Bovine Herpesvirus Type 4 Glycoprotein L Is Nonessential for Infectivity but Triggers Virion Endocytosis during Entry

Céline Lété,a Bénédicte Michail,a Philip G. Stevenson,b Alain Vanderplasschen,a and Laurent Gilletb

The core entry machinery of mammalian herpesviruses comprises glycoprotein B (gB), gH, and gL. gH and gL form a heterodimer with a central role in viral membrane fusion. When archetypal alpha- or betaherpesviruses lack gL, gH misfolds and progeny virions are noninfectious. However, the gL of the rhadinovirus murid herpesvirus 4 (MuHV-4) is nonessential for infection. In order to define more generally what role gL plays in rhadinovirus infections, we disrupted its coding sequence in bovine herpesvirus 4 (BoHV-4). BoHV-4 lacking gL showed altered gH glycosylation and incorporated somewhat less gH into virions but remained infectious. However, gL− virions showed poor growth associated with an entry defect. Moreover, a major part of their entry defect appeared to reflect impaired endocytosis, which occurs upstream of membrane fusion itself. Thus, the rhadinovirus gL may be more important for driving virion endocytosis than for incorporating gH into virions, and it is nonessential for membrane fusion.

The Herpesviridae family contains numerous important pathogens that are classified in three subfamilies (Alpha-, Beta-, and Gammaherpesvirinae). These enveloped viruses enter cells by fusing their envelopes with host cell membranes either by direct fusion at the plasma membrane or by pH-dependent or -independent endocytosis, depending on the virus and on the target cell (4). While most enveloped viruses rely on a single fusogenic protein for entry, herpesviruses are more complex. Indeed, they use a core fusion machinery, composed of glycoprotein B (gB) and the gH/gL heterodimer, that is conserved across the three subfamilies (10). Most of the herpesviruses employ also one or more additional receptor-binding or regulating proteins specific to subfamilies or genera. This complexity explains why herpesvirus entry, and particularly the fusion mechanism, is still poorly understood.

The X-ray structure determinations of herpes simplex virus 1 (HSV-1) and Epstein-Barr virus (EBV) gB (5, 26) suggested that it is a class III viral fusion protein as is glycoprotein G of vesicular stomatitis virus (VSV) (44–45) or baculovirus gp64 (29). However, in contrast to those proteins, gB cannot function on its own and requires the gH/gL heterodimer. This complex is a major target of virus-neutralizing antibodies in several herpesviruses (17, 43), emphasizing its role in virus entry. It has been proposed that gH has features characteristic of class I viral fusogens (14–16). However, the recently published structures of HSV-2 and EBV gH/gL (9, 39) suggested that these proteins do not likely act as cofusogen but rather regulate fusion by gB. These structures also revealed extensive contacts between gL and the N-terminal domain of gH. Interestingly, the sequences of gL and the gH N terminus vary substantially among herpesviruses and it appears that the gH-gL pairs in each herpesvirus have coevolved to form tight complexes with possible specific functions (9).

Bovine herpesvirus 4 (BoHV-4) belongs to the Gammaherpesvirinae subfamily, Rhadinovirus genus, together with the notable human pathogen Kaposi’s sarcoma-associated herpesvirus (KSHV). Until recently, little was known about gH and gL in these viruses beyond the fact that they are virion-associated components (33, 41). However, we recently showed that gL was not essential for murid herpesvirus 4 (MuHV-4), another rhadinovirus. MuHV-4 lacking gL both incorporates gH into virions and remains infectious, although it shows some attenuation relative to the wild type (WT) (23). This result is quite surprising compared to results for all alpha- and betaherpesviruses tested to date, in which gL proved to be indispensable (7, 13, 27, 31, 46). This is also intriguing, as rhadinovirus gH/gL is a major neutralization target (17) protected by various antibody evasion mechanisms (24, 36).

In order to know if MuHV-4 gL properties are shared among rhadinoviruses, we disrupted the gL coding exon (ORF47) in the BoHV-4 genome. BoHV-4 lacking gL remained infectious but displayed a growth deficit. This appeared to be associated with impaired cell endocytosis rather than cell binding. Indeed, gL deletion severely altered the trafficking of the virion containing endosomes during entry.

MATERIALS AND METHODS

Cells and virus. 293T (ATCC CRL-11268), Madin-Darby bovine kidney (MDBK; ATCC CCL-22), embryonic bovine lung (EBL); German Collection of Microorganisms and Cell Culture (DSMZ) ACC192), bovine turbinate (BT; ATCC CRL-1390), and embryonic bovine trachea (EBTr; ATCC CCL-44) cells were cultivated in Dulbecco’s modified Eagle medium (Invitrogen) containing 10% fetal calf serum (FCS), 2% penicillin/streptomycin (Invitrogen), and 1% nonessential amino acids (Invitrogen). The BoHV-4 V.test strain and the V.test BAC G-derived bacterial artificial chromosome (BAC) clone were described elsewhere (22, 49).

Antibodies. Five mouse monoclonal antibodies (MAbs) raised against BoHV-4 were used in the present study (12). Their specificities were unraveled on 293T cells transfected with the vectors encoding gB-glycophosphatidylinositol (gB-GPI), gH-GPI, or gL-GPI (36). The epitopes depending on the gH-gl heterodimer were reconstituted by co-
expressing gH-GPI and gL-GPI (Fig. 1). MAb 35 recognizes gB, as previously stated (34). For some Western blots, we used serum of a rabbit infected intravenously with 10⁸ PFU of the BoHV-4 V.test strain and collected 63 days postinoculation. Lysosome-associated membrane protein 1 (LAMP-1) was detected with rabbit polyclonal antibody (PAb; ab24170; Abcam).

Indirect immunofluorescent staining of adherent cells. Cells were fixed and permeabilized with acetone 95% for 10 min at 20°C or with paraformaldehyde (4% [wt/vol]) for 10 min on ice and Tween 20 (0.1% [vol/vol]) in phosphate-buffered saline (PBS; anti-LAMP-1 stainings). After the cells were washed with PBS, immunofluorescent staining (incubation and washes) was performed with PBS containing 10% (vol/vol) FCS. Samples were incubated at 37°C for 45 min with the different mouse anti-BoHV-4 MAbs or anti-LAMP-1 rabbit polyclonal. After three washes, samples were incubated at 37°C for 30 min with Alexa Fluor 488 or 568 goat anti-mouse (GAM) IgG (2 µg/ml; Invitrogen) or with Alexa Fluor 568 goat anti-rabbit (GARb) IgG (2 µg/ml; Invitrogen). When stated in the figure legends, nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole) or To-Pro-3 iodide (Invitrogen; 1 nM PBS). Fluorescence was then visualized with a Nikon Eclipse TE2000-S microscope and a Leica DC300F charge-coupled-device (CCD) camera system or with a Leica true confocal scanner (TCS) SP laser scanning microscope.

Production of BoHV-4 gL STOP and revertant strains. We disrupted the BoHV-4 V.test strain gL coding sequence (42) (GenBank accession number JN133502; genomic coordinates 60731 to 60309) by introducing
stop codons into the coding sequence for the gL signal peptide (gL STOP). BoHV-4 recombinants were produced using BAC cloning and prokaryotic recombination technologies as described before (22). The V.test BAC G plasmid was used as the parental plasmid (22). The V.test BAC G plasmid was produced using a two-step galactokinase (galk gene product) positive/negative selection in bacteria (52) (Fig. 2). The first recombination process (galk positive selection) consisted of introducing the galk gene in ORF47 (genomic coordinate 60693), coding for gL, resulting in the V.test BAC G ORF47 galk plasmid. Recombination was achieved using the galk cassette. It consisted of the galk gene flanked by 50-bp sequences corresponding to ORF47 regions (60644 to 60669 and 60694 to 60744 of the BoHV-4 V.test strain genome). This cassette was produced by PCR using the pgalK vector (52) as the template and fl-galK (5'-ATGGATTTCTGATAGAATTTACAAATGTTGATATTGAAGATG-3' and rev-galK (5'-ACAATATATACAAATGTGAGATATCTATGTTTTTTGTCTTTGCTGTTA TGAATTTTGACAGCAGTCTCGTCTCCCT-3') as forward and reverse primers, respectively. The second recombination process (galk negative selection) consisted of replacing the galk gene sequence with a gL STOP cassette to generate the BoHV-4 V.test strain gL STOP plasmid. The gL STOP cassette consisted of a synthetic double-stranded DNA (Eurogentec) corresponding to genomic coordinates 60742 to 60643 with the introduction of 36 nucleotides coding for in-frame stop codons and restriction sites responding to genomic coordinates 60742 to 60643 with the introduction of 36 nucleotides coding for in-frame stop codons and restriction sites after genomic position 60692 (Fig. 2). These 36 nucleotides do not insert stop codons in any of the five other frames of the genome. The V.test BAC G plasmid was produced similarly from BoHV-4 V.test strain gL STOP plasmid. The first recombination process (galk positive selection) was identical to the one described above. The second recombination process (galk negative selection) consisted of restoring ORF47 to generate a revertant plasmid. This cassette was produced by PCR using the BoHV-4 V.test strain genome as the template and gl-zone-rec-sens (5'-AAGGTA CCCCATGATCTAATGTGTTGGTG3'-3') and gl-zone-rec-rev (5'-AAAGGCGAAGTTGATTATG3'-3') as forward and reverse primers, respectively. Reconstitution of infectious virus from BAC plasmids was obtained by transfection in MDBK cells. The viruses used (BAC+<sup><sup>+</sup></sup>) express enhanced green fluorescent protein (eGFP) from a human cytomegalovirus (HCMV) IE1 promoter located in the BAC cassette (22), so eGFP expression provides a convenient marker of infection. Typically, eGFP is detectable as soon as 8 h postinfection.

**Southern blot.** Southern blot analysis of viral DNA digested with BamHI was performed with probe corresponding to the gL surrounding genome as the template and gL-zone-rec-sens and gL-zone-rec-rev as forward and reverse primers, respectively (genomic coordinates 60687 to 61134).

**Western blot.** Virions or infected cells were lysed and denatured by heating (95°C, 5 min) in SDS-PAGE sample buffer (31.25 mM Tris-HCl [pH 6.8], 1% [v/v] SDS, 12.5% [v/v] glycerol, 0.005% [v/v] bromophenol blue, 2.5% [v/v] mercaptoethanol). Proteins were resolved by electrophoresis with Mini-Protean Tris-glycine Extended (TGX) precast 4 to 15% resolving gels (Bio-Rad) in SDS-PAGE running buffer (25 mM Tris-base, 192 mM glycine, 0.1% [v/v] SDS) and transferred to polyvinylidene difluoride membranes (Immobilon-P transfer membrane, 0.45-μm pore size; Millipore). The membranes were blocked with 3% nonfat milk in PBS/0.1% Tween 20 and then incubated with primary antibody in the same buffer. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG PAB or rabbit anti-mouse IgG PAB (Dako Corporation), followed by washing in PBS/0.1% Tween 20, development with enhanced chemiluminescence (ECL) substrate (GE Healthcare), and exposure to X-ray film.

**Oligosaccharide digestion.** All reagents were obtained from New England BioLabs (NEB). Samples were denatured in glycoprotein denaturing buffer (0.5% SDS, 40 mM dithiothreitol [DTT]) for 10 min at 100°C and then digested for 3 h at 37°C with 500 NEB units of endo-β-N-acetylglucosaminidase-H (Endo-H) or 250 NEB units of peptide-N-glycosidase F (PNGase F), and/or 250 NEB units of neuraminidase, β-4-galactosidase, and O-glycanase in G7 reaction buffer (50 mM sodium phosphate, pH 7.5) with 1% NP-40. Reactions were stopped by the addition of Laemmli sample buffer, and proteins were analyzed by immunoblotting as described below.

**Virus purification.** BoHV-4 virions grown on MDBK cells were purified as follows. After removal of the cell debris by low-speed centrifugation (1,000 × g, 10 min), virions present in the infected cell supernatant were harvested by ultracentrifugation (100,000 × g, 2 h) through a 30% (w/v) sucrose cushion and then centrifuged through two successive 20 to 50% (w/v) sucrose potassium tartrate gradients in PBS (100,000 × g, 2 h). Virions were finally washed and concentrated in PBS (100,000 × g, 2 h).

**Growth curves.** The growth kinetics of mutant and revertant viruses were compared to those of the WT. Cell cultures were infected at a multiplicity of infection (MOI) of 0.01 (multistep assay). After 1 h of adsorption, the cells were washed and then overlaid with minimum essential medium containing 5% FCS. Supernatants of infected cultures or infected cells were harvested at successive intervals, and the amount of infectious virus was determined by plaque assay on MDBK cells.

**Flow cytometry.** Cells exposed to eGFP<sup>+</sup> viruses were washed in PBS and analyzed directly for green channel fluorescence with a three-laser Becton Dickinson fluorescence-activated cell sorter (FACS)Aria.

**Viral genome detection by real-time PCR.** DNA was purified from infected cells or from 10<sup>6</sup> purified virions of the different strains using a QIAamp DNA minikit (Qiagen). Real-time PCRs were performed to determine viral and cellular genome copies. Briefly, a 138-bp fragment corresponding to BoHV-4 ORF7 was amplified with the forward primer ORF7 sens (5'-CAGCGAAAAAGTTGGGTCTTC-3') and the reverse primer ORF7 rev (5'-TTGAGGCGCTTGATATTGTC-3'). The PCR products were quantified by hybridization with a TaqMan probe (genomic coordinates 10073 to 10096, 5'-6-FAM-TGACACCCATCCCCATCCATTTTTT-3', where FAM is 6-carboxyfluorescein and BHQ-1 is Black Hole Quencher-1 dye) and converted to genome copies by comparison with a standard curve of BoHV-4 V.test strain WT DNA, amplified in parallel. PCR amplifications were performed under the following conditions: initial activation of the Taq polymerase (Bio-Rad) at 95°C for 3 min followed by 50 cycles at 95°C for 15 s and 58°C for 40 s. Cellular DNA was quantified in parallel by amplifying part of the bovine actin gene (forward primer, 5'-CGCGATCCGAAACTACCT-3'; reverse primer, 5'-CAACCGACTCTGCTACCCCT-3'). The latter PCR products were quantified with Sybr green (Invitrogen), and the copy number was calculated by comparison with a standard curve of cloned bovine actin template. PCR amplifications were performed under the following conditions: initial activation of the Taq polymerase (Bio-Rad) at 95°C for 3 min followed by 45 cycles at 95°C for 30 s, 56°C for 45 s, and 72°C for 45 s. PCR amplifications and fluorescence reactions were carried out with an iCycler system (Bio-Rad).

**RESULTS**

**Identification of gH-specific MAbS.** As demonstrated for other herpesviruses, BoHV-4 gL and gH associate both in virions and in infected cells (33). We have previously characterized monoclonal antibodies recognizing gL alone (MAB 16) or the gH/gL heterodimer (MAB 33) (36). As the absence of gL could interfere with gH stability and recruitment into virions, we first looked for MAbS recognizing a gL-independent gH epitope. Anti-BoHV-4 MAbS were therefore screened for gH, gL, or gH/gL specificity as described in Materials and Methods. Interestingly, similarly to MuHV-4 (17), some epitopes of the gH/gL heterodimer were reconstituted by coexpression of gH-GPI and gL-GPI, suggesting that gL lies close to the virion membrane in the rhadinovirus gH-gL heterodimer. Cells expressing the extracellular part of gB were used as the control (Fig. 1). MABs 20, 30, and 15 recognized
FIG 2 Generation of a gl-deficient BoHV-4 mutant. (A) Schematic representation of the strategy followed to produce the recombinant BoHV-4 strain. The gl-deficient BoHV-4 mutant was derived from a cloned BoHV-4 BAC by the galK counterselection method. The gl coding sequence (ORF47) was disrupted by inserting stop codons near the end of the coding sequence for its predicted signal peptide (gl STOP). The mutation incorporated a new BamHI restriction site. (B) Verification of the molecular structure. Viral DNA was digested with BamHI, resolved by agarose gel electrophoresis, and hybridized with a ³²P-labeled probe, corresponding to nucleotides 60087 to 61334. Open arrows show the restriction fragment that contains gl STOP. The 7,137-bp wild-type (WT) band becomes 6,553 bp for the gl STOP mutant. Black arrows show the remaining 620-bp fragment. Marker sizes (in kbp) are indicated on the left. (C) Glycoprotein expression by the gl-deficient BoHV-4 strain. MDBK cells were infected with the WT, the WT BAC, gl STOP, and gl STOP Rev BoHV-4 (MOI of 0.5 PFU per cell). Thirty-six hours postinfection, the cells were fixed, permeabilized, and stained with MAb 15 (gB), MAb 20 (gL), MAb 30 (gH/gL), MAb 6 (gH), or MAb 9 (gH). An Alexa 568-conjugated goat anti-mouse secondary antibody was then used. Nuclei were counterstained with DAPI.
gL, the heterodimer gH/gL, and gB, respectively. MAbs 6 and 9 were shown to recognize gH independently of gL.

**Generation of a gL-deficient mutant.** We tested the functional importance of gL for BoHV-4 replication by disrupting ORF47. In the BoHV-4 V. test strain, ORF47 is predicted to be located from genomic coordinates 60731 to 60309 (42). It encodes a predicted 137-amino-acid protein with a 22-amino-acid leader sequence. As a second in-frame ATG is present at genomic coordinates 60701 to 60699, we disrupted this coding sequence by inserting stop codons at genomic coordinate 60892 to generate the BoHV-4 gL STOP strain as described in Materials and Methods. This terminated ORF47 translation 9 amino acids before the end of its predicted signal sequence without any associated deletion (Fig. 2A). A revertant strain, called gL STOP Rev, was finally constructed to validate the gL STOP mutant. The predicted molecular structures of the recombinant strains were confirmed by BamHI restriction mapping (data not shown) and Southern blotting (Fig. 2B) and, further, by DNA sequencing (data not shown).

The gL STOP mutant produced infectious virus after BAC DNA transfection into MDBK cells. Immunostaining of infected cells (Fig. 2C) confirmed the lack of expression of gL-dependent epitopes, as seen with MAb 20 (anti-gL) and MAb 30 (anti-gH/L) staining. In contrast, MAB 6 and 9 stainings showed that gL-independent gH epitopes were normally expressed. Taken together, these results showed that BoHV-4 gL is nonessential.

**Characterization of gH in the gL− mutant.** To analyze the influence of the absence of gL on other structural proteins, Western blot analyses of infected cells were performed. Immunoblotting with a polyclonal serum raised against whole virus showed no observable difference between gL STOP and WT or gL STOP Rev strain-infected cells (Fig. 3A). There was also no evidence of a difference in gB or gp180, another viral envelope glycoprotein involved in entry (35). In contrast, while anti-gH MAbs 6 and 9 detected an ~120-kDa protein in both WT and gL STOP Rev strain-infected cells, they both detected a higher band in gL STOP strain-infected cells.

This difference could reflect differences in gH glycosylation. BoHV-4 V.test strain gH has no potential O-glycosylation site (Fig. 3B) but 16 potential N-glycosylation sites (Fig. 3C). More specifically, the absence of gL causes a postbinding deficit. The gL-dependent entry deficit could be due to impaired cell binding or impaired cell penetration of virions. We first investigated cell binding by quantifying the number of viral genomes associated with the cells after various times of infection. Surprisingly, we did not observe reduced binding of gL STOP virions at early time points after infection either in MDBK (Fig. 5A) or in EBL (Fig. 5B) cells—quite the contrary. The apparent better binding of gL STOP virions observed at these early time points could be explained by the noninfectious-to-infectious particle ratio that was approximately 10-fold higher for gL− viruses than for gL+. Indeed, when protein contents of purified virions are compared (Fig. 5C and D), viral protein contents for the same numbers of PFU are about 10-fold higher for gL− virus stocks than for the WT and Rev. Genome-to-PFU ratios confirmed that there were proportionally more noninfectious particles in gL− stocks than in gL+. stocks

Downloaded from http://jvi.asm.org/ on September 23, 2017 by guest
FIG 3 Western blot analysis. (A) MDBK cells were mock infected or infected with the WT BAC, gL STOP, and gL STOP Rev BoHV-4 strain (1 PFU/cell). Forty-eight hours later, cells were scraped and immunoblotted for virion components, using either a rabbit serum raised against whole virus (total BoHV-4) or MAbs specific for gp180, gB, and gH, as indicated. In the anti-gp180 blot, open and filled triangles indicate the specific 180-kDa protein and a background band, respectively. In the anti-gB blot, open and filled triangles indicate uncleaved and furin-cleaved gB, respectively. (B) Prediction of O-glycosylation sites for the complete BoHV-4 V.test strain gH protein sequence using the NetOglyc 3.1 algorithm. The shaded regions indicate the signal peptide and transmembrane region. The red line indicates the significance threshold. (C) Prediction of N-glycosylation sites for the complete BoHV-4 V.test strain gH protein sequence using the NetNglyc 1.0 algorithm. The shaded regions indicate the signal peptide and transmembrane region. The red line indicates the significance threshold. (D) MDBK cells were mock infected or infected with the WT BAC, gL STOP, and gL STOP Rev BoHV-4 strain (1 PFU/cell). Forty-eight hours later, cells were scraped and deglycosylated with Endo H or PNGase F and then immunoblotted for gH (MAb 9). (E) Purified virions were subjected to Western blotting with rabbit serum raised against whole virus (total BoHV-4) or MAbs specific for gp180, gB, and gH, as indicated. In the anti-gB blot, open and filled triangles indicate uncleaved and furin-cleaved gB, respectively. In the anti-gH blot (MAb 6), open and filled triangles indicate the specific 120-kDa gH protein and a background band, respectively. The position of an MM standard (kDa) is shown.
In other words, we underestimate the number of gL STOP virions when standard plaque assay titration is used. At later time points, genome copy numbers increased significantly in WT-infected cells (Fig. 5A and B), reflecting likely viral DNA replication. In contrast, we did not observe a similar increase in gL STOP-infected cells. Taken together, these results suggest that the entry deficit of gL STOP virions is mainly postbinding.

We tested penetration of the different viral strains in MDBK cells (Fig. 6A). The results obtained showed that after 4 h, most of the WT and gL STOP Rev virions had penetrated the cells.
contrast, after 6 h, most of the gL STOP virions were still sensitive to an acid wash, indicating that they were still at the cell surface. These results showed that the gL STOP mutant appeared to be mainly impaired in cell penetration. This deficit could not be overcome by forcing fusion of these cell surface blocked virions. Indeed, polyethylene glycol (PEG) treatment did not significantly enhance gL− virion entry compared to that of gL+ virions (Fig. 6B).

**gL-deficient virions still require endocytosis but are less sensitive to inhibitors of endosomal acidification.** Gammaherpesvi-
ruses have been reported variously to fuse with the plasma membrane or with endosomal membranes (2, 17, 40). Wild-type BoHV-4 infection of MDBK cells was blocked by inhibitors of clathrin-mediated endocytosis or lysosomal acidification (Fig. 6C). As gL STOP virions display a penetration deficit, we tested their sensitivity to the same drugs. Surprisingly, infection by gL-deficient virions remained as sensitive to chlorpromazine as WT or gL STOP Rev strains (0.5 PFU/cell). NH4Cl treatment, which mainly blocks endocytosis as previously observed (19), gave similar results (Fig. 6C). Interestingly, gL STOP virions were less sensitive to bafilomycin B1, which specifically blocks endosome acidification through inhibition of vacuolar-type H\(^+\)/H\(_{1001}\)-ATPase, than those of WT or gL STOP Rev strains (Fig. 6C). gL-deficient virions therefore appeared to still require endocytosis but to be less dependent on low pH.

**gL-deficient virions show abnormal distribution during entry.** We finally tracked the entry of gL STOP virions into MDBK and EBL cells by immunofluorescence (Fig. 7) with MAb 35 that recognizes gB (34). We bound WT, gL STOP, and gL STOP Rev virions to MDBK or EBL cells for 3 h at 4°C before washing off any unbound virion. We then analyzed virion localization either directly or after incubation at 37°C for 3 h. As previously shown (Fig. 5), we did not observe any gL-related binding deficit in either MDBK or EBL cells (Fig. 7A, 0h post-binding). After 3 h, most of the endosomes containing WT or gL STOP Rev proteins reached the nuclear margin. In contrast, gL-deficient virions remained scattered mainly near the plasma membrane (Fig. 7A, 3h post-binding). We observed that WT BoHV-4 virions, like MuHV-4 virions (19), reach LAMP-1 \(/H\(_{11001}\) late endosomes (Fig. 7B). In contrast, gL- virions remained scattered throughout the cytoplasm but failed to progress to LAMP-1 \(/H\(_{11001}\) late endosomes (Fig. 7B). This result suggests that the entry deficit associated with the absence of gL is downstream of cell binding but upstream of membrane fusion.

**DISCUSSION**

Most enveloped viruses devote just one protein to cell binding and membrane fusion. Herpesviruses are more complex and engage cells via multiple glycoproteins. gL is one of the three glycoproteins, the others being gH and gB, that form the core entry machinery conserved for the **Herpesviridae** family. All known functions of gL are directly associated with its dimerization with gH. Indeed, gL is a chap-
FIG 7  
gL-deficient virions show altered glycoprotein distribution during entry. (A) MDBK or EBL cells were exposed to WT BAC, gL STOP, or gL STOP Rev BoHV-4 strains (3 h, 4°C, 10 PFU/cell) and then washed in PBS and either fixed immediately (0 h postbinding) or incubated first (3 h, 37°C) to allow virion endocytosis (3 h postbinding). The cells were then permeabilized and stained for BoHV-4 glycoprotein antigen (MAb 35, anti-gB). Nuclei were counterstained with To-Pro-3. Equivalent data were obtained in three further experiments. (B) BoHV-4 virions were bound to MDBK cells (3 h, 4°C, 10 PFU/cell) with or without bafilomycin (5 nM). The cells were then washed in PBS to remove unbound virions and further incubated with or without drug (3 h, 37°C) to allow virion endocytosis. The cells were then fixed, permeabilized, and stained for gB plus LAMP-1.
The absence of gL could also result in a fusion deficit. However, the PEG-induced fusion experiment would have, at least partially, rescued gL− virions, as observed for PRV gL− mutants (31). That was not the case (Fig. 6B). A fusion deficit also does not explain why endosomes containing glycoproteins of gL− virions do not migrate to the nuclear margin, as observed for WT and gL STOP Rev infection in either EBL or MDBK cells (Fig. 7A). Moreover, fusion blockage of WT virions, as observed with bafilomycin-treated cells, is not associated with a defect in endosome migration in contrast to what is observed with gL− virions (Fig. 7B). Taken together, these results show that the major part of the gL− virion defects appeared to reflect impaired endocytosis and altered endosome migration, which occur upstream of membrane fusion itself.

During entry, the crowded and highly packed cytoplasm inevitably creates a big challenge for trafficking of herpesvirus particles (8). Rhadinoviruses could bypass this problem by eliciting endocytosis and endosome migration to the nuclear margin. While complexing cellular surface receptors is likely to be sufficient to induce endocytosis, endosome targeting probably requires virus signaling to the cell. As endosomes containing glycoproteins of gL−deficient BoHV-4 virions show abnormal distribution after incubation at 37°C (Fig. 7), we propose that BoHV-4 gL could be involved in such signaling. Treatment with chlorpromazine (Fig. 6C) shows that both gL+ and gL− virions enter the cells through clathrin-mediated endocytosis. However, the process is much slower in the absence of gL (Fig. 7A).

One hypothesis could therefore be that interaction of some gL-dependent epitope with a cell surface receptor induces de novo formation of clathrin-coated pits as observed for some other viruses, such as influenza A virus (47) or VSV (11). In the absence of gL, virions could be internalized by random ongoing endocytic activities. That would explain why they remain at the cell surface for longer periods of time (Fig. 6A) but still require endocytosis (Fig. 6C). As the ongoing endocytic activities are certainly different between cells, this could explain the differences that we observed in gL− virion infectivity between cells.

This potential role of gL could, at least partially, explain why the gH/gL heterodimer is a major neutralization target in rhadinoviruses (17), although HS binding is redundant. Moreover, it would explain why gH/gL targeted neutralization of MuHV-4 WT virions did not affect virus binding or membrane fusion but blocked the accumulation of perinuclear capsids (D. Glauser and P. G. Stevenson, unpublished results).

Taken together, our results show that gL is nonessential in BoHV-4 although it is involved in viral entry. We propose that one of the major functions of gL in rhadinoviruses is to trigger virion endocytosis.

ACKNOWLEDGMENTS

C.L., B.M., and L.G. are Research Fellows and Research Associates of the “Fonds de la Recherche Scientifique—Fonds National Belge de la Recherche Scientifique” (F.R.S.-FNRS), respectively. P.G.S. is a Wellcome Trust Senior Clinical Fellow (GR076956MA).

This work was supported by the following grants: ARC “GLYVIR,” starting grant from the University of Liège (D-09/11), and an Incentive Grant for Scientific Research from the F.R.S.-FNRS (F.4510.10).

REFERENCES

forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. 66:2240–2250.


