Ovine Herpesvirus 2 Glycoproteins B, H, and L Are Sufficient for, and Viral Glycoprotein Ov8 Can Enhance, Cell-Cell Membrane Fusion

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ABSTRACT Ovine herpesvirus 2 (OvHV-2) is a gammaherpesvirus in the genus *Macavirus* that is carried asymptotically by sheep. Infection of poorly adapted animals with OvHV-2 results in sheep-associated malignant catarrhal fever, a fatal disease characterized by lymphoproliferation and vasculitis. There is no treatment or vaccine for the disease and no cell culture system to propagate the virus. The lack of cell culture has hindered studies of OvHV-2 biology, including its entry mechanism. As an alternative method to study OvHV-2 glycoproteins responsible for membrane fusion as a part of the entry mechanism, we developed a virus-free cell-to-cell membrane fusion assay to identify the minimum required OvHV-2 glycoproteins to induce membrane fusion. OvHV-2 glycoproteins B, H, and L (gB, gH, and gL) were able to induce membrane fusion together but not when expressed individually. Additionally, open reading frame Ov8, unique to OvHV-2, was found to encode a transmembrane glycoprotein that can significantly enhance membrane fusion. Thus, OvHV-2 gB, gH, and gL are sufficient to induce membrane fusion, while glycoprotein Ov8 plays an enhancing role by an unknown mechanism.

IMPORTANCE Herpesviruses enter cells via attachment of the virion to the cellular surface and fusion of the viral envelope with cellular membranes. Virus-cell membrane fusion is an important step for a successful viral infection. Elucidating the roles of viral glycoproteins responsible for membrane fusion is critical toward understanding viral entry. Entry of ovine herpesvirus 2 (OvHV-2), the causative agent of sheep associated-malignant catarrhal fever, which is one of the leading causes of death in bison and other ungulates, has not been well studied due to the lack of a cell culture system to propagate the virus. The identification of OvHV-2 glycoproteins that mediate membrane fusion may help identify viral and/or cellular factors involved in OvHV-2 cell tropism and will advance investigation of cellular factors necessary for virus-cell membrane fusion. We found that OvHV-2 glycoproteins B, H, and L are sufficient for, and viral glycoprotein Ov8 can significantly enhance, cell-cell membrane fusion.

KEYWORDS glycoproteins, membrane fusion, ovine herpesvirus 2

Ovine herpesvirus 2 (OvHV-2) is a gammaherpesvirus in the genus *Macavirus*. Sheep (*Ovis*) are the carrier of the virus and a well-adapted host. Hosts in the order Artiodactyla, such as bison, cattle, and cervids, that are poorly adapted to OvHV-2, can develop sheep-associated malignant catarrhal fever (SA-MCF) when infected with OvHV-2 (1). SA-MCF is a usually fatal disease characterized by lymphoproliferation and

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vasculitis (2). *In vivo*, the virus has tropism for at least two cell types, CD8⁺ T cells and epithelial cells, specifically alveolar type II epithelial cells in sheep lungs (3, 4). There is no treatment for or vaccine against this disease. Studies of OvHV-2 in general, including entry into host cells, have been hindered by the lack of a cell culture system to propagate the virus.

Herpesviruses enter cells via attachment of the virion to the cell surface, binding of one or more viral glycoproteins to cellular receptors, and subsequent fusion of the viral envelope with a cellular membrane, either an endosomal membrane or the plasma membrane (5–7). Glycoprotein B (gB), glycoprotein H (gH), and glycoprotein L (gL) are conserved among all herpesviruses, including OvHV-2, and are considered the core fusion machinery (7–9). Although these glycoproteins are sufficient to mediate membrane fusion for some herpesviruses, others need additional glycoproteins to mediate fusion. For instance, gB, gH, and gL are sufficient to mediate membrane fusion for Kaposi's sarcoma-associated herpesvirus (KSHV) (10), while herpes simplex virus 1 (HSV-1) requires gB, gH, gL, and the alphaherpesvirus-specific gD to induce membrane fusion (11). Also, gH and gL alone from KSHV, varicella-zoster virus (VZV), and human cytomegalovirus (HCMV) mediate low levels of detectable fusion (10, 12, 13).

In addition to the glycoproteins necessary for membrane fusion, herpesviruses also use other virion envelope glycoproteins to mediate attachment and binding to cells. Among gammaherpesviruses, Epstein-Barr virus (EBV) gp350/220 binds to C3d on B cells (14) and KSHV K8.1 glycoprotein and murid herpesvirus 4 (MuHV-4) gp150 interact with heparan sulfate (15–18). Open reading frames (ORFs) encoding these proteins are all located between ORFs 50 and 52, conserved among all gammaherpesviruses, in the virus genomes (8). The ORF encoding OvHV-2 Ov8, which has no homology to other herpesvirus glycoproteins, is predicted to be spliced and encode a glycoprotein and is positioned between ORFs 50 and 52 (8, 9). The function of putative glycoprotein Ov8 is not known. However, based on the position of the ORF in the genome, Ov8 may play a role in viral attachment or binding similar to the EBV, KSHV, and MuHV-4 proteins. Identifying OvHV-2 glycoproteins that play roles in membrane fusion is an important step toward understanding the viral entry mechanism. In this study, we identified the minimum OvHV-2 glycoproteins sufficient for inducing membrane fusion. In addition, we characterized a novel glycoprotein, Ov8, and found that it can enhance membrane fusion. These findings will advance the knowledge of OvHV-2 biology and open avenues for further research on viral glycoproteins and their interactions with target cells.

RESULTS

OvHV-2 glycoprotein expression in CHO-K1 cells. OvHV-2 gB and gH/gL expression in transiently transfected CHO-K1 cells using the same plasmids as those used in this study has been previously demonstrated via Western blot analysis (19). In that study, gB and gH were detected in lysates from cells transfected with plasmids encoding gB and gH individually, but gL was not detected in cells transfected with the gL plasmid alone. However, gL was detected in lysates from cells transfected with both gH and gL plasmids (19). The same experiments were done in this study and confirmed the previous results (data not shown).

Because cell-cell fusion requires cell surface expression of the involved proteins, flow cytometry was used to determine whether OvHV-2 gB and gH/gL were present on the surface of transfected CHO-K1 cells (Fig. 1). The mean fluorescence intensity (MFI) of cells transfected with the gB expression plasmid and probed with anti-gB rabbit polyclonal hyperimmune serum was significantly higher ($P < 0.001$) than that of cells transfected with the empty pJP007 vector (Fig. 1, gB and Vector α gB), thus showing that OvHV-2 gB was present on the cell surface. The MFI of cells transfected with gH or gL expression plasmids alone and probed with anti-gH/gL rabbit polyclonal hyperimmune serum was not significantly greater than that of the control, with P values of 0.991 (Fig. 1, gH and Vector α gHgL) and 0.995 (Fig. 1, gL and Vector α gHgL), respectively. This indicates that gH and gL individually were not expressed on the cell surface.

FIG 1 OvHV-2 glycoprotein expression in transfected CHO-K1 cells. Flow cytometry was used to demonstrate OvHV-2 glycoproteins on the surface of CHO-K1 cells transfected with plasmids encoding OvHV-2 gB, gH, or gL alone and gH/gL together. Cells transfected with vector plasmid pJP007 were used as a background control (Vector αgB and Vector αgH/gL). Bars represent the mean fluorescence intensity of cells probed with rabbit polyclonal hyperimmune sera against gB (gB and Vector αgB) or gH/gL (gH, gL, gH/gL, and Vector αgH/gL) and then incubated with Alexa Fluor 488 donkey anti-rabbit secondary antibody. Results are averages from three independent experiments. Error bars represent the standard errors of the means. *, $P = 0.01$; **, $P < 0.001$.

However, when cotransfecting gH and gL expression plasmids and probing with anti-gH/gL rabbit polyclonal hyperimmune serum, the MFI was significantly higher than that of the control ($P = 0.01$), indicating cell surface expression of the proteins (Fig. 1, gH/gL and Vector αgH/gL). The flow cytometry data suggest that OvHV-2 gH and gL must be coexpressed for proper trafficking, similar to the case with some other herpesviruses (20, 21).

Cell-cell fusion mediated by OvHV-2 gB and gH/gL. Glycoproteins B, H, and L form the core fusion machinery of many herpesviruses; however, for some processes, such as HSV-1 and EBV entry into B cells, these three glycoproteins are insufficient to mediate membrane fusion (5, 6). For OvHV-2 it is not known whether gB and gH/gL are sufficient or whether additional proteins are needed to induce membrane fusion. Previous studies have successfully demonstrated the role of several herpesvirus glycoproteins in membrane fusion using a virus-free *in vitro* cell-cell fusion assay (10, 11, 22–25). Therefore, a virus free cell-cell fusion assay was used to examine which OvHV-2 glycoproteins mediate membrane fusion.

Coculture of effector (CHO-K1) cells expressing gB and gH/gL along with the T7 RNA polymerase, mixed with target (MDBK) epithelial cells, expressing luciferase under the control of the T7 promoter resulted in luciferase expression levels significantly higher ($P < 0.0001$) than those of coculture of effector cells transfected with the empty pJP007 vector control with target cells (Fig. 2A, gBgH/gL and Vector). Effector cells expressing gB or gH/gL alone cocultured with target cells yielded higher background levels but did not result in luciferase expression significantly different from that of the empty pJP007 vector ($P = 0.479$ and 0.569 , respectively) (Fig. 2A, gB, gH/gL, and Vector). Low levels of fusion mediated by KSHV gB and gH/gL alone have been observed (10). It is possible that, similar to the case with KSHV, a low level of fusion occurred with OvHV-2 gB and gH/gL alone but did not rise to the level of statistical significance. When a fetal sheep fibroblast line (A113) was used as the target, coculture with effector cells expressing gB and gH/gL resulted in luciferase expression significantly higher than that of the control ($P < 0.0001$) (Fig. 2B, gBgH/gL and Vector). Similar to the results with MDBK target cells, luciferase expression from coculture of effector cells expressing gB or gH/gL alone with A113 cells was not statistically different from that of the control

FIG 2 Cell-cell membrane fusion induced by OvHV-2 glycoproteins. Fusion between CHO-K1 cells expressing OvHV-2 glycoproteins and MDBK (A) or A113 (B) cells was detected by luciferase activity. Results are averages from 4 independent experiments and are presented as luciferase counts per second (cps). Error bars are SEM. Vector, plasmid pJP007. *, $P < 0.0001$. (C) Fusion between CHO-K1 cells expressing OvHV-2 glycoproteins and MDBK cells was visualized using epifluorescence microscopy. Control, CHO-K1 cells transfected with plasmid pJP007. The middle row shows white boxed areas enlarged 134%.

($P = 0.186$ and 0.383 , respectively) (Fig. 2B, gB, gHgL, and Vector). These results showed that OvHV-2 gB and gH/gL are sufficient to mediate membrane fusion for two different target cell types.

To visualize cell-cell fusion, effector cells (CHO-K1) transfected with OvHV-2 glycoprotein-expressing plasmids or the empty pJP007 plasmid, plus a green fluorescent protein (GFP)-expressing plasmid, were cocultured with target MDBK or A113 cells that were stained with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil). Fluorescence microscopy revealed multiple fusion events, identified by colocalization of the GFP (green) in effector cells, Dil (red) in target cells, and Hoechst (blue) staining of nuclei associated with the presence of more than two nuclei within one membrane. Figure 2C illustrates fusion between effector cells expressing OvHV-2 gB and gH/gL and MDBK cells (Fig. 2C, gBgHgL and gBgHgL enlarged). No fusion was observed when effector cells transfected with vector pJP007 were cocultured with MDBK (Fig. 2C, Control). Fluorescence microscopy on effector cells cocultured with A113 cells yielded results similar to those with MDBK cells (data not shown). Because syncytium formation is not a pathognomonic feature of SA-MCF caused by OvHV-2, membrane fusion detected in cell-cell fusion assays may not be as robust as for

syncytium-causing viruses. The fluorescence microscopy data clearly confirmed the results obtained in the luciferase cell-cell fusion assays showing that OvHV-2 gB and gH/gL together mediate membrane fusion.

OvHV-2 ORF Ov8 encodes a glycoprotein. The cell-cell fusion assay demonstrated that gB, gH, and gL are sufficient for membrane fusion, but other OvHV-2 glycoproteins might still have a role in the process. The OvHV-2 unique ORF Ov8 is located between conserved ORFs 50 and 52, similarly to the genes encoding MuHV-4 gp150, EBV gp350/220, and KSHV K8.1 (8). Of these proteins, only EBV gp350/220 has been evaluated specifically for a role in fusion, and it was found to have little to no effect on fusion with both Daudi cells, a B-cell line, and human embryonic kidney cells (HEK-293-P) (26, 27). However, all of these glycoproteins interact with cell surface molecules; therefore, based on the positional analogy with these genes, Ov8 was selected for study (14–18).

The Ov8 open reading frame consists of 2,369 nucleotides, bases 81538 to 83911 of the reference sequence (GenBank accession no. [NC_007646.1](#)), and is predicted to contain two exons of 1,219 bp (81,538 to 82,756) and 203 bp (83,704 to 83,906) separated by a 947-bp intron (8). Computational analysis of the predicted spliced gene using Vector NTI Advance v11.5 (Invitrogen) predicted a protein of 473 amino acids (aa) with a molecular mass of 51 kDa and 15 potential N-glycosylation sites. Amino acid sequence analysis using TMHMM v2.0 (28, 29) identified a single transmembrane domain and a short cytoplasmic tail (Fig. 3A). Alternatively, translation of Ov8 from an mRNA, without splicing, to the first in-frame stop codon at nucleotides 83683 to 83685 would give rise to a protein of 704 aa with a molecular mass of 74 kDa, 17 potential N-glycosylation sites, and no transmembrane domain. Although transcripts from the Ov8 open reading frame have been detected in OvHV-2-infected lymphoblastoid cells (30), no studies have looked at the encoded protein(s).

Plasmid pJP007-Ov8 was constructed by cloning an amplicon generated from OvHV-2 genomic DNA. Therefore, transcription of more than one mRNA was possible in transfected cells. Reverse transcriptase PCR (RT-PCR) followed by gel electrophoresis was used to examine Ov8 transcript expression in transfected CHO-K1 cells (Fig. 3B). A single band of approximately 1,500 bp was detected in the RT-containing reaction from pJP007-Ov8-transfected cells (Fig. 3B, lane 2). This size corresponds to the predicted 1,422-bp cDNA from a spliced Ov8 RNA versus a predicted cDNA of 2,148 bp from an unspliced RNA terminating at the first in-frame codon. No bands were visible in the reactions without RT from pJP007-Ov8-transfected cells (Fig. 3B, lane 3) or from pJP007 vector-transfected cells with and without RT (Fig. 3B, lanes 4 and 5). Plasmid pJP007-Ov8 DNA was used as a positive control for the PCR (Fig. 3B, lane 6). These results indicate that the Ov8 mRNA in pJP007-Ov8-transfected CHO-K1 cells is spliced. Sequencing of the amplified cDNA showed that it was identical to the reference genome, with consensus donor GT and acceptor AG nucleotide pairs (data not shown).

Ov8 protein expression in transfected CHO-K1 cells was examined using Western blot analysis with a mouse monoclonal antibody (MAb) against the N-terminal FLAG epitope appended to Ov8; under reducing (treatment with a reducing buffer and heating) conditions, four specific bands of molecular masses ranging from 80 to 150 kDa were detected in cell lysates (Fig. 3C, lane 1). Because anti-FLAG MAb-labeled bands were larger than the predicted 51 kDa, we hypothesized that Ov8 was heavily glycosylated. To determine whether the shift in size of Ov8 from the predicted 51 kDa to about 80 to 150 kDa was due to glycosylation, cell lysates were treated with various glycosidases and examined with Western blot analysis (Fig. 3D). Treatment to remove most glycans, using a deglycosylation mix, resulted in a reduction of Ov8 to about 51 kDa (Fig. 3D, lane 2). The same size reduction was seen when Ov8 was treated with peptide-N-glycosidase F (PNGase F) (Fig. 3D, lane 3) but there was no effect on protein migration when Ov8 was treated with O-glycosidase or α -2-3 neuraminidase (Fig. 3D, lanes 4 and 5). These results together indicate that Ov8 is primarily N-glycosylated when

FIG 3 Characterization of Ov8 in transfected CHO-K1 cells. (A) Predicted amino acid sequence of Ov8. Bold blue letters indicate potential N-glycosylation sites. Underlined letters indicate the predicted transmembrane domain, and red italic letters indicate the predicted cytoplasmic tail. (B) RT-PCR was used to examine transfected CHO-K1 cells for Ov8 transcript expression. Lane 1, DNA ladder, in kilobases. Lanes 2 and 3, PCR products from pJP007-Ov8-transfected cells with and without RT, respectively. Lanes 4 and 5, PCR products from pJP007 vector-transfected cells with and without RT, respectively. Lane 6, PCR product using plasmid pJP007-Ov8 DNA as the template. (C) Protein extracts from CHO-K1 cells transfected with plasmids encoding OvHV-2 FLAG-tagged Ov8 (lane 1) or empty vector pJP007 (lane 2) were resolved using SDS-PAGE and analyzed by Western blotting using an anti-FLAG MAb. (D) Cell lysates from CHO-K1 cells expressing Ov8 were left untreated (lane 1) or treated with deglycosylation mix (lane 2), PNGase F (lane 3), α 2-3 neuraminidase (lane 4), or O-glycosidase (lane 5) and analyzed by Western blotting using an anti-FLAG MAb. (E) Nonreduced, nondenatured protein extracts from CHO-K1 cells transfected with pJP007-Ov8 (Native, Ov8) or pJP007 (Native, pJP007) were resolved under native conditions and analyzed by Western blotting using an anti-FLAG MAb. Arrowheads (Native, Ov8) indicate different Ov8 oligomeric structures. As a control for the experiment, nonreduced, nondenatured protein extracts from CHO-K1 cells expressing Nipah virus glycoprotein G were resolved using native PAGE (Native, NiVG) or SDS-PAGE (Seminative, lane NiVG). Reduced, denatured protein extracts were resolved using SDS-PAGE (Reduced, NiVG). NiV G protein was analyzed by Western blotting using an anti-HA MAb. Arrowheads (Seminative, NiVG) indicate NiV G protein monomers, trimers, and tetramers. pJP007 lanes in each blot are lysates from cells transfected with the vector plasmid. Molecular mass marker sizes, in kilodaltons, are indicated on the left sides of panels C to E.

expressed in CHO-K1 cells and the slower-migrating bands seen in Fig. 3C, lane 1, are likely posttranslational modifications, including variable glycosylation of Ov8. These assays did not detect O-glycan or sialic acid modifications, but their presence cannot be ruled out because of the relatively small effect they may have on the size of glycoproteins.

Some viral glycoproteins involved in fusion, such as HSV-1 gB and Nipah virus G protein, form oligomers (31, 32). To determine whether Ov8 formed oligomers, extracts from cells transfected with pJP007-Ov8 were resolved under native (untreated and native PAGE) conditions, followed by Western blotting (Fig. 3E). Multiple bands ranging from 160 to >720 kDa were observed, demonstrating higher-order oligomeric forms (Fig. 3E, Native, Ov8). As a positive control, the NiV G glycoprotein was run under native, seminaive (untreated and SDS-PAGE), and reducing conditions, followed by Western blotting. Seminaive conditions showed higher-order oligomeric forms corresponding to tetramers, dimers, and monomers, as previously published (Fig. 3E, Semi Native and Reduced, NiVG) (31). NiV G glycoprotein oligomers did not resolve under native conditions, a phenomenon that has been seen for some proteins (33).

OvHV-2 Ov8 is expressed on the cell surface and increases membrane fusion.

Flow cytometry was used to determine whether Ov8 expressed alone and with gB and gH/gL was expressed on the surface of transfected CHO-K1 cells (Fig. 4A). The mean fluorescence intensity of Ov8 either alone (Fig. 4A, Ov8) or with gB and gH/gL [Fig. 4A, Ov8(All)] was significantly higher ($P < 0.0001$) than background (Fig. 4A, Vector α FLAG), indicating that Ov8 was expressed at the cell surface. The cell surface expression of gB [Fig. 4B, gB(All)] and gH/gL [Fig. 4B, gHgL(All)] in the presence of Ov8 was also confirmed. Detecting the N-terminally tagged Ov8 on the cell surface also shows that Ov8 is likely a type I transmembrane protein with its N terminus localized extracellularly and C terminus intracytoplasmically (34).

To determine whether Ov8 had an effect on cell-cell fusion, Ov8 was expressed in effector cells along with gB and gH/gL, cocultured with MDBK or A113 cells, and luciferase activity was measured (Fig. 4C and D). The addition of Ov8 led to a significant increase in the luciferase activity compared to those of gB and gH/gL in MDBK cells ($P = 0.009$) (Fig. 4C, gBgHgLOv8 and gBgHgL). Effector cells expressing Ov8 and gB or Ov8 and gH/gL alone were also cocultured with MDBK cells; luciferase expression was not different from that of the vector control (Fig. 4C, gBOv8 and gH/gLOv8). The relatively high background in the MDBK cell fusion assay could be due to non-viral-protein-mediated fusion, as clumps of cells were seen following coculture, and/or luciferase gene expression might not be tightly controlled in these cells. Ov8 did not significantly affect fusion with A113 cells ($P = 0.703$) compared to fusion with gB and gH/gL (Fig. 4D, gBgHgLOv8 and gBgHgL). Cell-cell fusion was visualized using fluorescence microscopy; the multinucleated cells with colocalized green and red fluorescence observed were qualitatively similar to fusion seen when gB and gH/gL without Ov8 were expressed (data not shown).

DISCUSSION

Although significant progress has been made in the last decade in understanding SA-MCF caused by OvHV-2 (1), the lack of a cell culture system for the virus limits the type of work that can be done to study basic biological properties of OvHV-2, including virus entry into host cells and cell tropism. To begin addressing these knowledge gaps, we used a cell-cell fusion assay to identify OvHV-2 glycoproteins involved in membrane fusion. Glycoprotein B and gH/gL were shown to be sufficient to induce membrane fusion. While this result is perhaps not surprising, it was important to determine because gH/gL alone from KSHV, VZV, and HCMV are known to mediate low levels of membrane fusion (10, 12, 13).

We next examined whether Ov8, a predicted glycoprotein unique to OvHV-2, had an effect on cell-cell fusion mediated by gB and gH/gL. Effector cell fusion with MDBK cells was increased when Ov8 was also expressed, while fusion with A113 cells was unaffected. One possible mechanism for Ov8 enhancement of fusion includes binding to a cellular receptor and helping maintain the cell membranes in apposition, thus increasing cell-cell fusion. Another possibility is that binding of Ov8 to a cellular receptor results in a conformational change or exposure of a masked domain of gH that triggers a series of events resulting in membrane fusion, as proposed for HSV-1, EBV, and HCMV (7, 25).

FIG 4 Cell surface protein expression and membrane fusion in the presence of Ov8. (A) Flow cytometry was used to demonstrate OvHV-2 FLAG-tagged Ov8 on the surface of CHO-K1 cells transfected with the plasmid encoding Ov8 alone (Ov8), with plasmids encoding gB, gH/gL and Ov8 together [Ov8(All)], and the empty vector (Vector αFLAG). Cells were probed with anti-FLAG MAb conjugated to phycoerythrin. Cells transfected with vector pJP007 were used as a background control. (B) Flow cytometry was used to demonstrate cell surface gB expression on cells transfected with plasmids encoding gB, gH/gL, and Ov8 [gB(All)] using gB-specific polyclonal rabbit hyperimmune serum and gH/gL expression on cells transfected with gB, gH/gL, and Ov8 [gHgL(All)] using gH/gL polyclonal rabbit hyperimmune serum. Cells transfected with the vector pJP007 and incubated with the αgB (Vector αgB) rabbit serum and αgH/gL (Vector αgH/gL) rabbit serum were used as controls. (C) Fusion between CHO-K1 cells expressing OvHV-2 gB and gH/gL (gBgHgL), gB, gH/gL and Ov8 (gBgHgL-Ov8), gB and Ov8 (gBOv8), and gH/gL and Ov8 (gHgLOv8) and MDBK cells was detected by luciferase activity. (D) Fusion between CHO-K1 cells expressing OvHV-2 gB and gH/gL (gBgHgL) or gB, gH/gL, and Ov8 (gBgHgL-Ov8) and A113 cells was detected by luciferase activity. Vector, cells transfected with plasmid pJP007. Results are averages from 4 independent experiments and presented as luciferase counts per second (cps). Error bars show SEM. *, $P = 0.047$; **, $P = 0.009$; ***, $P = 0.0005$; ****, $P < 0.0001$. ns, not significant.

No change in fusion was detected between effector and A113 cells when Ov8 was expressed along with gB and gH/gL. Ov8's lack of effect on fusion using A113 cells could be due to the absence of a cellular receptor, or cell-cell fusion with these cells could be maximal without Ov8 and any additional binding by Ov8 would not be detected in this assay.

Determining the mechanism by which Ov8 increases cell-cell fusion with MDBK cells is an important future direction. Work is under way to determine whether Ov8 binds a cellular receptor and, if so, to identify said receptor. By increasing knowledge about

OvHV-2 basic biology, these findings will help advance investigation of cellular factors necessary for mediating virus-cell membrane fusion and may help identify viral and/or cellular factors involved in OvHV-2 cell tropism.

MATERIALS AND METHODS

Plasmids. Plasmid pJP007, which encodes a FLAG epitope at the 5' end of a multiple-cloning site, was provided by Li Lin (Laboratory of Cardiovascular Sciences, National Institute on Aging, Baltimore, MD) (35). Plasmids pT7EMCluc, encoding the firefly luciferase gene under the control of the T7 promoter, and pCAGT7, encoding the T7 RNA polymerase, were gifts from Patricia Spear (Northwestern University, Chicago, IL) and were described previously (10, 36). Plasmids encoding OvHV-2 gB, gH, and gL with C-terminal V5 epitopes were described previously (19). A pcDNA3.1 plasmid expressing Nipah virus glycoprotein G (pcDNA3.1 NiVG) was described previously (37).

Large-scale plasmid preparation was done using the HiSpeed plasmid maxikit (Qiagen) according to the manufacturer's protocol.

Construction of OvHV-2 Ov8 expression plasmid. The ORF that encodes Ov8 was PCR amplified using sense primer 5'-ACT GGA TCC ATG GAT AAC GCT ACC TTA-3' and antisense primer 5'-GCC TCT AGA TTA CTC GGT TAA TAC ACA G-3'. BamHI and XbaI endonuclease restriction recognition sites were added to the 5' ends of the sense and antisense primers, respectively (bold and underlined in the preceding sequences). The PCR mixture consisted of a 0.5 μ M concentration of each primer, genomic DNA extracted from nasal secretions of sheep infected with OvHV-2 as a template, and 1 $\times$ HotStarTaq Plus master mix (Qiagen). Cycling conditions were as follows: 95°C for 5 min, 35 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 2.5 min, and a final extension at 72°C for 7 min. An amplicon of the correct size (2,369 bp) was detected using agarose gel electrophoresis, purified (QIAquick gel extraction kit; Qiagen), digested with BamHI and XbaI, and ligated to the BamHI and XbaI sites of plasmid pJP007. The resulting plasmid was sequenced to confirm the identity and orientation of the insert (Eurofins MWG Operon LLC) and designated pJP007-Ov8.

Cell lines. CHO-K1 and MDBK cells were obtained from the American Type Culture Collection (ATCC) and maintained in minimal essential medium (MEM) α with GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, 100 μ g/ml of streptomycin, and 1 μ g/ml of amphotericin B (complete MEM) at 37°C and 5% CO₂.

Isolation of sheep fetal lung cells and establishment of an immortalized cell line. The immortalized sheep lung cell line used in this study was derived as follows. A pregnant OvHV-2 uninfected yearling ewe (ID number A113), housed and handled in accordance with Washington State University IACUC-approved protocol 2232, was euthanized for tissue collection. Lungs were removed aseptically from the fetus, lavaged to remove alveolar macrophages, and perfused to remove as much blood as possible. A single cell suspension was prepared by mincing the lungs with razor blades and incubating the tissue in Dulbecco's modified Eagle medium/F-12 nutrient mix (DMEM/F-12; Gibco) containing 10 μ g/ml of DNase I (Sigma), 1 mg/ml of protease XIV (Sigma), and 0.025% collagenase Ia (Sigma) at 37°C for 2 h. Digested lung was filtered sequentially through gauze and then 100- μ m and 40- μ m cell strainers and centrifuged at 290 $\times$ g and 4°C for 10 min, and the cell pellet was suspended in sterile 0.87% Tris NH₄Cl (pH 7.4) for 2 min at room temperature to lyse red blood cells. Remaining cells were recovered by centrifugation, suspended in DMEM/F-12, and incubated at 37°C for 1 h in a plastic tissue culture flask to allow cells to adhere. Nonadherent cells were collected, centrifuged, suspended in DMEM/F-12 (containing 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 μ g/ml of amphotericin B), and seeded into 4 wells of a 24-well plate coated with 10 μ g/ml of fibronectin (Sigma) and 1 μ g/ml of rat tail type I collagen (BD Bioscience). Cells were incubated at 37°C in 5% CO₂.

To establish an immortalized cell line, the fetal lung cells were transfected with plasmid pBABE-puro-hTERT (38) using Lipofectamine LTX with Plus reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Transfected cells were selected using puromycin (0.5 μ g/ml), and limiting dilution was used to generate cell clones. One clone, A113OvFL1.4B5 (referred to as A113), was selected for use in this study. Cells had typical fibroblast morphology.

Cell transfection. CHO-K1, MDBK, and A113 cells were seeded in 6-well tissue culture plates at 0.5 $\times$ 10⁶/well with complete MEM and incubated overnight at 37°C and 5% CO₂ to give 80 to 90% confluence. All transfections were done with Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol except for the cell-cell fusion assay using the A113 cells, which was done with Lipofectamine LTX with Plus reagent (Thermo Fisher Scientific). Total DNA was kept at 2.5 μ g/well. As a control for transfection efficiency, one well was transfected with a plasmid carrying the green fluorescent protein (GFP) gene (pmaxGFP; Lonza). Cells were incubated for 24 h and then processed for subsequent assays.

Western blotting. CHO-K1 cells transfected with plasmids encoding OvHV-2 Ov8, an empty pJP007 vector, or pcDNA3.1 NiVG were lysed using Pierce IP lysis buffer (Thermo Scientific) according to the manufacturer's instructions, and cellular debris was removed by centrifugation (18,407 $\times$ g at 4°C for 10 min). Samples were treated with 50 mM Bond-Breaker tris(2-carboxyethyl)phosphine (TCEP) solution, at neutral pH (Thermo Scientific), and heated at 95°C for 10 min (reducing conditions) or left untreated (semireducing conditions) and separated using SDS-polyacrylamide gel electrophoresis (PAGE) (NuPAGE Novex 4 to 12% bis-Tris gels; Invitrogen). For native conditions, untreated samples were separated using the NativePAGE gel system (Invitrogen) according to the manufacturer's instructions.

Proteins were transferred to a nitrocellulose membrane using an iBlot 2 gel transfer device (Thermo Scientific), and the membrane was blocked using 0.05% phosphate-buffered saline (PBS)-Tween 20

(PBS-T) with 5% milk. Membranes were incubated with mouse monoclonal anti-FLAG M2-horseradish peroxidase (HRP) antibody (Sigma-Aldrich) diluted 1:500 in PBS-T with milk for 1 h to detect FLAG-tagged Ov8. To detect hemagglutinin (HA)-tagged NiVG, the membrane was incubated with mouse monoclonal anti-HA.11 epitope tag antibody (Biolegend) diluted 1:1,000 in PBS-T with milk, followed by a 1-h incubation with HRP-conjugated donkey anti-mouse IgG antibody (Abcam). Lysates from cells transfected with empty vector pJP007 were probed similarly, as a negative control. The membranes were washed and processed for detection by chemiluminescence (HyGlo Quick spray; Denville Scientific) and developed on X-ray film.

Flow cytometry. Twenty-four hours following transfection, cells were harvested with 10 μ M EDTA and 3.75×10^5 cells from each transfection reaction were aliquoted into 4 wells of a 96-well plate. Cells were pelleted by centrifugation ($582 \times g$ for 5 min) and the supernatant was decanted. Cells transfected with pJP007-Ov8 were incubated with mouse monoclonal anti-DDDDK (FLAG) tag antibody conjugated to phycoerythrin (Abcam) diluted 1:250 in 10% FBS in PBS (FACS buffer) for 1 h on ice. Cells transfected with empty pJP007 vector were used as a negative control. Cells transfected with gB, gH, and/or gL plasmids were treated with rabbit polyclonal hyperimmune sera against OvHV-2 gB and/or gH/gL (19) diluted 1:250 in FACS buffer for 1 h on ice. This was followed by two cycles of washes with FACS buffer and incubation with a donkey anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (Life Technologies). Cells transfected with empty vector pJP007 incubated with anti-gB or anti-gH/gL rabbit polyclonal hyperimmune serum followed by anti-rabbit secondary antibody were used as controls. Unbound antibodies were washed out, and the cells were fixed and stored in 0.5% paraformaldehyde until analysis. Bound antibodies were detected and mean fluorescence intensities recorded using a Guava EasyCyte benchtop flow cytometer (Millipore).

RT-PCR. Total RNA was purified from CHO-K1 cells transfected with pJP007-Ov8 using the TRIzol PLUS RNA purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions. One-third of the purified RNA was reverse transcribed to cDNA using the SuperScript IV VILO master mix with ezDNase (Thermo Fisher Scientific) according to the manufacturer's instructions, and 1 μ l of cDNA was used as the template for Ov8-specific PCR. The PCR conditions and primers were as described above for plasmid pJP007-Ov8 construction. A no-RT control was used to confirm that there was no DNA contamination. A single amplicon of approximately 1,500 bp was detected by agarose gel electrophoresis and sequenced (Eurofins MWG Operon LLC).

Ov8 deglycosylation. Cell lysates of CHO-K1 cells transfected with pJP007-Ov8 were treated with a protein deglycosylation mix, peptide-N-glycosidase F (PNGase F), α -2-3 neuraminidase, or O-glycosidase (New England BioLabs) according to the manufacturer's protocols. Treated and untreated cell lysates were run on NuPAGE Novex 4 to 12% bis-Tris gels (Invitrogen) under reducing conditions. Following transfer to a polyvinylidene difluoride membrane, Western blotting was performed to detect FLAG-tagged Ov8 as described above.

Luciferase cell-cell fusion assay. Effector (CHO-K1) and target (MDBK and A113) cells were grown in 6-well plates overnight at 37°C and 5% CO₂ to 80 to 90% confluence. The effector cells were transfected with individual or combinations of plasmids carrying OvHV-2 gB, gH, gL, and Ov8 genes and with plasmid pCAGT7. Total DNA amounts for transfections were kept constant at 2.5 μ g by adding empty pJP007 plasmid as necessary. The target cells were transfected with the pT7EMCLuc plasmid. At 18 to 20 h after transfection, the cells were washed with PBS, lifted with 10 μ M EDTA, pelleted at $201 \times g$ for 5 min, and resuspended in MEM α medium with 10% FBS. Assays were performed in triplicate. Target and effector cells were cocultured at 37°C and 5% CO₂ for 12 h. As a negative control, effector cells transfected with empty pJP007 and pCAGT7 and target cells transfected with pT7EMCLuc were cocultured similarly. After 12 h, cells were washed with PBS and lysed using 400 μ l of 1 $\times$ cell lysis buffer (Promega), and 190- μ l quantities of cell lysates were transferred to a 96-well plate (Corning; black flat). Just before reading, 100 μ l of luciferase assay substrate (Promega) was added and the plate was read in a luminometer (Wallac 1450 Microbeta Trilux liquid scintillation and luminescence counter; PerkinElmer). Luciferase activity was recorded as counts per second.

Fluorescence microscopy. Effector cells (CHO-K1) were transfected with plasmids that encode OvHV-2 glycoproteins and a plasmid carrying the GFP gene (pmaxGFP, Lonza). Target cells (MDBK and A113) were stained with Dil Vybrant multicolor cell-labeling kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. Target and effector cells were cocultured for 24 h and then fixed with 2% paraformaldehyde for 10 min at room temperature. Effector cells transfected with pJP007 and the GFP-expressing plasmid and cocultured with Dil-stained target cells were used as a negative control. Fixed cells were stained with Hoechst 33342 solution (Thermo Fisher Scientific) and then visualized using epifluorescence microscopy at a magnification of $\times 20$ (Nikon Eclipse Ti), and photomicrographs were taken using NIS Elements Basic Research software v3.06 (Nikon).

Statistical analysis. The cell-to-cell membrane fusion assay was repeated four times. The differences in luciferase counts between effector cells transfected with OvHV-2-expressing plasmids or empty vector pJP007 were analyzed using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple-comparison test. The same statistical analysis was used for flow cytometry data. A *P* value of <0.05 was considered statistically significant.

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We declare no conflict of interest.

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