Collaboration between a Soluble C-Type Lectin and Calreticulin Facilitates White Spot Syndrome Virus Infection in Shrimp

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White spot syndrome virus (WSSV) mainly infects crustaceans through the digestive tract. Whether C-type lectins (CLs), which are important receptors for many viruses, participate in WSSV infection in the shrimp stomach remains unknown. In this study, we orally infected kuruma shrimp Marsupenaeus japonicus to model the natural transmission of WSSV and identified a CL (designated as M. japonicus stomach virus–associated CL [MjsvCL]) that was significantly induced by virus infection in the stomach. Knockdown of MjsvCL expression by RNA interference suppressed the virus replication, whereas exogenous MjsvCL enhanced it. Further analysis by GST pull-down and coimmunoprecipitation showed that MjsvCL could bind to viral protein 28, the most abundant and functionally relevant envelope protein of WSSV. Furthermore, cell-surface calreticulin was identified as a receptor of MjsvCL, and the interaction between these proteins was a determinant for the viral infection–promoting activity of MjsvCL. The MjsvCL–calreticulin pathway facilitated virus entry likely in a cholesterol-dependent manner. This study provides insights into a mechanism by which soluble CLs capture and present virions to the cell-surface receptor to facilitate viral infection. *The Journal of Immunology*, 2014, 193: 2106–2117.
inoculum was delivered through the shrimp oral tract to model natural infection, and the expression of CLs in the stomach was analyzed. Among the CLs identified as induced by WSSV, one that was mainly expressed in the stomach and responded most highly to WSSV infection drew our attention and was subjected to further investigation. The function of this CL, designated as Marsupenaeus japonicus stomach virus–associated CL (MjsvCL), in virus infection was studied by both knockdown and overexpression of the protein. Moreover, the mechanism by which MjsvCL participates in WSSV infection was explored. The identification of a viral infection promoting CL in shrimp in this study will be helpful for understanding the molecular pathogenesis of WSSV and WSSV–shrimp interactions.

Materials and Methods

Animals

Healthy kuruma shrimp M. japonicus (~6–7 g) obtained from a farm in Rizhao, Shandong, China, were cultured in air-pumped artificial seawater and fed a commercial diet daily in the laboratory. Animals were randomly selected to extract genomic DNA from the gills using the MagExtractor Genomic DNA Purification Kit (Toyobo, Shanghai, China) according to the manufacturer’s instructions. The extracted DNA was tested by PCR using primers (WSSVRF, R) listed in Table I to ensure that the shrimp were WSSV free.

Preparation of viral inoculum

The gills (1 g) of WSSV-infected shrimp (moribund shrimp artificially infected in the laboratory for virus amplification) were homogenized in 10 ml sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) and centrifuged at 3000 × g for 5 min at 4°C. After passing the resulting supernatant through a 0.45-μm filter, part of the filtrate (100 μl) was used to extract genomic DNA to determine the virus titer by quantitative real-time PCR (qRT-PCR; see later). The remaining filtrate was frozen at −80°C and diluted to the appropriate titer with PBS before use. The gills from WSSV-free shrimp were treated in the same way as the control.

Quantification of viral copy numbers

A WSSV vp28 gene fragment was amplified using the WSSVRF and R primers (Table I) and then inserted into the pBlueScript vector to generate a recombinant plasmid. The plasmid sample, for which the copy number had been calculated, was serially diluted 10-fold. The genomic DNA extracted from the viral inoculum (100 μl) or the tissue (10 mg) together with the plasmid samples were analyzed by qRT-PCR. The reaction mix consisted of 10 μl of the IQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 2 μl 1:100 diluted DNA, and 4 μl forward and reverse primers (0.5 μM each). qRT-PCR was performed in the CFX96 Real-Time System (Bio-Rad) with the following procedure: 94°C for 3 min, 40 cycles of 94°C for 15 s and 60°C for 1 min, and a final dissociation protocol from 65°C to 95°C. Results from the plasmid samples were used to generate a standard curve to quantify viral copy numbers in the inoculum or tissues.

Oral infection of shrimp and sample collection

The oral infection was performed by delivering 20 μl viral inoculum (~2.5 × 107 virions/ml) into the shrimp oral cavity with a sterile flexible micro-syringe. Thereafter, the shrimp were maintained with the ventral side up for 5 s, rinsed in seawater, and then cultured in clean seawater. Shrimp treated similarly with virus-free inoculum was used as controls. At 6, 12, 24, 48, and 72 h postinfection, the stomach was collected from experimental shrimp, rinsed with sterile water, dried with blotting paper, and divided into three parts. The first part was weighed and used to extract the genomic DNA to determine the virus titer in the tissue to confirm the viral replication. The other two parts were used to extract the total RNA and proteins separately. The stomach from control shrimp was also collected for total RNA extraction. At least six shrimp were pooled for each sample.

Screening WSSV-sensitive genes in shrimp stomach

qRT-PCR was performed to screen for WSSV-sensitive genes in the shrimp stomach at 6 and 12 h postinfection. Total RNA was extracted from the earlier-mentioned stomach samples with TRIzol (Invitrogen, Carlsbad, CA). The first-strand cDNA was synthesized with a cDNA Synthesis Kit (M-MLV version; Takara, Dalian, China) following the manufacturer’s instructions. Fourteen CLs (10096, 14495, 15188, 18420, 18765, 18947, 22501, 25392, 34742, 31681, 31996, 35574, 40058, and 47086), which were obtained from a transcriptome sequencing of kuruma shrimp stomach, were selected for the screening. PCR was performed as described earlier using primers listed in Table I. Gene expression in each experimental stomach sample was normalized to that in the control and calculated as the fold change of expression induced by WSSV infection. Results are presented as the mean value ± SD.

Identification of MjsvCL cDNA

The CL 40058 (MjsvCL) was characterized as a WSSV-sensitive gene. The fragment encoding the full open reading frame of MjsvCL was obtained from a transcriptome sequencing of kuruma shrimp by the Beijing Genomics Institute (BGI, Shenzhen, China). A pair of primers (MjsvCLGFS/R) was used to amplify the full-length cDNA of MjsvCL to confirm the correctness of the sequence. The SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. SMART (http://smart.embl-heidelberg.de/) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) programs were used to analyze the domain architecture.

Analysis of MjsvCL expression profiles

Total RNA samples were extracted from hemocytes, heart, hepatopancreas, gills, stomach, intestine, lymphoid organ, hematopoietic tissue, epidermis, pleopod, eyestalk, and muscle of three uninfected shrimp to synthesize cDNA. qRT-PCR was then performed to detect the distribution of MjsvCL transcripts, with β-actin as the reference gene. The temporal expression of MjsvCL in the stomach postinfection was also determined by qRT-PCR. Data were analyzed by Student’s t test and expressed as the mean ± SD from three independent experiments.

The distribution and temporal expression of MjsvCL protein were evaluated by Western blot. Generally, the tissues were homogenized in PBS supplemented with 5 mM EDTA and 0.5 mM PMSF. The homogenate was centrifuged at 12,000 × g for 15 min at 4°C, and the resulting supernatant was diluted by 12.5% PAGE (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane and subsequently blocked with 5% nonfat milk (in PBS) for 1 h with gentle rotation. The membrane was then immersed with 1:300 diluted MjsvCL antiserum (see later) in the blocking milk solution with rotation. After three washes with PBST containing 0.02% Tween 20 (PBS-T), the membrane was incubated with 1:10,000 diluted HRP-conjugated goat anti-rabbit Abs in the blocking milk solution for 3 h with rotation. The membrane was then washed three times with PBST, and the target bands were visualized by oxidizing 4-chloro-1-naphthol in the presence of H2O2 in PBS. Expression of β-actin was determined as the fold change of expression induced by WSSV infection. Data were analyzed by Student’s t test and expressed as the mean ± SD from three independent experiments.

To determine the presence of any soluble MjsvCL protein in the circulating plasma, an immunoprecipitation assay was performed with the MjsvCL Ab to concentrate the target protein to a detectable level. The hemolymph from WSSV-infected shrimp (0, 6, 12, 24, 48, and 72 h) was collected and centrifuged at 800 × g for 5 min. The supernatant was centrifuged again at 20,000 × g for 20 min to obtain the plasma. Ten shrimp were used for each sample. The plasma was diluted with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% Nonidet P-40, 1 mM EDTA, 0.5 mM PMSF, pH 8.0) and precleared with 50 μl protein A beads (GenScript, Nanjing, China) with agitation for 40 min at 4°C. After removing the beads by centrifugation at 12,000 × g for 10 min, 5 μl MjsvCL antiserum was added to the supernatant. The mixture was incubated overnight with agitation at 4°C. Protein A beads (10 μl) were then added to isolate the Ab with agitation at 4°C for 1 h. The beads were collected, washed four times using RIPA buffer with each wash lasting 10 min, resuspended in SDS-PAGE sample buffer (15 μl), and boiled for 10 min before Western blot analysis using the MjsvCL Ab.

RNA interference

A partial MjsvCL cDNA fragment was amplified by PCR with primers linked to the T7 promoter (Table I) and used as the template to produce dsRNA with an in vitro T7 Transcription Kit (Takara, Dalian, China). The control GFP dsRNA was synthesized in the same way with primers listed in Table I. To test whether the expression of MjsvCL could be suppressed, we injected the MjsvCL dsRNA (20 μg) into each shrimp i.m. at the fourth abdominal segment with an equal amount of GFP dsRNA injected as the control. At 24 h postinfection, MjsvCL expression in the stomach and muscle was analyzed by qRT-PCR and Western blot to confirm the RNA

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interference (RNAi) efficiency. At least three shrimp were used for testing of RNAi efficiency.

After validating that MjsvCL expression could be silenced by the dsRNA injection, 30 shrimp were used for RNAi by injecting each with 20 μg MjsvCL dsRNA, and another 30 shrimp were injected with GFP dsRNA as the control. Oral injection of all shrimp was performed 24 h later using the method described earlier. The genomic DNA and proteins were extracted from the stomach and gills at 24 and 48 h postinfection. Virus titers and VP28 expression in the tissues were determined by qRT-PCR and Western blot. At least 10 shrimp were pooled for each sample. Results were analyzed by Student t test and expressed as the mean ± SD from three independent repeats.

Recombinant protein and antisera production

The fragment encoding the mature MjsvCL was amplified by PCR with primers in Table I. The resulting DNA was digested with BamHI and Xhol, and ligated into the pGEX-4T-1 vector (GE Healthcare, Piscataway, NJ). The recombinant plasmid was introduced into Escherichia coli Rosetta (DE3) cells. Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at 28°C for 10 h after the OD600 of the culture reached 0.8. The recombinant protein was purified by affinity chromatography using GST-resin (GenScript, Nanjing, China). To remove the majority of contaminating endotoxins, we washed the column thoroughly with cold PBS containing 0.1% Triton X-114 (Sigma-Aldrich, St. Louis, MO) and then PBS at 4°C (20). The proteins were finally eluted by a buffer containing 10 mM glutathione (Sigma-Aldrich) and 50 mM Tris-HCl (pH 7.5), dialyzed in PBS three times and adjusted to the concentration of 1 mg/ml. For some experiments (injection or application to the hemocyte culture), the GST tag of the recombinant protein was cleaved by incubation with thrombin overnight. The fraction was then adjusted to the concentration of 1 mg/ml. For some experiments (injection or application to the hemocyte culture), the GST tag of the recombinant protein was cleaved by incubation with thrombin overnight. The fraction was then adjusted to the concentration of 1 mg/ml. For some experiments (injection or application to the hemocyte culture), the GST tag of the recombinant protein was cleaved by incubation with thrombin overnight. The fraction was then adjusted to the concentration of 1 mg/ml. For some experiments (injection or application to the hemocyte culture), the GST tag of the recombinant protein was cleaved by incubation with thrombin overnight. The fraction was then adjusted to the concentration of 1 mg/ml. For some experiments (injection or application to the hemocyte culture), the GST tag of the recombinant protein was cleaved by incubation with thrombin overnight. The fraction was then adjusted to the concentration of 1 mg/ml. For some experiments (injection or application to the hemocyte culture), the GST tag of the recombinant protein was cleaved by incubation with thrombin overnight. The fraction was then adjusted to the concentration of 1 mg/ml.

Generally, the stomach was homogenized in 50 ml PBS and then sonicated at 300 Hz for 3 min on ice. The homogenate was centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was incubated with 20 μg rGST-MjsvCL with rotation for 3 h at 4°C, whereas the other half was incubated with an equal amount of the rGST tag as a control. Each incubated mixture was then passed through a GST-resin column (1 ml) with a flow speed of 0.5 ml/min at 4°C. A generous amount of PBS was then added to wash the column. The bound proteins were eluted with 10 mM glutathione (Sigma-Aldrich) in 50 mM Tris-HCl (pH 7.5) and separated by SDSPAGE in a 12.5% gel. Protein bands that were present in the experimental group but absent in the control group were cut out from the gel and sent to be commercially analyzed by mass spectrometry (MS/MS; Bioschip, Tianjin, China). The MS/MS results were searched against proteins from the database Crustacea in GenBank.

Characterization of calreticulin

The recombinant partial calreticulin (CRT) protein was expressed in E. coli cells with a construct using the pET32a (+) vector (Novagen) and primers listed in Table I. The protein, expressed in soluble form and purified with Ni-NTA beads (Novagen), was used to inject a rabbit to prepare the antisera. CRT expression profiles were analyzed by RT-PCR (CRTRTF, R; Table I) and Western blot in the same way as for MjsvCL.

To determine whether CRT is expressed on the hemocyte surface, we collected hemocytes from unchallenged and virus-challenged shrimp by centrifuging the hemolymph at 800 × g for 10 min. Approximately 107 hemocytes were washed with cold marine PBS (PBS with 0.45 M NaCl) and incubated with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in marine PBS for 1 h at 4°C. Cells were washed with cold marine PBS five times to quench the unreacted agent and then lysed in RIPA buffer for 30 min. The lysate was centrifuged at 12,000 × g for 10 min at 4°C to obtain the supernatant, to which 20 μl streptavidin-agarose beads (Pierce) were added to immunoprecipitate the biotinylated cell-surface proteins with gentle rotation for 1 h at 4°C. The beads were washed with RIPA buffer five times, boiled in SDS-PAGE sample buffer, and analyzed by Western blot with the CRT Ab and β-actin Ab as the control.

To label the stomach cell-surface proteins, we performed perfusion following a previously described procedure (22) with slight modifications. In brief, the intact esophagus and stomach were isolated carefully. Marine PBS was administered through the posterior portal with a microsyringe and allowed to flow out through the anterior portal for 5 min. Thereafter, a sulfo-NHS-LC-biotin solution was used to perfuse the esophagus and stomach for 15 min as with the marine PBS, followed by perfusion with marine PBS again to wash away the unreacted reagent. The stomach was collected, washed with marine PBS, and then homogenized in RIPA buffer. Biotinylated proteins were detected as described earlier. At least three shrimp were used to prepare each sample.

To confirm the interaction between CRT and MjsvCL, we performed co-IP as described earlier, with slight modifications. The protein pool used in this study was the stomach homogenate from healthy shrimp. The β-actin Ab and IgG were used as negative controls.
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100 IU/ml penicillin, and 100 µg/ml streptomycin was distributed into a 24-well plate at 10^5 cells/well and cultured at 28°C. The virus (WSSV, isolated from the ventral sinus and immediately mixed with an equal volume of precooled anticoagulant. The mixture was centrifuged at 800 g for 10 min to remove the anticoagulant and centrifuged at 20,000 g for 30 min to wash the virus. The supernatant was centrifuged at 100,000 g for 30 min to pellet the virus and pellet was resuspended in 100 µl of water-soluble cholesterol (Sigma-Aldrich). After incubation for 1 h, the virus infection–promoting activity of MjsvCL was evaluated as described earlier.

## Results

### MjsvCL responds to oral WSSV infection

To screen the WSSV-sensitive CLs in the shrimp stomach, which is the first tissue that the virus encounters during natural transmission and experiences the most injury from the infection, we delivered the viral inoculum through the oral cavity to model the natural infection. The inoculated virus could successfully infect shrimp through the stomach and replicated quickly from 12 to 72 h postinfection, with the viral titer of >10^6 copies/mg tissue (Fig. 1A). The expression of all CLs (identified from a transcriptome analysis) expressed in the stomach was examined at the early stage (6, 12 h) of virus infection (Table I). Among the 14 CLs detected, the expression of 5 CLs was upregulated, whereas that of 4 CLs was downregulated by viral infection (Fig. 1B). The expression of CL-40058 was induced most highly, with a change of ~3- and 7-fold at 6 and 12 h postinfection, respectively. This gene was then selected for further study and designated as MjsvCL (GenBank accession no. KF712277; http://www.ncbi.nlm.nih.gov/nuccore/KF712277). Analysis of the MjsvCL sequence suggested that it contains a signal peptide and a CTLD (Fig. 1C). Interestingly, MjsvCL was found to share some similarity with mammalian MBLs, similar to some CLs of other crustaceans (Supplemental Fig. 2) (24). Although lacking the collagen-like domain, MjsvCL contains a region rich in glutamates and asparagines (Q/N-rich region) between the signal peptide and CTLD (Fig. 1C). The unique arrangement of MjsvCL prompted us to further study its properties during virus infection.

### MjsvCL transcripts were distributed mainly in the stomach and muscle, with very little expression in the lymphoid organ, hematopoietic tissue, heart, epidermis, and pleopod (Fig. 1D, upper panel). Consistent expression profiles of the MjsvCL protein were observed by Western blot (Fig. 1D, lower panel). The virus induced the expression of MjsvCL from the very beginning of infection, and the induction persisted until 72 h postinfection (Fig. 1E). More MjsvCL protein was synthesized in the stomach postinfection, accompanied by the increase in VP28 protein, which could be regarded as the marker for virus replication (Fig. 1F). Moreover, soluble MjsvCL protein could be detected in the shrimp circulating plasma, and the amount was increased by WSSV infection (Fig. 1G). The obvious induction by WSSV suggested that MjsvCL plays a role during viral infection.

### MjsvCL promotes viral infection

To study the function of MjsvCL in viral infection, we silenced the expression of MjsvCL by RNAi. The expression of MjsvCL could be suppressed at 24 h after dsRNA injection at both the transcriptional (Fig. 2A, upper panel) and the translational (Fig. 2A, lower panel) levels in the stomach and muscle (Fig. 2A), as revealed by qRT-PCR and Western blot. The viral inoculum was then introduced to the oral cavity of shrimp preinjected with MjsvCL or GFP dsRNA. Results showed that the replication of...
WSSV was inhibited in the MjsvCL knockdown shrimp. Viral titers in the stomach and gills at 24 and 48 h were both lower than those in the control shrimp (Fig. 2B). The VP28 expression in the experimental samples also was weaker than that of corresponding controls (Fig. 2C), further confirming that the viral replication was suppressed upon silencing of MjsvCL expression. These data indicated a positive role of MjsvCL for the viral infection.

rMjsvCL was expressed (Fig. 2D) and injected into shrimp to generate an “overexpression” model to verify the RNAi results. As shown in Fig. 2E and Supplemental Fig. 4, injection of the viral inoculum together with exogenous MjsvCL could enhance the viral replication, compared with injection with the rGST tag or rGFP. The virus titer in the overexpression shrimp was much greater in the overexpression group than in the control shrimp in the stomach, gills, and muscle at both 24 and 48 h postinfection (Fig. 2E). In addition, the VP28 expression was much greater in the overexpression group than in the control shrimp in several tissues detected (Fig. 2F). Together, these results led to the conclusion that MjsvCL plays a role in promoting virus infection and reproduction in the host.

**MjsvCL binds to the most abundant and functionally relevant envelope protein of WSSV**

Many CLs have been demonstrated to possess virus-binding abilities. Therefore, to explore the mechanism of MjsvCL-mediated promotion of viral infection, we tested the binding ability of this protein to the virus. rMjsvCL was first used to perform a pull-down assay to capture the VPs from virus-infected tissues. Results showed that rGST-MjsvCL could interact with VP28, the most abundant envelope protein of WSSV, whereas the control rGST tag could not (Fig. 3A). These data indicated that MjsvCL may function as a WSSV-binding molecule through interaction with VP28.

To confirm the protein interactions found by the earlier pull-down assay, we performed co-IP using virus-infected tissue extracted containing the pool of VPs and MjsvCL. As shown in Fig. 3B, native MjsvCL immunoprecipitated together with VP28. In addition, a positive MjsvCL signal was detected in the sample immunoprecipitated by the VP28 antiserum.

**Viral infection–promoting activity of MjsvCL is dependent on MjsvCL–CRT interaction**

The earlier results provided a partial explanation for the function of MjsvCL in promoting WSSV invasion by binding to the virus. However, the underlying mechanism by which MjsvCL facilitates virus infection after recognition still needed to be determined. With this purpose, potential interacting proteins of MjsvCL were identified by pull-down assays and subsequent MS/MS analysis. Among the seven candidates analyzed, only three were successfully identified, probably because of the lack of shrimp genomic data identified, probably because of the lack of shrimp genomic data (Fig. 4A). Nevertheless, the CRT protein drew our attention, because it was found to be: 1) clearly induced by WSSV infection in many organisms (25–27); 2) present in the extracellular matrix or on the cell surface, as well as in the endoplasmic reticulum as a chaperone (28); and 3) the receptor of mammalian MBL (29). This background information prompted us to further study the potential role of CRT in the MjsvCL-mediated virus infection. Tissue distribution analysis suggested that CRT is expressed in all
MjsvCL and CRT was confirmed by a co-IP assay, with the portantly, CRT was found on the cell surface of shrimp hemocytes tissue detected (Fig. 4B), and the expression profile confirmed that its expression is induced by virus infection (Fig. 4C). More importantly, CRT was found on the cell surface of shrimp hemocytes and stomach cells (Fig. 4D, 4E). Furthermore, the interaction of MjsvCL and CRT was confirmed by a co-IP assay, with the β-actin Ab and IgG used as negative controls (Fig. 4F). These results validated cell-surface CRT as a receptor of MjsvCL.

To determine how the MjsvCL–CRT interaction is involved in virus infection, we examined whether these two proteins would bridge. These three proteins can form a complex with MjsvCL as the bridge.

To determine whether the function of MjsvCL is dependent on CRT, the expression of CRT was influenced by dsRNA preinjected shrimp, in the viral infection–promoting activity of MjsvCL was influenced by CRT knockdown. Expression of the WSSV igel gene was detected as a marker. As shown in Fig. 5C, the expression of igel promoted by MjsvCL was 6.7-fold higher than that by the rGST tag when the tests were performed in the GFP-dsRNA preinjected shrimp, in which the expression of CRT was not influenced (Fig. 5C, left).

### Table I. Primers used for this study

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Results were analyzed by Student t scan the Western blot bands from three independent repeats. Relative expression levels of VP28/promotes virus infection, which can be determined by analyzing Our results thus far indicated that the MjsvCL–CRT pathwayOur results thus far indicated that the MjsvCL–CRT pathway

cholesterol by the interaction with CRT. these results suggested that the function of MjsvCL is determined

displayed a blocking effect against viral infection, in total con-

still harboring a capacity for virus binding, the truncated MjsvCL

the interaction with CRT. As shown in Fig. 5D, the truncated

region (Fig. 5D, involved in WSSV infection but not containing the Q/N-rich

region could not pull down CRT, similar to the results with an-

MjsvCL containing only the CTLD but lacking the Q/N-rich

region (Fig. 5D,). This result indicated that the Q/N-

expression of ie1 by M

neral region of MjsvCL mediates the interaction with CRT. (4) Promotion of VP28 expression by exogenous MjsvCL. (Upper panel) Western blot of VP28 expression; (lower panel) ImageJ was used to scan the Western blot bands from three independent repeats. Relative expression levels of VP28/β-actin are shown, and the value of the first lane was set as 1. Results were analyzed by Student t test. **p < 0.01. (C) Suppression of VP28 expression by silencing MjsvCL. (Upper panel) Western blot of VP28 expression; (lower panel) ImageJ was used to scan the Western blot bands from three independent repeats. Relative expression levels of VP28/β-actin are shown, and the value of the first lane was set as 1. Results were analyzed by Student t test and expressed as the mean ± SD. *p < 0.05, **p < 0.01, compared with control group. (D) Expression of rMjsvCL in E. coli. The protein was expressed with the pGEX-4T-1 vector in Rosseta (DE3) cells and purified by affinity chromatography. The GST tag was cut by thrombin. (E) Promotion of virus replication by exogenous MjsvCL, rMjsvCL (2 µg) or rGST tag as the control was injected i.m. in normal shrimp together with the viral inoculum (5 × 10^7 copies). The virus titer and VP28 expression were determined in the shrimp stomach, gills, and muscle at 24 and 48 h postinfection. Four shrimp were used to prepare each sample, and results are expressed as the mean ± SD derived from three independent repeats. Data were analyzed by Student t test. *p < 0.05, **p < 0.01. (F) Promotion of VP28 expression by exogenous MjsvCL. (Upper panel) Western blot of VP28 expression; (lower panel) ImageJ was used to scan the Western blot bands from three independent repeats. Relative expression levels of VP28/β-actin are shown, and the value of the first lane was set as 1. Results were analyzed by Student t test and expressed as the mean ± SD. *p < 0.05, **p < 0.01, compared with control group.

panel). By contrast, the CRT silencing decreased the viral infection–promoting activity of rMjsvCL to only 1.4-fold, compared with the effect of the rGST tag (Fig. 5C, right panel). This result indicated that the viral infection–promoting ability of MjsvCL relies on its receptor CRT.

To clarify whether the dependence of MjsvCL function on CRT was due to the CRT expression or MjsvCL–CRT interaction, we determined the critical region of MjsvCL responsible for WSSV infection. (A) Knockdown of MjsvCL expression. Shrimp were injected with MjsvCL dsRNA (20 µg) i.m., and equal amounts of GFP dsRNA served as a control. RNA and protein samples were collected at 24 h postinjection to check the silencing efficiency by qRT-PCR and Western blot with β-actin as a reference. Data are representative of two independent repeats. **p < 0.01. ***(p < 0.001. (B) Suppression of virus replication by silencing MjsvCL expression. Shrimp were preinjected with MjsvCL dsRNA (20 µg), and GFP dsRNA served as a control. The infection was performed 24 h later. Genomic DNA and protein samples from stomach and gills were obtained at 24 and 48 h postinfection to determine the virus titer. At least 10 shrimp were used for each sample, and results are expressed as the mean ± SD. Data were analyzed by Student t test. **p < 0.01. (C) Suppression of VP28 expression by silencing MjsvCL. (Upper panel) Western blot of VP28 expression; (lower panel) ImageJ was used to scan the Western blot bands from three independent repeats. Relative expression levels of VP28/β-actin are shown, and the value of the first lane was set as 1. Results were analyzed by Student t test and expressed as the mean ± SD. *p < 0.05, **p < 0.01, compared with control group. (D) Expression of rMjsvCL in E. coli. The protein was expressed with the pGEX-4T-1 vector in Rosseta (DE3) cells and purified by affinity chromatography. The GST tag was cut by thrombin. (E) Promotion of virus replication by exogenous MjsvCL, rMjsvCL (2 µg) or rGST tag as the control was injected i.m. in normal shrimp together with the viral inoculum (5 × 10^7 copies). The virus titer and VP28 expression were determined in the shrimp stomach, gills, and muscle at 24 and 48 h postinfection. Four shrimp were used to prepare each sample, and results are expressed as the mean ± SD derived from three independent repeats. Data were analyzed by Student t test. *p < 0.05, **p < 0.01. (F) Promotion of VP28 expression by exogenous MjsvCL. (Upper panel) Western blot of VP28 expression; (lower panel) ImageJ was used to scan the Western blot bands from three independent repeats. Relative expression levels of VP28/β-actin are shown, and the value of the first lane was set as 1. Results were analyzed by Student t test and expressed as the mean ± SD. *p < 0.05, **p < 0.01, compared with control group.

MjsvCL–CRT enhancement of virus infection is dependent on cholesterol

Our results thus far indicated that the MjsvCL–CRT pathway promotes virus infection, which can be determined by analyzing the expression of the WSSV ie1 gene. Because ie1 is an immediate early gene, its expression can be used to monitor viral entry into host cells. A previous study had shown that WSSV enters shrimp cells through caveolae-mediated endocytosis by treating shrimp cells with MβCD, which effectively inhibits this process by removal of cholesterol in the cell membrane (8). To determine whether MjsvCL–CRT–mediated viral entry is dependent on cholesterol, we used MβCD in this study to treat shrimp hemocytes (Fig. 6A). The MβCD treatment only slightly influenced the viability of the hemocyte culture, with an ∼75% survival rate upon treatment with the inhibitor at 2.5 mM (Fig. 6B). The dose-dependent blocking effect of virus infection by MβCD was first determined. The results showed that the expression of ie1 was gradually suppressed along with the increase in amount of the inhibitor, with full inhibition of ie1 expression observed at the dose of 2.5 mM (Fig. 6C). Further study found that the ability of rMjsvCL to facilitate the ie1 expression, normalized to the effect of control rGST tag, was inhibited by increasing amounts of MβCD (Fig. 6D). In other words, when membrane cholesterol was not fully removed, rMjsvCL in excess could still exert its function to a limited extent; however, full removal of cholesterol completely restrained the viral infection–promoting activity of MjsvCL. Furthermore, cholesterol replenishment, which could rescue the WSSV infectivity in MβCD-treated cells (Fig. 6E), also restored the viral infection–promoting activity of MjsvCL (Fig. 6F). These data suggested that the MjsvCL–CRT interaction facilitates viral entry into shrimp cells, a process that is dependent on cholesterol.
A mixture was maintained at 4˚C overnight. Protein A beads (30 μl) were added for further incubation for 1 h. The beads were washed and boiled, and then incubated with 30 μl antiserum to MjsvCL or VP28. The resins were eluted, and the resulting samples were analyzed by Western blot using antiserum against VP28 (right panel). Data are representative of two independent repeats. (B) Confirmation of the interaction between MjsvCL and VP28 by a co-IP assay. RIPA buffer was used to homogenize the infected gills and stomach to obtain the pool of VPs and MjsvCL. The resultant homogenate was precleared with protein A beads and then incubated with 30 μl antiserum to MjsvCL or VP28. The mixture was maintained at 4˚C overnight. Protein A beads (30 μl) were added for further incubation for 1 h. The beads were washed and boiled, and the resultant samples were probed in a Western blot with Abs of interest. Antiserum that was originally prepared for another shrimp CL but could not recognize any shrimp or VP was used as the control (IgG). Data are representative of two independent repeats. IB, immunoprecipitates were detected with the Ab specific for each panel; IP, proteins were immunoprecipitated with the Ab specific for each lane.

FIGURE 3. MjsvCL interaction with WSSV envelope protein VP28. (A) Determination of interactions of rMjsvCL with VP28 by pull-down assay. The homogenate of infected gills (3 ml), containing the pool of VPs, was incubated with 2 μg rGST-MjsvCL or rGST tag (left panel) for 3 h at 4˚C. GST resin (20 μl) was added to extract the recombinant protein and the interacting proteins. The resins were eluted, and the resulting samples were analyzed by Western blot using antiserum against VP28 (right panel). Data are representative of two independent repeats. (B) Confirmation of the interaction between MjsvCL and VP28 by a co-IP assay. RIPA buffer was used to homogenize the infected gills and stomach to obtain the pool of VPs and MjsvCL. The resultant homogenate was precleared with protein A beads and then incubated with 30 μl antiserum to MjsvCL or VP28. The mixture was maintained at 4˚C overnight. Protein A beads (30 μl) were added for further incubation for 1 h. The beads were washed and boiled, and the resultant samples were probed in a Western blot with Abs of interest. Antiserum that was originally prepared for another shrimp CL but could not recognize any shrimp or VP was used as the control (IgG). Data are representative of two independent repeats. IB, immunoprecipitates were detected with the Ab specific for each panel; IP, proteins were immunoprecipitated with the Ab specific for each lane.

Discussion
Compared with i.m. injections, oral delivery of WSSV more closely reproduces the natural route of infection in shrimp. The digestive tract is first targeted after oral infection by WSSV, and the stomach is one of the most highly infected organs (30). This information prompted us to clarify whether any CL, which may be involved in virus infection, participates in the WSSV invasion in the stomach. We successfully identified a WSSV-sensitive CL, MjsvCL, from the stomach of shrimp and further studied its function. Our results implicated its positive role in virus infection, unlike many virus-induced host proteins that have been found to be inhibitory against WSSV (11–14, 31, 32). The unique function of MjsvCL suggests that it can be manipulated for preventing viral infection, which would greatly benefit the aquaculture industry.

Shrimp have an open vascular system, allowing soluble proteins to be spread to the whole body through the circulating plasma. Although the MjsvCL protein was mainly detected in the stomach and muscle of shrimp, its secretion to the plasma suggested that its function is not limited to only the several tissues in which it is expressed. This reasoning also suggested that the discovery in this study is not stomach specific, but rather a common mechanism among shrimp tissues. Nevertheless, the function of MjsvCL in the stomach is particularly important, because the virus first targets this organ before systemic dissemination to other tissues. Therefore, the observed decrease in infectivity of WSSV in the gills after knockdown of MjsvCL may be attributed to both the suppressed viral dissemination from the stomach and the decreased secretion of MjsvCL into the plasma. In addition, although MjsvCL expression was induced from 6 and 12 h postinfection, its high basal expression, compared with those of other shrimp CLs (X.W. Wang and J.X. Wang, unpublished observations), may be sufficient to promote WSSV infection at the early stage. Moreover, the newly synthesized and secreted MjsvCL would help the virus to disseminate from the stomach and infect other tissues.

The basis of the viral infection–promoting activity of MjsvCL was also studied, and the data showed that this activity was related to the ability of MjsvCL to bind to the most abundant envelope protein of WSSV. For enveloped viruses, envelope proteins play vital functions during infection, especially for viral entry. For example, DENV attaches to the host cell by the binding of its E (envelope) protein to a cell-surface receptor (33). The E protein also mediates the fusion between viral and host membranes, which is critical to the release of genetic materials when the low-pH condition triggers the conformational change of the E protein in the endosomes (34). Furthermore, some inhibitors targeting the E protein can effectively block the infection of DENV (35). Studies on WSSV also have shown that envelope proteins determine at least the attachment to cell membranes (36, 37). Specifically, VP28 can allow the binding and rapid entry of the virus into shrimp cells (10). The Ab against VP28 has been shown to neutralize WSSV infection (9), and immunization with DNA vaccines encoding VP28 or recombinant VP28 could protect the host from WSSV infection (38–40). Because VP28 is the most abundant protein in the envelope, the binding of MjsvCL to VP28 is an effective strategy for the host to capture the virions. Usually, the recognition by viral receptors is made through binding glycans present on envelope proteins (41, 42). However, proteomic analyses of WSSV structural proteins found that none of them is glycosylated (43, 44), suggesting that the recognition of VP28 by MjsvCL is through a protein–protein interaction.

As a soluble receptor secreted out of the cells, the collaboration of MjsvCL with receptors is needed to present the virus to the cells, as in the case of other soluble CLs important for viral infection (18). Through a pull-down screening assay, CRT was found to interact with MjsvCL, and the interaction was further confirmed by a co-IP assay (Fig. 3). CRT is an important quality-control chaperone, mainly distributed in the endoplasmic reticulum (45). However, increasing lines of evidence have suggested that CRT is also localized to the cell surface and extracellular matrix, and plays diverse roles in biological processes, such as Ag processing and presentation, phagocytosis, and cell–cell adhesion (28). Some mammalian soluble CLs have been found to interact with the CRT present on the cell surface. For example, MBL can target apoptotic cells for phagocytosis by binding CRT, which is expressed on the surface of macrophages, and, in turn, bind to CD91 (29). This mechanism is also shared by other collectin family members, such as surfactant protein A and surfactant protein D (46). Similar to some MBL-like proteins identified in decapod crustaceans, MjsvCL also shares considerable similarity with human MBL (24). However, the mode of interaction of MjsvCL with CRT is unknown due to its lack of the collagenous region, which has been demonstrated to be the binding site of CRT. It is worth noting that MjsvCL contains a Q/N-rich region instead of the collagen domain. Interestingly, the truncated rMjsvCL lacking this region...
could not bind to CRT and exhibited a protective role for the host against WSSV infection, similar to the effect of two other CLs (MjLdlLec1, 2), which could not interact with CRT. These results partly indicated that the CRT binding site may be located within the Q/N-rich region of MjsvCL, and the interaction with CRT determined the virus infection–promoting ability of MjsvCL. This finding can also be supported by evidence that those other shrimp CLs (LvCTL1 and MjLecs), which do not contain the CRT-interacting region, show virus-blocking activities in contrast with the effect of MjsvCL (Supplemental Fig. 3) (12, 14). However, to confirm whether the Q/N-rich region is a determinant for the MjsvCL–CRT interaction still requires further structural analysis.

Nevertheless, it was interesting to uncover that CRT may be related to the function of MjsvCL during WSSV infection, because this protein has long been found to be inducible by WSSV infection in shrimp. For example, a previous study found that WSSV could consistently promote the synthesis of CRT by 3-fold, regardless of the temperature in a culture of Penaeus monodon (25). Similarly, another report found that CRT protein expression in the stomach of L. vannamei was significantly upregulated by WSSV infection (27). This study confirmed the inducible expression and cell-surface exposure of CRT. The increased amount of CRT, particularly on the cell surface, upon virus challenge implicated its important role in WSSV infection. Based on previous studies, several functions of CRT during the WSSV infection process have been proposed: 1) to modulate the intracellular calcium homeostasis, which is critical for both host defense and virus infection cycle (47); 2) to mediate the folding and translocation of large amounts of newly synthesized VPs (46); and 3) to form a complex with gC1qR to prevent virus-infected cells from apoptosis, thus maintaining a niche for viral replication (48). This study identified a new function of cell-surface CRT during WSSV infection by recruiting extracellular MjsvCL and determined its viral infection–promoting activity, as monitored by the expression of the WSSV ies1 gene. Because the expression of WSSV ies1 represents an early stage of the virus infection, CRT may be considered to have the novel function of facilitating viral entry.

FIGURE 4. Interaction of MjsvCL with cell-surface CRT. (A) Identification of MjsvCL-interacting proteins from host cells. The stomach homogenate (20 ml) was incubated with 20 μg rGST-MjsvCL or rGST tag with rotation at 4°C for 3 h. The mixture was then passed through a GST-resin column. The bound proteins were eluted and analyzed by SDS-PAGE. Bands of interest were excised and analyzed by MS/MS. Data are representative of two independent repeats. (B) Tissue distribution of CRT. The expression of CRT was detected by RT-PCR (upper panel) and Western blot (lower panel). Data are representative of two independent repeats. (C) Expression profiles of CRT in stomach after virus infection. (Upper panel) Western blot of CRT, representative of three independent repeats. (Lower panel) ImageJ was used to quantify the Western blot bands from three repeats. **p < 0.01. (D) Illustration of procedure for cell-surface protein detection. Hemocytes or stomach cells were labeled with sulfo-NHS-LC-biotin by incubation or perfusion and then lysed. The biotinylated cell-surface proteins were immunoprecipitated by streptavidin-agarose beads and analyzed by Western blot. (E) Detection of CRT on surface of hemocytes and stomach cells before and after WSSV infection. Data are representative of two independent repeats. (F) Confirmation of MjsvCL–CRT interaction by co-IP assay. The experiment was performed similarly to that for analyzing the interaction with VPs, except that the protein pool was obtained from healthy shrimp stomachs. The β-actin Ab and IgG were used as negative controls. Data are representative of two independent repeats.
Cholesterol has been found to be necessary for the infection of many types of viruses. For example, cholesterol depletion by treatment with MβCD was shown to result in a 100-fold suppression of West Nile virus infection, whereas addition of exogenous cholesterol restored the infection (49), similar to the finding in this study. Cholesterol is important for generating and maintaining the structure of caveolae, the critical endocytic vesicle used by many viruses, such as SV40 and tiger frog virus, to enter host cells via endocytosis (50, 51). By using MβCD to block infection and showing the colocalization of virions with early endosomes, a recent study claimed that WSSV also adopts caveolae-mediated endocytosis to break through the membrane barrier (8). Although our present findings did not definitively determine whether the MjsvCL–CRT–mediated viral entry occurs through caveolae-mediated endocytosis, our observations by depleting and restoring membrane cholesterol supported the possibility of such a mechanism.

In summary, our screening of CLs in the stomach of orally infected shrimp identified MjsvCL as a receptor of WSSV. The CTLD of MjsvCL confers the VP28-binding ability, and the Q/N-rich region links the MjsvCL–virus complex to the cell-surface CRT. CRT likely determines the function of MjsvCL in a cholesterol-dependent manner, possibly by linking the receptor–virus complex to caveolae-mediated endocytosis. The recognition by extracellular soluble MjsvCL allows the virions to be quickly captured and recruited to the cell membrane, thereby accelerating the infection process. In addition, the MjsvCL–virus complex can spread throughout the body in the circulating hemolymph to induce a systemic infection. This work is the first to report a viral infection–promoting CL in shrimp, which may be used as a target for prevention and control strategies against WSSV infection. Moreover, the present findings are helpful for understanding the entry mechanism of WSSV and its interaction with the host.
FIGURE 6. Dependence on cholesterol of MjsvCL-CRT pathway mediated facilitation of WSSV infection. (A) Culture and treatment of shrimp hemocytes in vitro. Shrimp hemocytes were collected and cultured in Leibovitz L-15 medium at 28°C for 10 h. MBCD was then incubated with the cells for 20 min. Scale bars, 20 μm. (B) Cell survival rate with MBCD treatment. Cells were treated with different doses of the inhibitor, washed, and stained with 2 mg/ml trypan blue for 2 min. The total and dead cells were counted to determine the cell survival rate. A total of 600 cells was counted. Data were subjected to one-way ANOVA and are presented as the mean ± SD from three independent repeats. Different characters above each column represent significant difference among columns and same characters above different columns show no significant difference. (C) Dependence of WSSV entry on cholesterol. Shrimp hemocytes were cultured in 24-well plates. MBCD was added to the final concentrations of 0.05, 0.1, 0.2, 0.5, 1, and 2.5 mM/well. Twenty minutes later, the viral stock (10^6 copies/well) was added for a 3-h infection period; then the expression of ie1 was detected by qRT-PCR. Data are representative of two independent repeats. (D) Blocking of viral infection–promoting ability of MjsvCL by MBCD treatment. Shrimp hemocytes were pretreated with MBCD at various concentrations. The viral stock (10^6 copies) was added to each well with or without MjsvCL (1 μg) or rGST tag. The expression of ie1 was detected 3 h later. The viral infection–promoting ability was determined by calculating the fold change in ie1 expression in the experimental group (MjsvCL) compared with that of the control group (rGST) for the same dose of inhibitor. Data were analyzed by one-way ANOVA, and results are expressed as the mean ± SD derived from three independent experiments. Different characters above each column represent significant difference among columns and same characters above different columns show no significant difference. (E) Restoration of WSSV infection by cholesterol replenishment in MBCD-treated cells. Cholesterol replenishment was performed by changing the MBCD-containing medium to fresh medium with 400 μg/ml water-soluble cholesterol for a 1-h incubation. Virus infection was performed and detected as described earlier. Data are representative of two independent repeats. (F) Restoration of WSSV infection–promoting ability of MjsvCL by cholesterol replenishment in MBCD-treated cells. After cholesterol replenishment was performed, the function of MjsvCL was analyzed as described earlier. Results are expressed as the mean ± SD derived from three independent experiments, and differences were calculated. The different characters above each column represent significant difference among columns and the same characters above different columns show no significant difference.

Disclosures

The authors have no financial conflicts of interest.

References


