACGACTGACA-3′ and antisense primer 5′-CTTGGTGACGCGTTGAAGTGC-3′ for the mCav-1 gene, sense primer 5′-CAAGATGTGCGCGCGTTAT-3′ and antisense primer 5′-CAGCTGACCCGGCTTGTC-3′ for the ORF001L gene, and MCP-specific forward primer 5′-CAATGTGACCCAGACTGACC-3′ and MCP-specific reverse primer 5′-ACCTCAGCTCCTGCC TTGTC-3′ for the mcp gene. The reaction volume and cycling parameters were the same as those described above. The data were collected and analyzed as described above.

**TCID50 assay.** MFF-1 cells were infected with ISKNV at an MOI of 5 for 1 or 4 h at 48 h after siRNA transfection. After being rinsed five times with PBS, cells were lysed, and the entry of virions into the cells was then determined by a 50% tissue culture infective dose (TCID50) assay by using the Reed-Muench method (30).

**RESULTS**

Mandarin fish Cav-1 interaction with ISKNV MCP using far-Western blotting. Cav-1 is widely known to act as a scaffolding protein and participates in various events within caveolae (23, 33). Previously, we have shown that ISKNV is internalized into MFF-1 cells through caveola-dependent endocytosis (25). Here, we wanted to determine if mCav-1, the principal protein of caveolae, interacts with ISKNV structural proteins by using a virus overlay assay (far-Western blotting). For this assay, purified ISKNV particles were isolated, loaded in triplicates onto three SDS-PAGE gels, and then separated via SDS-PAGE. One replicated gel was stained with Coomassie brilliant blue R-250 (Fig. 1B), whereas the other two replicated gels were transferred onto a PVDF membrane.
through wet electroblotting. The resulting PVDF membrane of two replicated samples (2 separated portions of the PVDF membrane) was hybridized with MBP–mCav-1 fusion proteins after renaturation (far-Western blotting). Two specific proteins (50 kDa and 30 kDa) were visualized by subsequent Western blotting by applying a specific rabbit anti-MBP antibody onto one replicate of the PVDF membrane (Fig. 1A, left). In parallel, the other replicate PVDF membrane was incubated with MBP alone to determine whether these two proteins interacted with MBP alone. This showed that only the 30-kDa and not the 50-kDa protein bound to MBP alone (Fig. 1A, right), suggesting that this 30-kDa protein may be nonspecific. The ISKNV structural proteins corresponding to the mCav-1-bound 50-kDa position were extracted from the replicate gel (Fig. 1B) and then sent for mass spectrometry fingerprint analysis. The mass spectrometry analysis results indicated that the protein bound to mCav-1 was ISKNV MCP.

Pulldown and co-IP assays confirm the interaction ISKNV MCP with mCav-1. Co-IP and pulldown experiments were conducted to confirm the interaction of ISKNV MCP with mCav-1. First, a GST pulldown assay was performed to confirm the specific interaction of MCP with mCav-1 in vitro. GST-MCP was used as a probe to identify MCP-interacting proteins in lysates that were derived from HEK293T cells transiently expressing Myc–mCav-1. The expression of the Myc–mCav-1, GST, and GST-MCP proteins was confirmed by Western blotting (Fig. 2A, lanes 3, 4, and 5, respectively). The Myc–mCav-1 protein specifically bound to GST-MCP (Fig. 2A, lane 2) but not to GST protein alone (Fig. 2A, lane 1). To eliminate false positives associated with the GST tag, we applied cell lysates from HEK293T cells expressing Myc-tagged MCP (Myc-MCP) onto the MBP-tagged mCav-1 fusion protein immobilized on amylase-conjugated agarose beads. The MBP-mCav-1 protein specifically bound to GST-MCP (Fig. 2A, lane 2) but not to GST protein alone (Fig. 2A, lane 1).
based pulldown assay revealed that purified MBP–mCav-1 (Fig. 2B, lane 2), but not MBP (Fig. 2B, lane 1), interacted with MCP. The expression of Myc-MCP, MBP, and MBP–mCav-1 proteins was also confirmed by Western blotting (Fig. 2B, lanes 3 to 5). These results showed that MCP interacted with mCav-1 in vitro.

We performed a co-IP experiment to confirm the pulldown results. A combination of two recombinant plasmids (pCMV-Myc-MCP and pFlag-CMV4-mCav-1) and their parental vectors were transiently cotransfected into HEK293T cells (Fig. 3A). Anti-Flag antibody was used for coimmunoprecipitation of Flag–mCav-1–associated proteins, followed by Western blotting using anti-Myc antibody. The precipitates were analyzed via Western blotting with anti-V5 antibody (top). The data showed that V5-MCP was associated with Myc–mCav-1(1–104) or Myc–mCav-1(139–181) from cotransfected HEK293T cells expressing both V5-MCP and Myc–mCav-1(1–104) nor Myc–mCav-1(139–181) was associated with Myc–mCav-1 from cotransfected HEK293T cells expressing both V5-MCP and V5-tagged MCP were subjected to immunoprecipitation with anti-Myc antibody (Fig. 3C). These results indicated that MCP interacted only with mCav-1ΔCSD, whereas no band was detected when V5-MCP and empty pcDNA3.1/V5-His vector, V5-MCP and pCMV-Myc vectors, or empty pcDNA3.1/V5-His and pCMV-Myc vectors were cotransfected (Fig. 3B, top, lanes 2 to 4). A reverse co-IP was performed to further confirm the interaction. Monoclonal anti-Myc antibody was used to immunoprecipitate the mCav-1ΔCSD binding partners, whereas a rabbit anti-V5 antibody was used to detect the presence of MCP. The anti-Myc antibody clearly pulled down a complex of MCP and mCav-1 from cotransfected HEK293T cells expressing both V5-MCP and Myc–mCav-1ΔCSD (Fig. 3D, bottom, lanes 3 and 4). The cells transfected with Myc–mCav-1ΔCSD and the empty pcDNA3.1/V5-His empty vector, V5-MCP and pCMV-Myc, and pCMV-Myc and pcDNA3.1/V5-His were used as controls. No protein from the control cell lysates was captured when they were immunoprecipitated with anti-Myc antibody (Fig. 3D, top, lanes 1 to 3). The expression of the Myc–mCav-1ΔCSD protein was detected by Western blotting with anti-Myc antibody, indicating the presence of Myc–mCav-1ΔCSD protein (Fig. 3D, bottom, lanes 3 and 4). Taken together, these results indicated that the interaction of MCP with mCav-1 was Cav CSD independent, suggesting that the
The amount of MCP localizing in the denser fractions increased, but the amount of MCP in the low-density raft fractions decreased (Fig. 4C). As references, β-tubulin and actin cellular proteins were used as cytoskeletal markers in the fractionation studies. Although actin and β-tubulin were predominantly present in the high-density fractions (Fig. 4A to C, lanes 10 to 12), certain amounts of these proteins also cosegregated with mCav-1 (Fig. 4A to C, lanes 5 and 6). As in the uninfected cells, mCav-1 migrated into fractions 5 and 6 (Fig. 4D, lanes 5 and 6), this may rule out the possibility that ISKNV infection itself affects mCav-1 partitions preferentially to detergent-resistant caveola microdomains. This is further supported by the fact that the uninfected fractionated MFF-1 cells also showed similar migration patterns of cellular proteins (Fig. 4D, middle and bottom). Therefore, MCP was localized in the caveolae in the early stages of ISKNV infection.

**Colocalization of MCP with mCav-1 in MFF-1 cells in early stages of ISKNV infection was confirmed by confocal immunofluorescence microscopy.** To demonstrate MCP colocalization with mCav-1 in the early stages of ISKNV infection, MFF-1 cells were infected with ISKNV at an MOI of 5, incubated on ice for 1 h, and then incubated at 27°C for 0 h to 4 h. After incubation, the cells were fixed, incubated with anti-mCav-1 and anti-MCP antibodies, and then analyzed upon immunofluorescent staining using confocal laser scanning microscopy. Several MCPs (red) representing the virus particles were found on the cell surface at 0 h; however, few virus particles were colocalized with mCav-1 (green) (Fig. 5B). The frequency of colocalization of MCP with mCav-1 markedly increased when ISKNV-bound cells were incubated for 1 h at 27°C, as MCPs were found not only on the cell surface but also in the cytoplasm (Fig. 5C). Significant colocalization was observed at 2 h p.i. (Fig. 5D). Virus particles that colocalized with mCav-1 were more prominently in perinuclear regions at 4 h p.i. (Fig. 5E). Immunolabeling and laser scanning microscopy showed that the colocalization of MCP with mCav-1 increased with time until 4 h p.i. No colocalization was observed with uninfected MFF-1 cells (Fig. 5A). These results suggested that MCP was colocalized with mCav-1, confirming that MCP is associated with mCav-1 in the early stages of ISKNV infection of MFF-1 cells. Next, we wanted to determine if this association of MCP with mCav-1 is required for the internalization of ISKNV into the cell.

**Inhibition of ISKNV intracellular entry through mCav-1 knockdown.** Cav-1 is a main caveola component, and its disruption prevents caveola-mediated internalization of BK virus (34). However, we did not know if the disruption of mCav-1 affects the internalization of ISKNV into the cell. At the outset, we found that a mCav-1 knockdown agent (mCav-1 siRNA 07) proved most effective against mCav-1 mRNA (data not shown). Thus, we needed to determine the dosage of mCav-1 siRNA 07 for appropriate inhibitory effects on mCav-1 expression. The results showed that the inhibition of mCav-1 expression increased with escalating doses of mCav-1 siRNA 07, indicating that the use of 50 nM mCav-1 siRNA 07 almost completely inhibited mCav-1 expression (Fig. 6A). The effect of mCav-1 knockdown on ISKNV binding to MFF-1 cells was investigated. We used 50 nM mCav-1 siRNA 07 to transfact MFF-1 cells in a monolayer culture for 48 h and then let ISKNV bind to the cells on ice for 1 h. The cells were lifted and washed to remove unbound ISKNV. Total cellular proteins were then extracted from the infected cells and subjected to Western blot analysis (Fig. 6B). This showed that the mCav-1 expression level was significantly lower in MFF-1 cells with
mCav-1 siRNA 07 than in those without mCav-1 siRNA 07 (with either normal control siRNA or mock) (Fig. 6B, bottom). In contrast, the amount of ISKNV ORF101L proteins across all the lanes was not changed (Fig. 6B, top), suggesting that mCav-1 knockdown did not affect the binding of ISKNV to the cells at 1 h p.i.

We then wanted to determine the effect of knocking down mCav-1 on viral internalization after 4 h p.i. The cells were transfected with 50 nM mCav-1 siRNA 07 for 48 h and then incubated with ISKNV on ice for 1 h. The infected cells were incubated at 27°C for 4 h to allow viral entry into cells. Equal aliquots of total proteins derived from the infected cells were subjected to Western blot analysis. Figure 6C shows that mCav-1 expression in the cells transfected with 50 nM mCav-1 siRNA 07 was almost completely abolished, while mCav-1 was well present in control cells either transfected with control siRNA or transfected without siRNA (mock) (Fig. 6C, bottom). No significant differences in mCav-1 expression were observed between the control cells without siRNA transfection (mock) and the cells transfected with control siRNA (Fig. 6C, bottom, middle versus right lane). As expected, the ORF101L protein levels of ISKNV in cells transfected with mCav-1 siRNA 07 (Fig. 6C, top, middle lane) were significantly lower than those in cells either transfected with control siRNA (Fig. 6C, top, middle lane) or transfected without siRNA (Fig. 6C, top, right lane).

Moreover, the infectious viral titers determined by the 50% tissue culture infective dose (TCID50) of siRNA-transfected or control cells were determined at different times p.i. At 48 h after transfection of siRNA, MFF-1 cells were infected with ISKNV at an MOI of 5 for 1 h or 4 h. After rinsing five times with PBS, cells were lysed, and the entry of virions into the cells was then determined by TCID50 infectivity titration determination using the Reed-Muench method. The results showed that the TCID50 of cells transfected with mCav-1 siRNA 07 was lower than that of cells either transfected with control siRNA or without siRNA transfection at 1 h p.i. (Fig. 6D, top) or 4 h p.i. (Fig. 6D, bottom). The expression level of the mCav-1 gene in cells transfected with mCav-1 siRNA 07 was significantly lower than that in cells either transfected with control siRNA or transfected without siRNA at 1 h p.i. (Fig. 6E, left) or 4 h p.i. (Fig. 6E, right). The amount of mcp genes (DNA) in cells transfected with mCav-1 siRNA 07 was significantly lower than that in cells either transfected with control siRNA or transfected without siRNA at 1 h p.i. (Fig. 6F, left) or 4 h p.i. (Fig. 6F, right). These results suggested that the levels of invading ISKNV mcp genes (DNA) in cells transfected with
mCav-1 siRNA 07 were lower than those in cells either transfected with control siRNA or transfected without siRNA. The above-described results appear to indicate that knocking down mCav-1 using siRNA impaired ISKNV internalization but did not change the virus’s ability to bind to cells.

To confirm that knocking down mCav-1 using siRNA impaired ISKNV internalization, we decided to determine the levels of virus production at 12 h, 24 h, and 48 h p.i., beyond 4 h p.i., as described above. The TCID50 assay results showed that the levels of virus in cells transfected with mCav-1 siRNA 07 were lower
than those in cells either transfected with control siRNA or transfected without siRNA at 12 h, 24 h, or 48 h p.i. (Fig. 7A). The qRT-PCR results showed that the expression levels of the mCav-1, ISKNV ORF001L (a viral early gene) (35), and mcp genes were significantly lower in cells transfected with mCav-1 siRNA 07 than those in cells either transfected with control siRNA or transfected without siRNA (mock) at 12 h, 24 h, or 48 h p.i. (Fig. 7B to D). This is consistent with evidence at the protein level, obtained by West-
ern blotting, showing that the ISKNV ORF101L expression level in MFF-1 cells transfected with mCav-1 siRNA 07 was significantly lower than that in cells either transfected with or transfected without control siRNA at 12 h, 24 h, or 48 h p.i. (Fig. 7E). Taken together, these results indicated that inhibition of ISKNV internalization by knocking down mCav-1 resulted in reduced viral gene expression and virus production.

DISCUSSION

In the present study, our findings include the following. (i) ISKNV MCP interacted directly with mCav-1 in vitro. (ii) This interaction was Cav-1 CSD independent. (3) MCP colocalized with mCav-1 in the early stages of ISKNV infection. (4) siRNA-mediated mCav-1 gene knockdown significantly inhibited the internalization of ISKNV, leading to reductions in viral gene expression and viral production. These results suggested that mCav-1 may play an important role in the early stages of ISKNV infection, which may be related to the interaction of mCav-1 and MCP during ISKNV infection.

Research on virus–host protein interactions can help us understand the mechanisms of viral pathogenicity and virus-induced immunological responses. Caveolae or lipid rafts, from which multiple signaling pathways originate, have a propensity to concentrate receptors and signal transduction molecules (36, 37). Increasing evidence gathered over the past years shows that several pathogens, such as bacteria, parasites, and viruses, use caveolae for their own benefit (38, 39). On the one hand, certain viruses, such as HIV, SV40, RSV, and echovirus 1, use caveolar entry into the host (16, 18, 40, 41). On the other hand, raft domains also provide sites for the assembly or budding of enveloped and nonenveloped viruses, such as influenza virus, HIV, measles virus, rotavirus, Ebola virus, and Marburg virus (9, 11, 17, 42, 43). The interaction of enveloped-virus structural proteins with lipid rafts has been well documented for viral infections such as RSV (44), Newcastle disease virus (45), HIV-1 (46), Ebola virus (47), Sendai virus (48), measles virus (11), and influenza virus (49). Our current study showed that viral MCP interacted with mCav-1, which is the first report that a viral structural protein interacted with a host protein in the family Iridoviridae. In the early 1980s, transmission electron micrographic observations of frog virus 3 (FV3) entry revealed that enveloped FV3 particles were internalized by adsorptive endocytosis via coated pits and then moved through endosomes and finally to lysosomes (5, 9). Recently, research on the uptake of tiger frog virus (TFV) (a ranavirus) into mammalian cells (HepG2) at 27°C showed a pH-dependent atypical caveola-dependent endocytosis (50). ISKNV internalizes into MFF-1 cells through a caveola-dependent endocytic pathway and may then colocalize with mCav-1 in infected MFF-1 cells (25). The results of siRNA assays suggested that in association with mCav-1, ISKNV is directed into cells via a caveola-dependent endocytosis pathway. Considering the importance and conservation of the MCP gene in the family Iridoviridae, we postulated that studying the interaction of MCP with mCav-1 could help us understand the ISKNV infection process to not only provide a valuable reference for other iridoviruses but also lay out the scientific basis for the prevention and treatment of viral diseases.

Cav-1 is the principal structural component of caveolar membranes involved in a wide range of cellular processes, such as cell cycle regulation, signal transduction, transcytosis, endocytosis, cholesterol homeostasis, and apoptosis (51–55). Increasing evidence indicates that Cav-1 acts as a scaffolding protein and has a caveolin scaffolding domain (CSD) responsible for binding to various proteins (7, 33). The CSD-bound proteins contain one of three binding motifs, namely, ΦXXXΦXXΦ, ΦXXXXΦXXΦ or ΦXXXΦXXΦΦΦ, where Φ is any one of the aromatic amino acid residues (W, F, or Y) and X is any other amino acid residue (56). Several proteins interact with CSD directly or indirectly through their binding motifs, such as the transmembrane envelope glycoprotein gp41 of HIV (57, 58), the heterotrimeric G protein, endothelial nitric oxide synthase (59), and various receptor tyrosine kinases (7, 60). Our previous studies suggested that ISKNV internalizes into MFF-1 cells through a caveola-dependent endocytic pathway and that the mCav-1 expression level significantly increases after ISKNV infection (25, 26). Considering the relationship between Cav-1 and viral infection, we hypothesized that ISKNV structure proteins (4) with Cav-1-binding motifs may interact with mCav-1. A bioinformatics study searching for these motifs showed that 27 ISKNV viral proteins contain Cav-1-binding motifs (data not shown), indicating that these ISKNV proteins may interact with mCav-1. Far-Western blotting was performed by using MBP–mCav-1 to determine whether viral structural proteins can bind to mCav-1. The far-Western blotting, in full accord with mass spectrometric analyses, showed that ISKNV MCP specifically bound to the MBP–mCav-1 protein (Fig. 1A). Interestingly, ISKNV MCP did not contain the Cav-1-binding motifs according to bioinformatics analysis. Furthermore, the interaction between MCP and the mCav-1 protein is independent of the classic CSD, as confirmed by co-IP (Fig. 3). Some proteins without CSD-interacting motifs have been shown to bind to Cav-1. For example, rotavirus nonstructural protein 4 interacts with Cav-1 residues 2 to 22 at the N terminus and residues 161 to 178 at the C terminus (12); the isoform of the membrane scission enzyme dynamin binds with Cav-1 residues 22 to 33 at the N terminus and 21 residues at the C terminus; and sterol carrier protein 2, which lacks a consensus Cav-1–binding domain, binds with Cav-1 at the N-terminal domain between aa 34 and 40 (61, 62). However, further studies are needed to determine the exact amino acid sequences of mCav-1 that are responsible for the interaction as well as the MCP domain that interacts with mCav-1.

The major steps involved in viral entry include viral binding or attachment, membrane fusion, entry pore formation, and viral penetration (43). Viral binding and internalization are distinct steps. Viral binding relies on underlying mechanisms as viral glycoprotein ligand spikes interact with and bind to cellular surface receptors (43, 63). Several animal viruses use different endocytic pathways for efficient cell internalization. Among these pathways, two have been proven to be the most frequently used, namely, clathrin-dependent entry and caveola-mediated endocytic pathways. Cav-1 expression can induce caveola formation (64); hence, the disruption of the Cav-1 gene results in caveola abolishment (29, 64, 65). Knocking down of Cav-1 by siRNA interferes with some viral infections, such as BK virus and human coronavirus 229E (34, 66). Our study on the effect of mCav-1 knockdown on ISKNV binding and internalization showed that siRNA-mediated mCav-1 knockout did not significantly change the binding of the virus to the cells (Fig. 6B) but specifically inhibited ISKNV internalization (Fig. 6C to F). These results indicate that mCav-1 is vital for ISKNV internalization into MFF-1 cells, implying that ISKNV is internalized through the caveola-mediated pathway. SV40 enters many cell types through a process that involves cell surface
caveolae (22, 65, 67). However, SV40 can enter cells devoid of caveolae through an alternative tyrosine kinase- and cholesterol-dependent endocytic pathway (68). In our studies, mCav-1 knockdown remarkably inhibited ISKNV infection, suggesting that caveola endocytosis might be the only way of ISKNV entry into MFF-1 cells, and there was no other alternative invasion pathway, at least under these experimental conditions.

We have also studied the functional consequence of inhibiting mCav-1 expression on the infection of ISKNV, because our results showed that MCP was associated with caveolae in the early stages of viral infection (Fig. 4), the colocalization of MCP and mCav-1 (Fig. 5), and the interaction between the MCP and mCav-1 in vitro (Fig. 2). We speculated that the inhibition of mCav-1 expression might destroy the interaction of MCP and mCav-1, resulting in inhibition of virus entry. As caveolin-1 is localized to the inner surface of the plasma membrane (36), we may have an intuitive understanding that MCP could not interact with mCav-1. Our results showed that mCav-1 interacted with MCP and played key roles in the early stages of virus infection. We presume that ISKNV binds initially to the extracellular face of the cell membrane and subsequently enters the host cells by caveolar endocytosis. After internalization, ISKNV virions may be engulfed in caveola. The virus envelope might then undergo fusion with internal membranes of caveola vesicles, making the MCP of the virus exposed in the vesicle lumen, where MCP might interact with mCav-1. The exact processes require further study. Megaloviruses have been assumed to replicate in the vesicle lumen, where MCP might interact with mCav-1. The membranes of caveola vesicles, making the MCP of the virus exposed in the vesicle lumen, where MCP might interact with mCav-1. The processes require further study.

REFERENCES


