Research Note: Duck plague virus glycoprotein I influences cell–cell spread and final envelope acquisition

Tian Liu,*,†,1 Mingshu Wang,*,†,1 Anchun Cheng,*,†,2 Renyong Jia,*,†,2 Ying Wu,*,†,‡ Qiao Yang,*,†,1 Dekang Zhu,†,1 Shun Chen,*,†,‡ Mafeng Liu,*,†,‡ Xinxin Zhao,*,†,‡ Shaqiu Zhang,*,†,‡ Juan Huang,*,†,‡ Sai Mao,*,†,‡ Xumin Ou,*,†,‡ Qun Gao,*,†,‡ Xingjian Wen,*,†,‡ Di Sun,*,†,‡ Yunya Liu,*,†,‡ Yanling Yu,*,†,‡ Ling Zhang,*,†,‡ Bin Tian,*,†,‡ Leichang Pan,*,‡ and Xiaoyue Chen,*,†,‡

*Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu City, Sichuan 611130, P.R. China; †Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Chengdu City, Sichuan 611130, P.R. China; and ‡Avian Disease Research Center, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu City, Sichuan 611130, P.R. China

ABSTRACT To determine the role of glycoprotein I (gI) in duck plague virus (DPV), a gI-deleted mutant (BAC-CHv-Dgi) and a gI-revertant virus (BAC-CHv-Dgi Rev) were constructed by using a markerless two-step Red recombination system implemented on the DPV genome cloned into a bacterial artificial chromosome (BAC). Mutants were characterized on duck embryo fibroblast (DEF) cells compared with wild-type virus. BAC-CHv-Dgi produced viral plaques on DEF cells that were on average approximately 57.2% smaller than those produced by BAC-CHv-Dgi Rev and wild-type virus. Electron microscopy confirmed that deleting of gI resulted in nucleocapsids accumulated around the cytoplasm vesicles and few of them could complete the final envelopment process. These results clearly indicated that DPV gI plays significant roles in viral cell-cell spread and viral final envelopment process.

Key words: DPV, gI, viral cell-cell spread, viral final envelopment

INTRODUCTION Duck plague virus (DPV) has been classified as a member of the subfamily Alphaherpesvirinae of the family Herpesviridae. It causes focal necrosis in the parenchymal organs, and massive petechial hemorrhaging in lymphoid and digestive tract, which leads to a large number of ducks death. Duck plague virus Chinese virulent strain was isolated from dead infected ducks in China and its entire genomic sequence has been determined a few years ago. However, compared with other alphaherpesviruses, not too many DPV gene functions have been characterized especially in terms of the viral life cycle. Glycoprotein I (gI) of alphaherpesviruses plays important roles in viral cell–cell spreading and viral secondary envelopment. There are 2 ways for alphaherpesviruses to enter into the cells, one occurs at apical cell surfaces which is called viral entry and the other occurs at cell junctions which is called viral cell-to-cell spread (Sattentau, 2008). These processes rely heavily on viral glycoproteins. Herpes simplex virus (HSV) glycoproteins gB, gD, and gH/gL are not only essential for virus entry into cells, but also for viral cell-to-cell spread (Uchida et al., 2013). However, HSV and pseudorabies virus (PRV) gI only plays an important role in promoting cell-to-cell spread (Farnsworth and Johnson, 2006). Alphaherpesvirus gI also plays an important role in viral assembly. It provides an essential but redundant function during the acquisition of the final virion envelope. Apparent defects of the production of enveloped virus particles were observed in HSV gI and gD mutant virus (Farnsworth et al., 2003).

Although functions of alphaherpesvirus gI have been extensively studied, few research reports have been explored on DPV gI. Thus, the aim of the present study was to determine the contribution of DPV gI in viral cell–cell spread and viral final envelopment.

MATERIALS AND METHODS

The animal studies were approved by Institutional Animal Care and Use Committee of Sichuan...
Agricultural University, Sichuan, China, and followed the National Institutes of Health guidelines for the performance of animal experiments.

**Cells**

Duck embryo fibroblast (DEF) cells were prepared from 9-day-old cherry valley duck embryos and grown in Minimal Essential Medium (MEM, Gibco) supplemented with 10% newborn calf serum (NBS, Gibco) and 100 U/mL penicillin and 100 µL/mg streptomycin at 37 in 5% CO₂.

**Construction of gI Mutant and Revertant Viruses**

The CHv strain was separated and preserved in our laboratory. Duck plague virus gI mutant was constructed by modifying the BAC plasmid containing the whole CHv genome in GS1783 Escherichia coli. Mutagenesis was based on a markerless two-step Red recombination (Tischer et al., 2010). Briefly, bacteria containing BAC with DPV strain CHv was mutated to remove gI-coding sequences. PCR primers which included the upstream or downstream 40 bp of the gI-coding sequences were used to amplify the kanamycin gene flanked by a I_SceI site of pEPKana-S (5'-GTGCGCCATATA-GACGATATATGGTTTTCAAAAATAGAATAG-GGATAACCGTTA ATCGAATTT-3' and 5'-TCA-TAACAACAAATTTACTTTACTTCATAGCTGATG-GAATTC TATTTTTGGAAACTCAATATATCG-TTAACAAAAACATTTACTTTTAGTCATACTGATGTGAATGTTACAACCAAT-3'). The target gene was sequenced with identification primers (5'-TGTGGGTGGTGCACATCTCAG-3' and 5'-GACGGGTAGTTCCAATCACT-CAGTCATCTACAT-3'). All correctly sequenced recombinant BAC plasmids were extracted using the QIAGEN Plasmid Midi Kit (QIAGEN, Germany) and then transfected into DEFs producing the virus BAC-CHV-ΔgI and BAC-CHV-ΔgI Rev.

**Antibodies**

Rabbit anti-CHV polyclonal antibody and goat anti-rabbit immunoglobulin G (IgG) antibody conjugated to streptavidin-biotin complex (SABC, BosterBio, CA) were used for plaque morphology analysis. Mouse anti-gI polyclonal antibody and goat anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP, Bio-Rad, CA) were used for Western blot assay to explore gI expression. Rabbit anti-gE polyclonal antibody and goat anti-rabbit IgG antibody conjugated to HRP (Bio-Rad) were used for Western blot assay to explore gE expression. Mouse anti-gI polyclonal antibody and Alexa-568 goat anti-mouse IgG (H + L) antibody were used for indirect immunofluorescence assay (Thermo Fisher Scientific).

**Western Blot**

Duck embryo fibroblast cells growing in 6-well dishes were infected with BAC-CHV and the gI mutant and revertant viruses for 48 h. The cells were washed with cold phosphate-buffered saline (PBS), and incubated in RIPA lysis buffer (Beyotime Biotechnology, China) for 30 min. The cell extracts were centrifuged at 12,000 × g for 30 min, and then the supernatants were collected. The proteins were then transferred to the polyvinylidene difluoride membrane and immunoblotted.

**Indirect Immunofluorescence Assay**

Duck embryo fibroblast cells growing in 6-well dishes with glass coverslips were infected with BAC-CHV and the gI mutant and revertant viruses at multiplicity of infection (MOI) of 0.01. After 48 h, the cells were washed with PBS and then fixed with 4% paraformaldehyde overnight at 4°C. The cells were washed with PBS containing 0.01% Tween 20 (PBS-T), permeabilized with 0.25% Triton X-100 in PBS for 30 min at 4°C, and washed 3 times with PBS-T before being incubated with 5% bovine serum albumin (BSA, Invitrogen, CA).
overnight at 4°C. The cells were then incubated with mouse anti-gl polyclonal antibody overnight at 4°C, washed 3 times with PBS-T, and incubated with Alexa-568 goat anti-mouse IgG (H + L) antibody for 1 h at 37°C. The cells were then washed with PBS-T for 5 times, incubated with DAPI for 15 min at 37°C and washed again with PBS-T. The cells were photographed using a Nikon ECLIPSE 80i inverted fluorescent confocal microscope with a SpotFlex image restoration system. Images were processed using Photoshop software.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Duck embryo fibroblast cells infected with BAC-CHv and BAC-CHv-ΔgI at MOI of 0.01 were collected at 24 h after infection for gD gene mRNA expression analyses. Total RNA was isolated using RNAiso Plus (Takara, Japan) and then reverse transcribed into cDNA (Takara). The cDNA was amplified after an initial denaturation for 30 s at 95°C, in 40 cycles of 10 s at 95°C and 30 s at 59.4°C in CFX Connect Real-Time PCR Detection System (Bio-Rad). The relative transcription levels of the gD gene were calculated using the 2^{-ΔΔCT} method. All experiments were repeated 3 times.

Plaque Morphology

The plaque morphology assay was performed as follows. Briefly, near-confluent (85–90%) DEF monolayer cells in 6-well plates were infected with BAC-CHv and the gl mutant and revertant viruses at MOI of 0.01. The viruses penetrated cells for 2 h at 37°C in 5% CO₂. Thereafter, the culture medium was removed, and the cells were washed 3 times with PBS. The DEF cells were incubated for 24 h in MEM containing 1% methylcellulose and 2% NBS. The culture medium was removed, and the cells were washed 3 times with PBS. The cells were then fixed with 4% paraformaldehyde overnight at 4°C, washed 3 times with PBS-T and permeabilized with 30% H₂O₂ and methanol at a ratio of 1 to 50 for 30 min. The cells were incubated with 5% BSA (Invitrogen) for 30 min and then incubated with rabbit anti-CHv polyclonal antibody and goat anti-rabbit IgG antibody conjugated to SABC (BosterBio). After washing 3 times with PBS-T, the cells were colorized by the addition of diaminobenzidine (DAB, BosterBio). Photographs of viral plaques were taken at 40 × magnification, and 100 randomly selected plaques were imaged for the viruses under consideration.

Electron Microscopy

Duck embryo fibroblast cells were infected with BAC-CHv and gl mutant virus at MOI of 5 for 20 h. Duck embryo fibroblast cells were washed with PBS, scraped and centrifuged at 900 × g for 15 min. The cells were then fixed in 2.5% glutaraldehyde, washed with PBS, fixed in 1.0% osmium tetroxide, and then subjected to stepwise dehydration in acetone. The samples were embedded in epoxy resin 618 and polymerized at 80°C for 72 h. Then, 50 nm ultrathin sections were prepared, collected on grids, stained with uranyl acetate and lead citrate, and examined with the Tecnai G2F20 transmission electron microscope.

RESULT AND DISCUSSION

In this study, gl gene mutant (BAC-CHv-ΔgI) and revertant (BAC-CHv-ΔgI Rev) strains were constructed successfully through using a bacterial artificial chromosome (BAC) clone of DPV CHv strain and a markerless two-step Red recombination technology.

The functions of the gl gene were explored in the case of comparing the wild-type BAC-CHv infection to BAC-CHv-ΔgI infection. After deleting gl, the abilities of DPV cell–cell spread were reduced. The plaque sizes of BAC-CHv, BAC-CHv-ΔgI, and BAC-CHv-ΔgI Rev were examined on DEF cells at 24 h after infection. Results showed that the plaque sizes of BAC-CHv were larger than BAC-CHv-ΔgI. The glI mutant formed plaques that contained 42.8% of the number of DEF cells compared with plaques formed by BAC-CHv (P ≤ 0.0001) (Figures 1A, 1B, and 1D). The gl revertant virus formed plaques that were similar in size to those of BAC-CHv (Figures 1C and 1D).

Furthermore, deleting glI displayed defects in DPV final envelopment. In BAC-CHv-infected cells, complete final envelopment processes including nucleocapsids binding onto cytoplasm vesicles and nucleocapsids budding into cytoplasm vesicles to acquire the final envelopment were detected (Figure 2A i, ii). In BAC-CHv-ΔgI-infected cells, nucleocapsids binding onto cytoplasm vesicles process could be detected; however, only a few number of nucleocapsids were be found to bud into vesicles and acquire the final envelopment. For exploring this issue much more comprehensively, we showed different cells to describe the same phenomenon (Figures 2B–2E iii–vi).

Alphaherpesvirus life cycle process involves complicated steps (Johnson and Baines, 2011). Different life cycle steps occurred in different cellular locations. Viral final envelopment always happened in transgolgi network (TGN). Glycoproteins enriched on TGN membrane and then interacted with tegument proteins to drive viral final envelopment. Thus, the collection of glycoproteins on TGN was a significant process for viral final envelopment. It has been reported that glycoproteins including gE/glI, gD, and gM were involved in this process in HSV- and PRV-infected cells. In HSV, gE/glI together with gD affect the production of enveloped virus particles (Farnsworth et al., 2003). Similarly, in PRV, extracellular and intracytoplasmic enveloped virus particles are absent in the absence of gE/glI and gM, and unenveloped capsids accumulate in the cytoplasm surrounded by tegument proteins in infected cells (Brack et al., 1999). However, the single deletion of gE or glI only slightly reduces the number of enveloped virions in both HSV- and PRV-infected cells. It means that gE/
gI acted redundantly with other glycoproteins like gD in HSV and gM in PRV to promote cytoplasmic envelopment. Herpes simplex virus and PRV gE and gI form a heterodimeric complex and reports showed that gE cytoplasmic (CT) domain contained tyrosine motifs to concentrate the complex to the TGN to promote assembly and egress. Reports also showed that HSV gI CT domain had no functions on promoting the complex located on TGN. The CT domain of gE and gI were added to gD to replace its CT domain and the location of gD chimeric proteins was characterized. Results showed that gD-gE<sub>CT</sub> chimeric protein located in TGN; however, gD-gI<sub>CT</sub> chimeric protein located on the apical and basolateral surfaces of infected cells. In HSV, the specific function of the gI gene was not clearly stated. In feline herpesvirus (FHV), deleting gI fully retained gE in the endoplasmic reticulum (ER). The authors in this article suggested that FHV gI may compete with molecular chaperons to release properly folded gE from ER to TGN. Results of the functions of DPV gE and gI were different from HSV and PRV. Previous study showed that deleting gE resulted in large capsids anchored around the cytoplasm vesicles with few of them budding into vesicles to acquire viral final envelopment (Liu et al., 2020). In present study, although gE gene retained in gI-deletion mutant, the same phenomenon was also detected (Figure 2). Furthermore, we also found that in BAC-CHv-ΔgI, the protein molecular mass of gE was lower than wild-type virus, which was similar to FHV. In FHV, deleting gI resulted in gE retained in ER and fail to produce mature gE in TGN, which led to the reduction of gE protein molecular mass. Thus, we suggested that the functions of DPV gI may be similar to FHV, and deleting DPV gI may result in gE fail to located to TGN to promote viral assembly and egress.

In present study, we also found that deleting DPV gI influenced viral cell-cell spread. HSV and PRV mutants deleting gE/gI or just the CT domain of gE were severely restricted for viral spread in epithelial tissues and in the nervous system. Numerous studies had demonstrated that TGN was a site where not only virus final envelopment occurred but also that involved in viral cell–cell spread. Articles showed that nascent enveloped virions sorted from TGN subdomains to cell junctions and then enhanced viral cell-to-cell spread. The gE CT domain could facilitate cell-to-cell spreading by trafficking virions through TGN to the lateral surfaces of polarized cells, and the extracellular domains of gE could bind ligands at cell junctions to promote virus entry into adjacent cells. If deleting DPV gI influenced the location of gE in TGN, it may inhibit the viral cell–cell spread process.

In summary, in this study, we found that DPV gI protein played important roles in viral cell-to-cell spread and virion final envelopment. It also provided us a message that DPV gI may affect the gE TGN location to influence virion final envelopment. In subsequent
experiments, we will further explore whether DPV gI could affect the localization of gE and influence viral assembly and egress.

**ACKNOWLEDGMENTS**

This research was supported by grants from the National Key Research and Development Program of China (2017YFD0500800), the China Agricultural Research System (CARS-42-17), Integration and Demonstration of Key Technologies for Goose Industrial Chain in Sichuan Province (2018NZ0005) and the China Agricultural Research System, Sichuan Veterinary Medicine and Drug Innovation Group of China Agricultural Research System (SCCXTD-2020-18)

Conflict of Interest: The authors declare that there are no conflicts of interest.

**REFERENCES**

Brack, A. R., J. M. Dijkstra, H. Gronzow, B. G. Klupp, and T. C. Mettenleiter. 1999. Inhibition of virion Maturation by Simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. J. Virol. 73:5364–5372.
Farnsworth, A., K. Goldsmith, and D. C. Johnson. 2003. Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm. J. Virol. 77:8481–8494.

Farnsworth, A., and D. C. Johnson. 2006. Herpes simplex virus gE/gI must accumulate in the trans-Golgi network at early times and then redistribute to cell junctions to promote cell-cell spread. J. Virol. 80:3167–3179.

Johnson, D. C., and J. D. Baines. 2011. Herpesviruses remodel host membranes for virus egress. Nat. Rev. Microbiol. 9:382–394.

Liu, T., M. Wang, and A. Cheng. 2020. Duck plague virus gE serves essential functions during the virion final envelopment through influence capsids budding into the cytoplasmic vesicles. Sci. Rep. 10:5658.

Sattentau, Q. 2008. Avoiding the void: cell-to-cell spread of human viruses. Nat. Rev. Microbiol. 6:815–826.

Tischer, B. K., G. A. Smith, and N. Osterrieder. 2010. En passant mutagenesis: a two step markerless red recombination system. Methods Mol. Biol. 634:421–430.

Uchida, H., J. Chan, I. Shrivastava, B. Reinhart, P. Grandi, J. C. Glorioso, and J. B. Cohen. 2013. Novel mutations in gB and gH circumvent the requirement for known gD Receptors in herpes simplex virus 1 entry and cell-to-cell spread. J. Virol. 87:1430–1442.