A 32 kDa viral attachment protein of lymphocystis disease virus (LCDV) specifically interacts with a 27.8 kDa cellular receptor from flounder (Paralichthys olivaceus)

Ying Zhong,¹ Chenjie Fei,¹ Xiaoqian Tang,¹ Wenbin Zhan¹,² and Xiuzhen Sheng¹,*

Abstract

The 27.8 kDa protein in flounder gill (FG) cells was previously proved to be a receptor specific for lymphocystis disease virus (LCDV) entry and infection. In this paper, a 32 kDa viral attachment protein (VAP) of LCDV specifically binding to the 27.8 kDa receptor (27.8R) was found by far-Western blotting coupled with monoclonal antibodies (MAbs) against 27.8R. The 32 kDa protein was confirmed to be encoded by the open reading frame (ORF) 038 gene in LCDV-C, and predicted to contain a putative transmembrane region, multiple N-myristoylation and glycosylation sites and phosphorylation motifs. The expression plasmid of pET-32a-ORF038 was constructed and the recombinant VAP (rVAP) was obtained. Rabbit polyclonal antibodies against the rVAP were prepared and could recognize the rVAP and 32 kDa protein in LCDV. Immunogold electron microscopy showed that the 32 kDa protein was located on the surface of LCDV particles. Immunofluorescence assay demonstrated that the rVAP could bind to the 27.8R on the cell membrane of the FG monolayer and the anti-27.8R MAbs could block the rVAP binding. Pre-incubation of the rVAP with FG cells before LCDV infection, or pre-incubation of LCDV with the antibodies against the rVAP, could significantly decrease the LCDV copy numbers (P<0.05) and delay the emergence of cytopathic effects in FG cells in a dose-dependent manner. These results indicated for the first time that the 32 kDa protein functioned as an attachment protein for the initial attachment and entry of LCDV, and the interaction of the 32 kDa VAP with the 27.8R-initiated LCDV infection.

INTRODUCTION

Lymphocystis disease virus (LCDV), belonging to the genus Lymphocystivirus in the family Iridoviridae, is the causative agent of lymphocystis disease, which has affected more than 140 different freshwater, brackish-water and seawater fish species worldwide, causing great economic losses to the fish-farming industry [1–3]. In China, lymphocystis disease is often observed in cultured fish, especially flounder Paralichthys olivaceus, an economically important fish species in the aquaculture industry. It rarely causes death, but the diseased fish become more susceptible to secondary infection by other micro-organisms, resulting in high mortality [4]. LCDV is a large icosahedral DNA virus with an outer envelope and a fringe of fibril-like external protrusions [5, 6], and the envelope plays an important role in viral infection, replication and pathogenesis [7]. The complete genomes of two LCDV isolates, LCDV-1 from the flounder P. flesus in Europe [8] and LCDV-C from P. olivaceus in China [9], have been sequenced and annotated, and the information concerning genome architecture and genetic characterization enables us to explore the mechanism underlying interaction between LCDV and the hosts. In addition, some works regarding the cellular receptors mediating LCDV infection [10, 11] and host responses to lymphocystis cell formation [12] have been reported recently. However, the viral proteins involved in the LCDV-host interaction are largely unknown. Studies on the attachment
proteins on the viral envelope and the cellular receptors in hosts are of great significance to clarify the molecular mechanism of viral infection and pathogenesis.

For enveloped viruses, the principal route of entry is via a combination of receptor binding and fusion. A specific binding of viral attachment proteins (VAPs) to the cellular receptors of host cells, which are adsorbed on the surface of target cells, results in structural changes to the virus envelope protein and release of the virus genetic material into cells [13]. So far, in the study of fish iridovirus, the infection mechanisms for the enveloped virus in grouper iridovirus and Rana grylio virus (RGV) have attracted more research data, and several viral envelope proteins and genes associated with viral infection of the host cells have been identified [14–17], but information on the envelope proteins associated with LCDV infection is scarce. In previous research, a 27.8 kDa protein in flounder gill (FG) cells was identified as the putative receptor for LCDV attachment and infection [10]. Monoclonal antibodies (MAbs) against the 27.8 kDa protein were able to efficiently inhibit LCDV infection of FG cells [18], and the 27.8 kDa receptor protein was co-localized with LCDV in FG cells post-LCDV infection [19]. Moreover, this receptor was widely detected in the tissues of P. olivaceus [20] and the turbot Scophthalmus maximus [21]. In this paper, the MAbs 2G11 and 3D9 against the 27.8 kDa receptor protein, coupled with far-Western blotting, were used to identify the VAP of LCDV, and a 32 kDa envelope protein that could specifically bind to 27.8 kDa receptor protein was identified and indicated to function as an attaching protein for initial entry of LCDV by using the recombinant VAP (rVAP) and anti-rVAP polyclonal antibody. This study will lead to a better understanding of the molecular mechanisms of the viral pathogenesis and LCDV–host interactions, and provide a potential antiviral agent that targets LCDV entry.

RESULTS
Identification of an LCDV protein that binds the 27.8 kDa cellular receptor
To identify the LCDV protein that binds the 27.8 kDa cellular receptor, far-Western blotting was conducted. Purified viral particles were subjected to SDS-PAGE, transferred to PVDF membranes, and then incubated with FG cell membrane protein and anti-27.8 kDa receptor protein MAbs. FG cell cytoplasmic protein and the membrane protein of epithelial papillosum cells of carp (EPC), instead of FG cell membrane protein, were used separately as negative controls. The results of far-Western blotting showed that only one band with a molecular mass of 32 kDa in LCDV could specifically bind to the 27.8 kDa receptor protein in FG cells, which was recognized by the anti-27.8 kDa receptor MAbs (Fig. 1, lane 2). There were no bands in the negative controls (Fig. 1, lanes 3 and 4), revealing that the 32 kDa protein was the viral attachment protein that reacted with the 27.8 kDa receptor protein. Western blotting of LCDV and anti-27.8 kDa receptor MAbs showed no bands, indicating that the purified LCDV from tissues was not contaminated by the 27.8 kDa receptor protein (Fig. 1, lane 5).

The 32 kDa protein was subjected to mass spectrometry (MS) analysis and the MS result was compared against the Mascot database. A protein score over 73 was considered significant (P<0.05) in this experiment. The results demonstrated that the 32 kDa protein was encoded by the open reading frame (ORF) 038 gene of LCDV-C, and the GenBank accession number was gi|51869993 (Fig. 2a, b). Sequence analysis showed that the LCDV-C ORF038 gene was of 933 bp and encoded a 310 amino acid protein, with a theoretical molecular mass of 34.349 kDa and an isoelectric point of 9.08. The DAS server predicted that a putative transmembrane region was formed by amino acid residues 291 to 308. The 32 kDa protein was predicted by ScanProsite to contain three N-myristoylation sites and three N-glycosylation sites, and 14 possible phosphorylation motifs, including seven protein kinase C phosphorylation sites, five casein kinase II phosphorylation sites and two tyrosine kinase phosphorylation sites (Fig. 2c).

Recombinant expression of VAP and characterization of polyclonal antibody against rVAP
A 933 bp gene segment was amplified from LCDV-HD samples by PCR, and was accordant with the ORF038 gene and showed 100% identity to the reported LCDV-C genome’s
Fig. 2. Mass spectrographic and bioinformatics analysis of the 32 kDa protein in LCDV. (a) Fingerprint of the 32 kDa protein. (b) Mass spectrographic analysis of the fingerprint of the 32 kDa protein using Mascot; a protein score greater than 73 was significant (P<0.05). Accession information was as follows: GenBank accession number: gi|51869993; score: 82; description: poxvirus proteins of hypothetical protein function (lymphocystis disease virus – isolate China). (c) LCDV-C ORF038 gene nucleotide sequence and the 32 kDa protein sequence. The putative N-myristoylation sites are shown in italic, the N-glycosylation residues are underlined and the phosphorylation motifs are marked in boldface. The putative transmembrane region is boxed.
relevant nucleotide sequence. The constructed recombinant expression plasmid pET-32a-ORF038 was transformed into \textit{E. coli} BL21 (DE3), and the expression of the rVAP was induced by isopropyl-\(\beta\)-D thiogalactopyranoside (IPTG) (Fig. 3, lanes 1 and 2). SDS-PAGE analysis showed the enhancement of a protein band with a molecular mass of approximately 50 kDa, which was consistent with the expected molecular mass of recombinant 32 kDa protein plus a his-protein tag of about 18 kDa. Purification of rVAP by his-tag chromatography yielded a single protein of the anticipated 50 kDa size (Fig. 3, lane 3).

The LCDV total protein and purified rVAP were subjected to SDS-PAGE and detected by the developed rabbit anti-rVAP antibody in Western blotting. Specific reaction bands were observed at a molecular mass of 32 kDa in LCDV and 50 kDa recombinant protein (Fig. 4, lanes 3 and 5), respectively, whereas no bands appeared in the negative controls (Fig. 4, lanes 4 and 6), showing that the developed rabbit polyclonal antibody had good specificity.

**Location of the 32 kDa protein in LCDV using immunogold electron microscopy**

The polyclonal antibody against rVAP was used as a probe to locate the 32 kDa protein in LCDV, and then detected with gold-labelled anti-rabbit IgG. The results for immunogold electron microscopy showed that the gold particles were located on the outermost surface of the LCDV particles; the visual background was clean and did not contain scattered gold particles (Fig. 5a). No gold particles were observed in the negative control using rabbit pre-immune serum as the primary antibody (Fig. 5b).

**Binding of rVAP to the 27.8 kDa receptor in FG cells**

To investigate if the VAP could bind to FG cells, FG cells were grown on circular coverslips in 24-well plates and incubated with fluorescein isothiocyanate (FITC)-labelled rVAP. Under an inverted fluorescence microscope, a green fluorescence signal was present on the surface of FG cells (Fig. 6a), suggesting that the rVAP could bind to FG cells. No positive fluorescence signal was observed in the negative control, which used FITC-labelled VP26 of white spot syndrome virus (WSSV) to replace FITC-labelled rVAP (Fig. 6b).

To determine whether the rVAP directly bound to the 27.8 kDa receptor, the FG cells were pre-incubated with mouse anti-27.8 kDa receptor protein MAbs, and then incubated with FITC-labelled rVAP or FITC-conjugated goat-
anti-mouse Ig. The results showed that no positive green fluorescence signal appeared on FG cells after incubation with FITC-labelled rVAP (Fig. 6c), but the negative control, which was pre-incubated with supernatant of myeloma cells instead of mouse anti-27.8 kDa receptor protein MAbs, showed a green fluorescence signal on the surface of the FG cells (Fig. 6d), revealing that pre-incubation with anti-27.8 kDa receptor protein MAbs was able to block the rVAP binding to the cellular receptor on FG cells. By contrast, when the FG cells were incubated with anti-27.8 kDa receptor protein MAbs and then FITC-conjugated goat-anti-mouse Ig, positive green fluorescence was observed on the FG cell membrane (Fig. 6e), but no green fluorescence signal was seen in the negative control, which used supernatant of myeloma cells instead of anti-27.8 kDa receptor protein MAbs (Fig. 6f), which suggests that the 27.8R was located on the FG cell membrane. FG cells were counterstained in red using Evan’s blue dye.

**DISCUSSION**

Virus infects host cells in steps through attachment/adsorption, penetration, uncoating, biosynthesis, assembly, release and maturation [22, 23]. Non-enveloped viruses penetrate
Fig. 7. Binding competition of rVAP with LCDV and virus neutralization assay in vitro. (a) The standard curve of qPCR assay for LCDV ORF038 gene copy numbers. (b) Pre-incubation of FG cells with increasing concentrations of rVAP before LCDV infection decreased the number of LCDV copies in FG cells in a dose-dependent manner. (c) Pre-incubation of LCDV with anti-rVAP polyclonal antibody prior to viral infection of FG cells decreased the number of LCDV copies in FG cells significantly with the increase of antibody concentration. Error bars represent standard deviations (SD). Different letters denote significant differences among groups (P<0.05).

Fig. 8. In vitro rVAP and anti-rVAP polyclonal antibody inhibiting LCDV infection into FG cells showing cytopathic effect (CPE) development. The CPE was monitored at 24 h (a–d, a1–d1) and 48 h (e–h, e1–h1). FG cell cultures were incubated with 100, 10 and 1 µg ml⁻¹ rVAP (a–c, e–g) before LCDV infection. FG cells without incubation of rVAP (0 µg ml⁻¹) were infected with LCDV to serve as the positive control (d, h), while FG cells without LCDV infection served as the negative control (i, j). LCDV was incubated with 200, 20 and 2 µg ml⁻¹ (a1–c1, e1–g1) anti-rVAP polyclonal antibodies and then used to infect FG cells; non-immunized rabbit serum replaced polyclonal antibodies for the positive control (d1, h1) and FG cells without LCDV infection served as the negative control (i1, j1). Arrows: dying cells; *: CPE. Bars, 100 µm.
cells by endocytosis under the mediation of clathrin, while the entry of enveloped viruses relies on the fusion of the virus envelope with the cytomembrane, which then promotes the penetration of the virus genome into host cells [24, 25]. Virus attachment consists of the specific binding of a viral attachment protein to a cellular receptor [26]. Using pull-down and far-Western overlay assays, it was found that the WSSV envelope proteins VP28, VP26 and VP24 interact with prohibitins [27]. The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus were confirmed to interact with the receptor CD163 by co-immunoprecipitation [28], and the VP1 capsid protein of enterovirus 71 was found to interact with the receptor nucleolin through immunoprecipitation [29]. For LCDV infection, a 27.8 kDa receptor protein in FG cells could specifically mediate LCDV entry, and the anti-27.8 kDa protein MAbs were able to efficiently inhibit LCDV infection of FG cells [10, 18], but no study on the attachment protein of LCDV has been reported. In this paper, a 32 kDa protein of LCDV specifically binding to the 27.8 kDa receptor protein was identified for the first time using far-Western blotting, a widely employed approach for detecting protein–protein interactions [30–32], coupled with MAbs against the 27.8 kDa receptor protein. The 32 kDa protein was confirmed to be encoded by the ORF038 gene of LCDV-C [9] and appeared to function as an attaching protein for LCDV infection via interaction with the 27.8 kDa receptor protein. Through bioinformatics analysis, the 32 kDa protein of LCDV was predicted to have multiple putative myristoylation, phosphorylation and glycosylation sites. Myristoylation plays an essential role in protein–protein interactions, and in picornavirus the capsid protein VP4 is also myristoylated, which allows the protein to be inserted into the cell membrane when the virus attaches to a receptor [33]. Viral glycoprotein is known to be responsible for cell tropism, spreading infection and pathogenicity, and the phosphorylation of viral proteins plays important roles in initiating virus infections [7, 34]. It is common for moieties to modify the acetyl and formyl groups, phosphate groups, carbohydrates and lipids by covalent binding to the protein [35]. The VP088 envelope protein relating to grouper iridovirus 2L, our studies on the ORF038 gene encoding the 32 kDa protein is essential for virus infection. Since a unique homologue and some degree of sequence similarity existed between the LCDV ORF038 gene, SGIV VP19 and RGV envelope protein 2L, our studies on the ORF038 gene encoding the 32 kDa VAP will provide new insights into its roles in iridovirus pathogenesis.

In this paper, the rVAP was produced through prokaryotic expression, and polyclonal antibody against rVAP, which could recognize the recombinant protein and a 32 kDa protein in LCDV-HD, was prepared. The 32 kDa protein was found to localize on the surface of LCDV-HD particles by using the anti-rVAP antibody, suggesting that the ORF038 gene encoding the 32 kDa protein is an envelope protein, which is consistent with the previous prediction of ORF038 in the LCDV-C complete genome sequence made by Zhang et al. [9]. Of course, Western blotting of detergent-extracted LCDV particles will help to establish the subviral location in the envelope of the 32 kDa protein, but further research is required. Although the banding pattern of LCDV in different experiments was not completely consistent in the present paper, immunogold electron microscopy and Western blotting of LCDV particles with MAbs against 27.8 kDa receptor indicated that the purity degree of LCDV particles was high, and that the viruses were not contaminated by 28.8R from tissues, so the difference might result from the experimental operation and conditions. An immunofluorescence assay showed that the 27.8 kDa receptor protein was located at the FG cell membrane, and that the rVAP could bind to FG cells, while pre-incubation of the anti-27.8 kDa receptor MAbs could block rVAP binding to FG cells. This indicated that rVAP can specifically bind to the 27.8 kDa receptor protein on the cell surface. Envelope proteins play important roles in the process of virus invasion of host cells [38]. Viruses attach to cells as a result of the binding of a viral attachment protein to a site on a host-cell receptor. The literature indicates that pre-incubation of the recombinant domain III from the envelope glycoprotein of West Nile virus can inhibit virus entry [39]. Further, pre-incubation of polyclonal antibody against envelope protein with grouper iridovirus can decrease the virus titre significantly [14]. In the present study, pre-incubation of rVAP with FG cells was able to decrease the LCDV copy numbers and delay the occurrence of CPE, while a positive correlation between the inhibition effect and the increasing rVAP concentration was observed. These results revealed that the rVAP attaching to FG cells could compete with LCDV particles for the 27.8 kDa receptor, resulting in the inhibition of LCDV infection. Similarly, pre-incubation of increasing concentrations of anti-rVAP antibodies with LCDV reduced the LCDV copies and delayed CPE development in FG cells in a dose-dependent manner, which suggested that blocking the 32 kDa protein could abrogate LCDV infectivity. These
results indicated that the 32 kDa protein is an attaching protein responsible for the initial attachment of LCDV, which is mediated by specific interaction with the 27.8 kDa cellular receptor. However, even in the presence of a higher concentration of rVAP or anti-rVAP antibodies, a slight CPE still occurred, which suggested that blocking the interaction of the 27.8 kDa receptor with 32 kDa viral attachment protein was unable to completely inhibit the LCDV infection of FG cells. Therefore, some other attachment proteins in LCDV or FG cell receptor proteins may exist and mediate virus infection. In previous research, a 37.6 kDa receptor protein was found in FG cells by virus overlay protein binding assay [11], so the interaction of the 37.6 kDa receptor protein with the viral attachment protein of LCDV requires further research. In addition, the number of LCDV genome copies in the respective control groups in the rVAP and anti-rVAP polyclonal antibody inhibiting assay showed an obvious difference; this was because the two experiments were not performed at the same time, and the status of the FG cells might have been different, resulting in the LCDV genome copy discrepancy, so such a difference in replication as assessed by qPCR was considered normal.

DNA vaccines and inactivated vaccines against LCDV have been reported that can offer immune protection against lymphocystis disease [40, 41]. Since rVAP and anti-rVAP antibodies can inhibit LCDV infection of FG cells, they might be promising inhibitors to block the LCDV–receptor binding as an antiviral target. More specifically, the rVAP might be developed into a subunit vaccine as a receptor-binding agent to interfere with LCDV entry, and the anti-rVAP antibodies might be used as a VAP-binding agent to neutralize LCDV. Further, the 32 kDa VAP eukaryotic plasmid might be developed as a DNA vaccine to prevent LCDV infection. Therefore, it is worth performing further studies.

In conclusion, our results showed that a 32 kDa viral attachment protein specifically interacting with the 27.8 kDa cellular receptor participated in the initial attachment of LCDV, and that it was encoded by the LCDV-C ORF038 gene. The rVAP was able to bind to FG cells and compete with LCDV particles for the 27.8 kDa receptor. LCDV infection was inhibited by pre-incubation of rVAP with FG cells or pre-incubation of anti-rVAP antibody with LCDV particles. These results provide a new insight into the detailed molecular mechanism of LCDV cell entry, and offer a new way to prevent LCDV infection.

**METHODS**

**Virus, cells and monoclonal antibodies**

The flounder *P. olivaceus*, with obvious lymphocystis nodules on the body surface, were obtained from a farm in Qingdao, Shandong province of China, and the virus strain named LCDV-HD (GenBank accession number: DQ279090) was isolated [42]. The LCDV particles were extracted from the nodules and purified as described by Cheng et al. [43]. In short, 10 g lymphocystis nodules were homogenized in 90 ml TNE buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.01 M EDTA, pH 7.4) at 4 °C. The suspension was centrifuged at 680 g and then at 1800 g for 20 min, respectively. The supernatant was collected and dissolved into 30 % (w/w) sucrose solution, and further ultra-centrifuged at 48 000 g for 2 h. The precipitation was resuspended with 4 ml TNE buffer and stirred overnight at 4 °C. Subsequently, the viral suspension was ultra-centrifugated at 48 000 g for 2 h in sucrose discontinuous density gradients (33, 40, 47, 52, 57 and 62 % in TNE buffer, w/w), and the virus band in the 47–52 % sucrose gradient was harvested with a syringe, followed by addition of TNE solution and centrifugation at 48 000 g for 2 h to remove residual sucrose. The pellet was finally resuspended in 200 µl TNE buffer and stored at −80 °C until use.

The FG cell line was grown in minimal essential medium (MEM, Gibco, Germany) supplemented with 10 % of fetal bovine serum (FBS, Gibco), 100 IU ml$^\text{−1}$ penicillin and 100 µg ml$^\text{−1}$ streptomycin (Gibco), and cultivated at 22 °C with 2 % CO$_2$. The FBS was reduced to 2 % in maintenance medium after the cells had been infected with LCDV. The EPC cell line, which proved to be non-susceptible to LCDV infection [44], was cultured in medium 199 (M199, Gibco), supplemented with 10 % FBS, 100 IU ml$^\text{−1}$ penicillin and 100 µg ml$^\text{−1}$ streptomycin as above, and cultivated at 22 °C.

The mouse anti-27.8 kDa receptor protein MAbs (3D9 and 2G11) had been produced by our laboratory earlier [18].

**Preparations of cell membrane protein**

The cell membrane protein of the FG cell and EPC cell lines was prepared as described by Wang et al. [10]. Monolayer cultures of FG cells and EPC cells were washed three times with phosphate buffered saline (PBS) and harvested using a cell scraper, then centrifuged at 1000 g for 10 min at 4 °C. The pellet was resuspended in ice-cold NP-40 lysis buffer (Beyotime, China) containing 1 % phenylmethanesulfonyl fluoride (PMSF, Beyotime) and cracked for 3 h at 4 °C. After differential centrifugation at 1000 g for 10 min, 10 000 g for 20 min and 100 000 g for 40 min, the pellet was resuspended with PBS. The supernatant was cytoplasmic protein. The protein concentrations were determined by the BCA protein assay kit (Beyotime) following the manufacturer’s instructions, adjusted to 1 mg ml$^\text{−1}$ and stored at −80 °C before use.

**Far-Western blotting and mass spectrometry analysis**

20 µl LCDV particles were mixed with an equal volume of protein loading buffer (Takara, Japan), denatured in boiling water for 5 min, and then subjected to SDS-PAGE; one was stained with Coomassie blue, and the others were transferred to polyvinylidifluoride (PVDF) membranes (Millipore, USA). The membranes were blocked overnight with 4 % bovine serum albumin (BSA, Sigma, USA) at 4 °C and washed three times with PBST (PBS containing 0.05 % Tween-20), followed by incubation with the cell membrane proteins of FG cells with a concentration of 1 mg ml$^\text{−1}$ for...
Recombinant expression and purification of viral attachment protein

Based on the results of MS analysis and sequence alignment of the identified VAP, the specific primers were designed according to the sequence of the LCDV-C ORF 038 gene, ORF038F: 5'-GGGATCCGATGTCTGTCATAG-GAT-3' and ORF038R: 5'-CCTCGAGGGCTAAAAAG TCAAATAA-3' (the underlined letters represent the additive restriction sites BamHI and XhoI). The LCDV DNA was extracted using the TIANamp Marine Animals DNA kit (Qiagen, Germany) and then the target gene was amplified. The synthesized gene was digested with BamHI and XhoI restriction enzymes (Takara, Japan), and the fragment was purified from restriction enzyme buffers by the Gel purification apparatus (Takara, Japan) separately and denatured for 5 min at 100 °C. The mixtures were first separated by SDS-PAGE and then transferred to PDVF membranes; the polyclonal antibodies against rVAP served as the primary antibody and the AP-conjugated goat-anti-rabbit IgG as the secondary antibody.

According to the positive band in the PDVF membrane, the relevant 32 kDa band was excised from the gels, reduced and alkylated by 10 mM dithiothreitol and 55 mM iodoacetamide for protein identification using MS by Shanghai Sangon Biotech (China). The sequence of VAP that specifically bound to the 27.8 kDa cellular receptor was obtained according to the results of amino acid sequence alignment, and then the identified VAP was subjected to bioinformatics analysis. The predictions for transmembrane domain, physical and chemical parameters were performed with the dense alignment surface (DAS) transmembrane prediction server [45] and ProtParam tool of ExPasy [46]. The protein motifs and potential post-translational modification sites were analysed with the ScanProsite database [14].

Immunogold electron microscopy

To confirm that the 32 kDa protein was located on the envelope of LCDV, 10 µl of purified LCDV was dropped onto a copper grid, which was covered with formvar membrane, and left to stand for 5 min to absorb excess virus suspension. After being washed three times with PBS, the membrane was blocked with 2% BSA at 37 °C for 1 h and washed again, and then rabbit polyclonal antibodies (1:2000) against rVAP were added and incubated at 37 °C for 1 h and washed as above. Following the addition of goat-anti-rabbit IgG (1:100) conjugated with 15 nm colloidal gold (Sigma, USA) and incubation at 37 °C for 45 min, the sample was washed three times with PBS and distilled water, stained with 2% phosphotungstic acid for 1 min and observed under an electron microscope. Rabbit pre-immune serum instead of polyclonal antibodies against rVAP served as negative control. The specificity of the developed antibody was determined with Western blotting. The LCDV particles and purified rVAP were mixed with protein loading buffer (Takara, Japan) separately and denatured for 5 min at 100 °C. The mixtures were first separated by SDS-PAGE and then transferred to PDVF membranes; the polyclonal antibodies against rVAP served as the primary antibody and the AP-conjugated goat-anti-rabbit Ig (Sigma, USA) served as the secondary antibody. Finally, the membranes were placed into substrate solution containing NBT/BCIP for staining.

β-D thiogalactopyranoside (IPTG, 1:100) (Solarbio, China). The bacteria suspension was centrifuged at 7000 g for 15 min, washed with PBS and then resuspended in binding buffer (100 mM Na₂HPO₄, 12 mM NaH₂PO₄, 500 mM NaCl, 8 M urea and 20 mM imidazole, pH 7.4). The rVAP was purified by elution buffer (100 mM Na₂HPO₄, 12 mM NaH₂PO₄, 500 mM NaCl, 8 M urea and 500 mM imidazole, pH 7.4) using His-trap Ni-NTA affinity chromatography (GE Healthcare, USA) and a protein purification apparatus (GE Healthcare) according to the manufacturer’s instructions.

Polyclonal antibody preparation and specificity analysis

The polyclonal antibody against the purified rVAP was prepared by immunizing a New Zealand white rabbit by injecting 1.2 mg recombinant proteins emulsified with complete Freund’s adjuvant (Sigma, USA, 1:1) at six sites in the rabbit backside, and three booster immunizations were given after 14 d, 21 d and 28 d. One week after the last immunization, blood was obtained from the rabbit heart and placed at room temperature for 1 h and then at 4 °C overnight. The samples were centrifuged at 10 000 g for 30 min, and the supernatant was collected and purified using the caprylic acid–ammonium sulfate precipitation method [47], before being completely dialysed in PBS at 4 °C and stored at −80 °C until use.

The specificity of the developed antibody was determined with Western blotting. The LCDV particles and purified rVAP were mixed with protein loading buffer (Takara, Japan) separately and denatured for 5 min at 100 °C. The mixtures were first separated by SDS-PAGE and then transferred to PDVF membranes; the polyclonal antibodies against rVAP served as the primary antibody and the AP-conjugated goat-anti-rabbit Ig (Sigma, USA) served as the secondary antibody. Finally, the membranes were placed into substrate solution containing NBT/BCIP for staining.

Based on the results of MS analysis and sequence alignment of the identified VAP, the specific primers were designed according to the sequence of the LCDV-C ORF 038 gene, ORF038F: 5’-CGGATCCGATGTCTGTCATAG-GAT-3’ and ORF038R: 5’-CCCTCGAGGGCTAAAAAG TCAAATAA-3’ (the underlined letters represent the additive restriction sites BamHI and XhoI). The LCDV DNA was extracted using the TIANamp Marine Animals DNA kit (Qiagen, Germany) and then the target gene was amplified. The synthesized gene was digested with BamHI and XhoI restriction enzymes (Takara, Japan), and the fragment was purified from restriction enzyme buffers by the Universal DNA purification kit (Tiangen, China) and ligated into BamHI/XhoI double-digested pET-32a prokaryotic expression vector using T4 DNA ligase (Takara, Japan). The recombinant plasmid pET-32a-ORF038 was transformed into competent cells of Escherichia coli BL21 (DE3) (Takara, Japan) and cultured in LB medium. The positive clones were further confirmed by colony PCR and DNA sequencing by Shanghai Sangon Biotech (China). The expression of rVAP was induced at an optical density (OD 600) of 0.6 at 37 °C for 8 h by adding 100 mM isopropyl-

4 h at 22 °C and the mixed anti-27.8 kDa receptor protein MAbs (2G11:3D9=1:1, v/v) was diluted 1:1000 in PBS for 1 h at 37 °C. After being washed with PBST, the membrane was incubated with alkaline phosphatase (AP)-conjugated goat-anti-mouse IgG (Sigma) diluted 1:4000 in PBS for 50 min at 37 °C, and placed into substrate solution (100 mM NaCl, 100 mM Tris and 5 mM MgCl₂, pH 9.5) containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates (NBT/BCIP, Sigma). The cytoplasmic protein of FG cells and the cell membrane protein of EPC cells, instead of the FG cell membrane protein, served as negative controls. To exclude the condition where the purified virus contains 27.8 kDa receptor protein from tissue proteins, Western blotting of LCDV and anti-27.8 kDa receptor MAbs was conducted. Briefly, one of the PDVF membranes was incubated with the mixed anti-27.8 kDa receptor protein MAbs as the primary antibody and AP-conjugated goat-anti-mouse IgG as the secondary antibody.

According to the positive band in the PDVF membrane, the relevant 32 kDa band was excised from the gels, reduced and alkylated by 10 mM dithiothreitol and 55 mM iodoacetamide for protein identification using MS by Shanghai Sangon Biotech (China). The sequence of VAP that specifically bound to the 27.8 kDa cellular receptor was obtained according to the results of amino acid sequence alignment, and then the identified VAP was subjected to bioinformatics analysis. The predictions for transmembrane domain, physical and chemical parameters were performed with the dense alignment surface (DAS) transmembrane prediction server [45] and ProtParam tool of ExPasy [46]. The protein motifs and potential post-translational modification sites were analysed with the ScanProsite database [14].
Recombinant viral attachment protein binding assay

For the rVAP binding assay, the FITC-labelled rVAP was prepared according to the method described by Liu et al. [48]. The purified rVAP was adjusted to a concentration of 1 mg ml\(^{-1}\) and mixed with 1 mg ml\(^{-1}\) FITC (Sangon Biotech, China) solution containing 0.85 g FITC powder dissolved in 0.85 ml dimethyl sulfoxide before being incubated at room temperature overnight in a dark place with intermittent rocking; the mixture was then poured into a HisTrap HP column and equilibrated in 20 volumes of PBS for 24 h. Finally, the concentration of FITC-labelled rVAP was adjusted to 1 mg ml\(^{-1}\) with PBS containing 1 % BSA. Monolayer FG cells were grown on circular coverslips (Solarbio, China) in 24-well plates and washed gently with PBS after the medium had been removed. Following the addition of 200 µl FITC-labelled rVAP and incubation at 4°C for 1 h in the dark, the FG cells were washed three times in PBS and observed with an inverted fluorescence microscope. Evan’s blue dye (Fluka, Lyon, France) was used to counterstain the cell protein. Recombinant pET28a-VP26 of WSSV produced by our laboratory [48] replaced LCDV rVAP as the negative control.

To investigate whether rVAP interacted directly with 27.8 kDa cellular receptors, the FG cells grown on circular coverslips in 24-well plates were first incubated with anti-27.8 kDa receptor protein MAbs (2G11: 3D9=1:1, v/v) with a concentration of 200 µg ml\(^{-1}\) at 4°C for 1 h. Following three washes in PBS, the FG cells were incubated with FITC-labelled rVAP or FITC-conjugated goat-anti-mouse Ig (Sigma, USA) as described above. FG cells were washed again and observed with an inverted fluorescence microscope. A supernatant of myeloma cells (P3-X63-Ag8U1, P3U1) instead of anti-27.8 kDa receptor protein MAbs served as the negative control.

In vitro competition assay between rVAP and LCDV

The FG cells were grown separately in 75 cm\(^2\) cell-culture flasks and 24-well plates at 22°C. After monolayer cultures of FG cells were washed twice with MEM without FBS [MEM (−)], the rVAP, diluted with MEM (−) to different concentrations (100 µg ml\(^{-1}\), 10 µg ml\(^{-1}\), 1 µg ml\(^{-1}\)), were incubated with FG cells at 22°C for 4 h. After the supernatant had been removed, the FG cells were inoculated with LCDV at 4 TCID\(_{50}\)/ml at 20°C for 2 h; the virus liquid was then removed and maintenance medium was added, and the FG cells were cultivated at 22°C with 2 % CO\(_2\). MEM (−) without rVAP was used to incubate the FG cells before LCDV inoculation as the negative control. The CPE in FG cells cultured in 24-well plates was monitored using an inverted microscope every 12 h. After 48 h, the cells of each treatment group in the culture flasks were collected and DNA was extracted using the TIANamp Marine Animals DNA kit (Qiagen, Germany), and real-time quantitative PCR (qPCR) was performed to detect LCDV copies.

In vitro virus neutralization assay

The FG cells were grown separately in 75 cm\(^2\) cell-culture flasks and 24-well plates at 22°C, and monolayer cultures of FG cells were washed twice with MEM (−). Rabbit polyclonal antibody against rVAP was diluted with MEM (−) to different concentrations (200 µg ml\(^{-1}\), 20 µg ml\(^{-1}\), 2 µg ml\(^{-1}\)) after being filtered through a 0.22 µm acrodic syringe filter, and incubated with LCDV at 4 TCID\(_{50}\)/ml at 20°C for 4 h; the mixture was then added into FG cells and incubated at 22°C for 2 h. After the mixture had been removed and the maintenance medium was introduced, the FG cells were cultivated at 22°C with 2 % CO\(_2\). The CPE in FG cells was monitored every 12 h using an inverted microscope. After 48 h, the DNA from the FG cells in each treatment group was extracted as described above, and qPCR was applied to detect LCDV copies. Rabbit pre-immune serum with the concentration of 200 µg ml\(^{-1}\) was used to incubate LCDV as the negative control.

Real-time quantitative PCR

To detect the number of LCDV copies in FG cells, the specific primers P1/P2 were designed according to the sequence of the LCDV ORF038 gene: 5’-TCT TGT TCA TTT ACT TCT CGGC-3’, 5’-TCT TCT CCT TTA GAT GAT TTC CC-3’. NCBI BLAST showed that the specificity of the primers was good and that the amplified target fragment would be 155 bp. The pET-32a-ORF038 plasmid in E. coli BL21 (DE3) was extracted, and the quality and quantity of plasmid were detected using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Wilmington, USA). The positive plasmid was then ten-fold diluted and a standard curve was established, with the copy number ranging from 10\(^{1}\) to 10\(^{9}\). The qPCR reaction system contained 10 µl SYBR green I (Roche, Switzerland), 50 ng sample DNA and 2 µl detection primer P1/P2, with sterile distilled water being added to make up a final volume of 20 µl. The reaction procedures were as follows: 10 min at 95°C, followed by 45 cycles of 10 s at 95°C, 10 s at 53°C and 20 s at 72°C. After the qPCR reaction, software automatically generated the C\(_t\) values for each sample and the standard curve of the standard products, and the LCDV copy numbers were calculated via the standard curve according to the C\(_t\) values. Each sample was tested in triplicate.

Statistical analysis

All data were expressed as mean±standard deviation. The statistical analysis was performed using Origin 8.0 software and one-way ANOVA. Differences were considered statistically significant when P<0.05.

Funding information

This study was supported by the National Science Fund of China (31472295 and 31672685), the Science and Technology Development Project of Shandong Province (2014GNC111015) and the Taishan Scholar Program of Shandong Province.

Conflicts of interest

The authors declare that there are no conflicts of interest.
Ethical statement
This study was carried out strictly in line with the procedures in the Guide for the Use of Experimental Animals of the Ocean University of China. In this study the methods used in the animal experiments were approved by the Institutional Animal Care and Use Committee of the Ocean University of China (permit number: 20150101). All possible effort was dedicated to minimizing suffering.

References
40. Zheng FR, Sun XQ, Xing MQ, Liu H. Immune response of DNA vac- cine against lymphocystis disease virus and expression analysis


---

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.