Bovine Herpesvirus 1 UL49.5 Interacts with gM and VP22 To Ensure Virus Cell-to-Cell Spread and Virion Incorporation: Novel Role for VP22 in gM-Independent UL49.5 Virion Incorporation

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ABSTRACT Alphaherpesvirus envelope glycoprotein N (gN) and gM form a covalently linked complex. Bovine herpesvirus type 1 (BHV-1) UL49.5 (a gN homolog) contains two predicted cysteine residues, C42 and C78. The C42 is highly conserved among the alphaherpesvirus gN homologs (e.g., herpes simplex virus 1 and pseudorabies virus). To identify which cysteine residue is required for the formation of the UL49.5/gM complex and to characterize the functional significance of the UL49.5/gM complex, we constructed and analyzed C42S and C78S substitution mutants in either a BHV-1 wild type (wt) or BHV-1 UL49.5 cytoplasmic tail-null (CT-null) virus background. The results demonstrated that BHV-1 UL49.5 residue C42 but not C78 was essential for the formation of the covalently linked functional UL49.5/gM complex, gM maturation in the Golgi compartment, and efficient cell-to-cell spread of the virus. Interestingly, the C42S and CT-null mutations separately did not affect mutant UL49.5 virion incorporation. However, when both of the mutations were introduced simultaneously, the UL49.5 C42S/CT-null protein virion incorporation was severely reduced. Incidentally, the anti-VP22 antibody coimmunoprecipitated the UL49.5 C42S/CT-null mutant protein at a noticeably reduced level compared to that of the individual UL49.5 C42S and CT-null mutant proteins. As expected, in a dual UL49.5 C42S/VP22Δ virus with deletion of VP22 (VP22Δ), the UL49.5 C42S virion incorporation was also severely reduced while in a gMΔ virus, UL49.5 virion incorporation was affected only slightly. Together, these results suggested that UL49.5 virion incorporation is mediated redundantly, by both UL49.5/gM functional complex and VP22, through a putative gM-independent novel UL49.5 and VP22 interaction.

IMPORTANCE Bovine herpesvirus 1 (BHV-1) envelope protein UL49.5 is an important virulence determinant because it downregulates major histocompatibility complex class I (MHC-I). UL49.5 also forms a covalently linked complex with gM. The results of this study demonstrate that UL49.5 regulates gM maturation and virus cell-to-cell spread since gM maturation in the Golgi compartment depends on covalently linked UL49.5/gM complex. The results also show that the UL49.5 residue cysteine 42 (C42) mediates the formation of the covalently linked UL49.5-gM interaction. Furthermore, a C42S mutant virus in which UL49.5 cannot interact with gM has defective cell-to-cell spread. Interestingly, UL49.5 also interacts with the tegument protein VP22 via its cytoplasmic tail (CT). The putative UL49.5 CT-VP22 interaction is essential for a gM-independent UL49.5 virion incorporation and is revealed when UL49.5 and gM are not linked. Therefore, UL49.5 virion incorporation is mediated by UL49.5/gM complex interaction and through a gM-independent interaction between UL49.5 and VP22.

KEYWORDS UL49.5/gM complex, gM maturation, gM and UL49.5 virion incorporation, novel UL49.5-VP22 interaction

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Bovine herpesvirus type 1 (BHV-1) is an important pathogen of cattle that can cause a severe respiratory tract infection, known as infectious bovine rhinotracheitis (IBR), and abortion in pregnant cows (1, 2). In addition, BHV-1 is an important component of the bovine respiratory disease complex (BRDC), also known as shipping fever (3, 4). The BHV-1 gene product, envelope protein Ul49.5, a glycoprotein N (gN) homolog of alphaherpesviruses, forms a disulfide-linked complex with envelope glycoprotein M (gM). Both proteins are nonessential although in the absence of either Ul49.5 or gM, virus yield is reduced significantly (5, 6). The Ul49.5 gene products of BHV-1, herpes simplex virus 1 (HSV-1), and equine herpesvirus 1 (EHV-1) are not glycosylated (6–8). The corresponding Ul49.5 gene product of pseudorabies virus (PRV) is glycosylated and is termed gN (9). In PRV, gN is not essential for gM maturation in the Golgi compartment and for gM virion incorporation, but gM is necessary for gN virion incorporation (10). In contrast, formation of the Ul49.5/gM complex is essential for BHV-1 gM maturation in the Golgi compartment (11, 12). Currently, it is not known whether BHV-1 gM and/or Ul49.5 is necessary for each other’s virion incorporation. Among the varicelloviruses, BHV-1, PRV, and EHV-1 Ul49.5 or its gN homologs bind to the transporter associated with antigen presentation (TAP) in virus-infected cells and thereby downregulates major histocompatibility complex class I (MHC-I) cell surface expression (11, 13). However, unlike the PRV and EHV-1 proteins, BHV-1 Ul49.5 not only binds but also degrades TAP (13).

BHV-1 Ul49.5 is a 9-kDa type I membrane protein (6). The predicted Ul49.5 open reading frame (ORF) encodes 96 amino acids (aa) and is composed of an N-terminal signal sequence of 22 aa, an extracellular luminal domain of 32 aa, a transmembrane (TM) domain of 25 aa, and a short cytoplasmic tail (CT) of 17 aa (14) (Fig. 1A). There are two predicted cysteine residues in the BHV-1 Ul49.5 ORF, C42 and C78. Alignment of BHV-1 Ul49.5 amino acid sequences with the corresponding Ul49.5 sequences of other alphaherpesviruses showed that C42, located within the luminal domain of BHV-1 Ul49.5, is highly conserved among alphaherpesviruses (Fig. 1B). The complex between Ul49.5 and gM is thought to be linked via disulfide bonds between cysteine residues. Since the BHV-1 Ul49.5 C42 is highly conserved among herpesviruses (Fig. 1B), we hypothesized that the BHV-1 Ul49.5/gM complex is mediated by the predicted Ul49.5 residue C42 and a predicted cysteine residue in the gM ORF (Fig. 1C).

It was previously postulated that BHV-1 Ul49.5 binds to TAP through its TM domain (11). However, it has not been possible to map the BHV-1 Ul49.5/TAP binding domain within the TM because even a short deletion within the BHV-1 Ul49.5 TM domain resulted in degradation of the protein (11, 15). Additionally, it was reported that in a stably transfected cell line, gM interferes with Ul49.5-mediated TAP inhibition and MHC-I downregulation function, indicating that gM might compete with Ul49.5 for TAP binding (12). Recently, we have reported that Ul49.5 residues 30 to 32 (RXE motif) within the luminal domain and the Ul49.5 CT residues together mediated maximum Ul49.5 TAP inhibition function without affecting the covalent Ul49.5/gM interaction (15). These findings raised the question of whether the C78 residue within the Ul49.5 TM domain, also conserved in the PRV gN, is important for Ul49.5-TAP interaction and thereby MHC-I downregulation.

The goal of this study was to determine whether one or both cysteine residues are required for the formation of covalently linked Ul49.5/gM complex, gM maturation, cell-to-cell spread of the virus, and Ul49.5 or gM virion incorporation. Additionally, we wanted to investigate whether the mutation of one or both cysteine residues affects the Ul49.5-mediated MHC-I downregulation function.

To this end, we have constructed several BHV-1 Ul49.5 mutants with residue C42 or C78 replaced individually or simultaneously with a serine (S) residue using a Ul49.5 CT-null or wild-type (wt) virus as a backbone. Further, we have constructed two BHV-1 VP22 deletion mutants, one with wt Ul49.5 and the other with a Ul49.5 C42S mutation, and analyzed their respective levels of Ul49.5 virion incorporation. Finally, we constructed a virus with a deletion of gM (gM-deleted) and determined its Ul49.5 virion incorporation. The results demonstrated the following: (i) that the Ul49.5 residue C42...
but not C78 is essential for formation of the UL49.5/gM covalently linked complex and gM maturation in the Golgi compartment; (ii) that the UL49.5 C42S and UL49.5 CT-null mutant proteins are incorporated in the respective mutant’s virion envelope but that the UL49.5 C42S lacking UL49.5 CT residues 80 to 96 is not or markedly reduced; (iii) that covalently linked UL49.5 and mature gM are incorporated in the virion of a VP22 deletion (VP22Δ) strain and that, however, unlinked UL49.5 and immature gM require VP22 for their virion incorporation; and (iv) that in the absence of UL49.5/gM complex, a gM-independent UL49.5-VP22 interaction mediated probably by UL49.5 CT residues 80 to 96 is essential for UL49.5 virion incorporation.

RESULTS

BHV-1 UL49.5 forms a disulfide-linked complex with gM, which is required for gM processing in the Golgi compartment (5). BHV-1 UL49.5 also downregulates MHC-I cell surface expression by interacting with TAP in the endoplasmic reticulum (ER) (11). To investigate whether one or both of the cysteine residues in UL49.5 affects UL49.5/gM interactions and gM processing, the C42S and C78S mutant proteins are incorporated in the respective mutant’s virion envelope but that the UL49.5 C42S lacking UL49.5 CT residues 80 to 96 is not or markedly reduced; (iii) that covalently linked UL49.5 and mature gM are incorporated in the virion of a VP22 deletion (VP22Δ) strain and that, however, unlinked UL49.5 and immature gM require VP22 for their virion incorporation; and (iv) that in the absence of UL49.5/gM complex, a gM-independent UL49.5-VP22 interaction mediated probably by UL49.5 CT residues 80 to 96 is essential for UL49.5 virion incorporation.

FIG 1 BHV-1 UL49.5 predicted amino acid sequence and conserved cysteine residues between the gN and gM homologs of alphaherpesviruses. (A) Predicted amino acid sequences of the BHV-1 UL49.5 open reading frame (ORF). Signal sequence and luminal (ecto), transmembrane, and cytoplasmic tail domains are shown; the two cysteine residues C42 and C78 are italicized. (B) Alignment of predicted amino acid sequences of BHV-1, PRV, EHV-1, and HSV-1 UL49.5/gN homologs. Note that C42 (boxed) is conserved in all four gN homologs and that C78 is conserved in BHV-1, PRV, and HSV-1 but not in EHV-1. (C) Alignment of predicted amino acid sequences of BHV-1, PRV, EHV-1, and HSV-1 gM homologs. Cysteines (C) that are not conserved are in bold. Conserved cysteines are boxed. Asterisks (*) indicate positions which have a single, fully conserved residue; colons (:) indicate conservation between groups of strongly similar properties; periods (.) indicate conservation between groups of weakly similar properties.
C42S and/or C78S mutation on the mutant UL49.5-mediated downregulation of MHC-I cell surface expression due to TAP inhibition were analyzed. Mutation of UL49.5 residue C42 but not C78 resulted in a growth defect and small-plaque phenotype. To examine whether the C42S or C78S substitution within UL49.5 affected viral replication kinetics and virus yield in infected MDBK cells, one-step growth curves of C42S, C78S/CT-null, C42S/CT-null, VP22Δ, C42S/VP22Δ, wt, and CT-null viruses were determined. The results showed that viral growth kinetics of CT-null and C78S/CT-null are almost identical to wt kinetics (Fig. 2). However, both the C42S and double C42S/CT-null mutant viruses replicated with a 10-fold-reduced virus yield compared to that of their parental wt and CT-null viruses (Fig. 2). As shown in Fig. 3A and B, average plaque sizes produced in MDBK cells by C42S and double C42S/CT-null mutant viruses were significantly smaller than those of their respective parental wt and CT-null viruses. However, C78S (Fig. 3B) and C78S/CT-null mutant viruses had approximately the same diameters as the parental wt and CT-null viruses (Fig. 3A and B). The double cysteine C42S/C78S mutant virus produced plaques very similar to those produced by C42S and double C42S/CT-null mutant viruses, suggesting that the C42S mutation affected plaque size (Fig. 3A and B). In the wt UL49.5-expressing, stable MDBK cell line (MDBK-UL49.5), the C42S and C42S/CT-null viruses produced plaques of wild-type size (Fig. 3A and B), and they replicated with a 5-fold-higher titer than in noncomplementing MDBK cells (data not shown). However, the virus yield was still 5-fold lower than that of the wild-type virus, which could be due to a low level of UL49.5 expression by the stable cell line (Fig. 3C). Therefore, these results indicated that the growth defects (smaller plaque phenotype and 10-fold lower yield) of the C42S, C42S/CT-null, and C42S/C78S mutant viruses were due to the replacement of the UL49.5 C42 residue with a serine residue and not due to another mutation elsewhere in the genome.

UL49.5 residue C42 but not C78 is required for the formation of covalently linked UL49.5/gM complex and gM maturation in the Golgi compartment. To determine whether UL49.5 residues C42, C78, or both are essential for covalently linked UL49.5-gM interactions and gM processing in the Golgi compartment, 35S-labeled C42S, C78S, C42S/CT-null, C78S/CT-null, and C42S/C78S/CT-null mutant proteins expressed in the respective mutant virus-infected cells were immunoprecipitated with anti-UL49.5 and anti-gM antibodies and analyzed by Western blotting. As controls, wt and CT-null virus-infected cell lysates were similarly analyzed. As shown in Fig. 4A, UL49.5-specific antibody immunoprecipitated 9-kDa UL49.5 wt, C42S, and C78S proteins, but 8-kDa UL49.5 CT-null, C42S/CT-null, C78S/CT-null, and C42S/C78S/CT-null proteins were immunoprecipitated from the corresponding wt and mutant viruses. In addition, the antibody coimmunoprecipitated 43-kDa mature gM-specific proteins from wt, CT-null,
C78S, and C78S/CT-null virus-infected cell lysates. However, the UL49.5-specific antibody coimmunoprecipitated 36-kDa immature gM-specific proteins from the C42S, C42S/CT-null, and C42S/C78S/CT-null mutant virus-infected cell lysates. For comparison, plaque morphologies of C42S, double mutant C42S/CT-null, and double mutant C42S/VP22Δ viruses produced in the wt UL49.5-expressing MDBK cell line (UL49.5-MDBK) are shown. Plaque sizes were measured at 48 hpi. (B) Bar graph showing comparative plaque sizes produced by BHV-1 wt, U49.5 C78S, C42S, gMΔ, CT-null, C78S/CT-null, C42S/CT-null, C42S/C78S/CT-null, VP22Δ, and C42S/VP22Δ viruses. For comparison, plaque sizes produced by U49.5 C42S and double mutant C42S/CT-null viruses in a wt UL49.5-expressing MDBK cell line (UL49.5-MDBK) are shown. Error bars represent standard errors of the means. *** P < 0.001. (C) Analysis of UL49.5 expression in a stable MDBK UL49.5-expressing cell line compared with the level in wt virus-infected MDBK cells, as determined by immunoblotting (IB) or by immunoprecipitation (IP) with anti-U49.5 antibody.

C78S, and C78S/CT-null virus-infected cell lysates. However, the UL49.5-specific antibody coimmunoprecipitated 36-kDa immature gM-specific proteins from the C42S, C42S/CT-null, and C42S/C78S/CT-null mutant virus-infected cell lysates unlike results with the wt and C78S mutant (Fig. 4A). Notably, a vastly reduced level of the 36-kDa immature gM was coimmunoprecipitated by the UL49.5-specific antibody. As expected, gM-specific antibody immunoprecipitated the 43-kDa mature gM from wt, CT-null, C78S, and C78S/CT-null virus-infected cell lysates. Similar to results with immunoprecipitation with the anti-UL49.5 antibody, a 36-kDa gM protein was also immunoprecipitated from the C42S, C42S/CT-null, and C42S/C78S/CT-null virus-infected cell lysates (Fig. 4B). In addition, the anti-gM-specific antibody coimmunoprecipitated the corresponding UL49.5-specific 9-kDa C42S and C78S proteins and the 8-kDa CT-null, C42S/CT-null, C78S/CT-null, and C42S/C78S/CT-null proteins. However, the levels of UL49.5 C42S, C42S/CT-null, and C42S/C78S/CT-null proteins coimmunoprecipitated with the anti-gM antibody were reduced compared with the levels of the wt, CT-null, and C78S/CT-null proteins (Fig. 4B).

We hypothesized that the 43-kDa proteins detected in the wt, C78S, and CT-null virus-infected lysates are the mature Golgi-processed gM proteins and that the 36-kDa band detected in the C42S virus-infected lysate is the immature gM. Therefore, we determined their endoglycosidase H (EndoH) sensitivity. As expected, results showed that the 43-kDa mature gM protein (Golgi apparatus-processed) was resistant to EndoH digestion (Fig. 4C), but the 36-kDa immature gM protein was EndoH sensitive.
To confirm that the UL49.5 C42S mutation disrupts the formation of the UL49.5/gM covalently linked complex, wt, C42S, VP22Δ, and C42S/VP22Δ virus-infected cell lysates were subjected to SDS-PAGE under both reducing and nonreducing conditions and analyzed by immunoblotting with either anti-UL49.5 or anti-gM antibodies. The results shown in Fig. 5 demonstrated that under reducing conditions (with dithiothreitol [DTT]), wt UL49.5 migrated as a 9-kDa band (Fig. 5A), while under nonreducing conditions (without DTT), a large portion of the wt UL49.5 comigrated with gM as a 52-kDa heterodimer (Fig. 5A). However, under both reducing and nonreducing conditions, the mutant UL49.5 C42S protein migrated by itself as a 9-kDa band (Fig. 5A).

Interestingly, under both reducing and nonreducing conditions, anti-UL49.5-specific antibody recognized two higher-molecular-mass proteins of approximately 92 kDa in all virus-infected cell lysates and an approximately 80-kDa protein in wt- and C42S-infected lysates. The 80-kDa band was not detected in VP22Δ and UL49.5 C42S/VP22Δ virus-infected cell lysates. These bands were more prominent under the nonreducing conditions. Both the 92-kDa and 80-kDa proteins were absent in the mock-infected sample (Fig. 5A). When the identical blot was immunoblotted with the gM-specific antibody, both proteins were absent (Fig. 5B). Thus, the 92-kDa band might represent a heterodimeric complex of the approximately 9-kDa UL49.5 protein plus the approximately 82-kDa TAP1 protein (UniProt accession number A6QPZ6). Currently, the identity of the 80-kDa protein is not known.

Further, as shown in Fig. 5B under nonreducing conditions, the anti-gM antibody recognized an additional approximately 72-kDa protein in the C42S and C42S/VP22Δ virus-infected cell lysates but not in the mock-, wt-, and VP22Δ-infected cell lysates. It is highly likely that the 72-kDa protein represents a covalently linked homodimer of the 36-kDa immature gM.
To validate further that the UL49.5 C42S mutation alone can be attributed to the disruption of the UL49.5/gM covalent interaction required for gM maturation, we analyzed gM maturation in the UL49.5 C42S mutant-infected MDBK-UL49.5-expressing cell line. As expected, the effect of the UL49.5 C42S mutation on gM maturation was complemented to its mature 43-kDa molecular mass by the UL49.5-expressing cell line. However, the rescue or complementation of the 43-kDa protein was at a reduced level (data not shown). As noted above with respect to UL49.5 C42S mutant virus yield, the complementation at a reduced level is due to a lower level of UL49.5 expression by the stable cell line, probably due to a lower copy number of the expressed UL49.5 gene than during wt virus infection. Therefore, these results indicated that the C42S mutation alone is responsible for the defective gM maturation and growth defect (small-plaque phenotype and 10-fold-lower virus titer) of the mutant UL49.5 C42S virus.

Taken together, the results indicated (i) that UL49.5 residue C42 but not C78 is required for the formation of a covalent UL49.5-gM complex, (ii) that gM was processed to the mature 43-kDa protein only when it was covalently linked to wt UL49.5, and (iii) that in the absence of covalently linked UL49.5/gM complex, the immature gM can form a covalently linked homodimer.

UL49.5 luminal domain residue C42 and CT residues 80 to 96 are redundantly essential for UL49.5 virion incorporation. To determine the effects of UL49.5 residue C42 and C78 substitutions with or without the additional UL49.5 CT-null mutation on mutant UL49.5 and gM virion incorporation, purified wt and CT-null, C42S, C78S, C42S/CT-null, C78S/CT-null, C42S/C78S/CT-null, and UL49.5Δ mutant virions were ana-
lyzed by immunoblotting with either UL49.5- or gM-specific antibodies (Fig. 6). The results showed that even though similar levels of UL49.5 and mutant UL49.5 proteins were present in each of the mutant virus-infected cell lysates (Fig. 4A), the double C42S/CT-null and triple C42S/C78S/CT-null mutant UL49.5 proteins were not incorporated or were markedly reduced in their respective virion particles. However, the wt UL49.5, CT-null, C78S, and C78S/CT-null mutant proteins were incorporated into their respective virion particles (Fig. 6A). Therefore, only the simultaneous C42S and CT-null mutations but not the individual C42S and CT-null mutations affected UL49.5 virion incorporation (Fig. 6A). These results indicated that both UL49.5 residue C42 and UL49.5 CT residues 80 to 96 are essential for UL49.5 virion incorporation.

Both C42S and UL49.5Δ mutant viruses incorporated immature gM in the virion envelope. The results presented in Fig. 6B showed that in the case of wt, CT-null, C78S, and double C78S/CT-null mutant viruses, both mature and immature gM proteins were incorporated into the virion envelope. The immature gM was incorporated into the envelope of C42S, double C42S/CT-null, triple C42S/C78S/CT-null, and UL49.5Δ viruses (Fig. 6B). Therefore, incorporation of the immature gM in the virion envelope appears to be independent of its covalently linked interaction with UL49.5.

The UL49.5 CT residues 80 to 96 most likely interact with VP22. VP22 in HSV-1 and PRV is well known for its interaction with a number of envelope proteins (gM, gE, and gD) (16) and tegument protein VP16 (17, 18). We hypothesized that UL49.5 CT might be interacting with VP22 and thus play a role in the UL49.5 C42S virion incorporation. Therefore, we determined whether the mutant UL49.5 proteins expressed by C42S, CT-null, and double C42S/CT-null mutant viruses are coimmunoprecipitated with anti-VP22 antibody (Fig. 7). Since VP22 also interacts with gE, which was not manipulated in the UL49.5 mutants, we compared the levels of UL49.5, gE, and VP22 that are coimmunoprecipitated by anti-VP22 antibody from the corresponding virus-infected cell lysates. The results showed that in both wt and UL49.5 mutant virus-infected lysates, VP22-specific antibody immunoprecipitated or coimmunoprecipitated similar levels of VP22 and gE (Fig. 7B and C). However, the anti-VP22 antibody coimmunoprecipitated a reduced level of dual UL49.5 C42S/CT-null mutant protein.
compared with the corresponding level of C42S and CT-null mutant proteins (Fig. 7A). Since VP22 also interacts with gM in alphaherpesviruses (16, 17), we determined additionally the level of VP22 coimmunoprecipitated with anti-gM antibody in the wt, UL49.5 C42S, CT-null, and C42S/CT-null mutant virus-infected cell lysates with the anti-gM antibody. As shown in Fig. 7, regardless of gM maturation status (Fig. 7E), the levels of VP22 coimmunoprecipitated by the anti-gM antibody from the corresponding virus-infected cell lysates were very similar (Fig. 7F). Taken together, these data indicated (i) that UL49.5 CT residues 80 to 96 most likely interact with VP22, which is revealed only in the absence of covalent UL49.5/gM complex, and (ii) that neither C42S nor double C42S/CT-null mutations affected the gM-VP22 interaction.

In the absence of covalent UL49.5/gM complex, incorporation of UL49.5 in the virion envelope is probably mediated by interaction of UL49.5 CT residues 80 to 96 and VP22. To determine whether VP22 interaction with UL49.5 CT residues 80 to 96 plays an essential role in mutant UL49.5 C42S protein virion incorporation, a double C42S/VP22Δ and VP22Δ mutant virus were constructed. We predicted (i) that mutant UL49.5 C42S protein virion incorporation in the presence of UL49.5 CT residues 80 to 96 and VP22 will not be affected, and (ii) that the C42S mutant protein containing UL49.5 CT residues 80 to 96 but expressed by a VP22Δ mutant virus would be defective. As predicted, a significantly reduced level of UL49.5 C42S, expressed in the backbone of a VP22Δ mutant virus, was incorporated into the virion envelope (Fig. 8). However, the
results also showed that the 36-kDa immature gM expressed by the C42S mutant virus was incorporated into the virion, but the immature gM in context of the UL49.5 C42S/VP22Δ virus was not. This raised the alternative possibility that lack of VP22-immature gM interactions led to the defective UL49.5 C42S incorporation into the virion. To exclude this possibility, we constructed a gM-deleted virus and determined whether UL49.5 expressed in the absence of gM is incorporated in the virion envelope. As shown in Fig. 9, UL49.5 expressed in the backbone of a gM-deleted virus was incorporated in the virion though at a slightly reduced level.

Neither the individual UL49.5 cysteine residue mutations nor the combined mutations had an effect on MHC-I cell surface expression in mutant virus-infected cells. To determine the effects of UL49.5 residue C42 and C78 substitutions on UL49.5-mediated TAP inhibition or MHC-I downregulation, we compared MHC-I cell surface expression in the C42S, C78S, and double C42S/C78S mutant virus-infected cells with that of wt virus-infected cells. Fluorescence-activated cell sorting (FACS) analysis results clearly showed that the C42S and C78S mutations, either individually or combined, did not abrogate UL49.5-mediated MHC-I downregulation (Fig. 10).

**DISCUSSION**

We conducted these studies to determine the following: (i) which of the two UL49.5 cysteine residues (C42 and C78) is required for formation of the covalently linked UL49.5/gM complex and gM maturation; (ii) whether one or both of the proteins play a role in each other’s virion incorporation; and (iii) how UL49.5 residue C42S and C78S mutations affect MHC-I downregulation. The results of this study determined (i) that the covalently linked UL49.5/gM complex is necessary for BHV-1 gM processing in the Golgi compartment; (ii) that the UL49.5 residue C42S substitution mutation and not the C78S mutation affected the formation of the covalently linked UL49.5/gM complex; and (iii) that the covalently linked UL49.5/gM complex is also necessary for efficient cell-to-cell spread of the virus and efficient virus replication. Notably, C42S mutant virus produced 30% smaller plaques and replicated with more than 10-fold-reduced virus yield. The results also showed the following: (iv) that in the absence of the covalently linked
UL49.5/gM complex, both immature gM and UL49.5 are incorporated into the virion envelope in a VP22-dependent manner; (v) that while the individual C42S or CT-null mutations had no effect on UL49.5 virion incorporation, dual C42S and CT-null mutations affected the UL49.5 C42S/CT-null virion incorporation; and (vi) that in both UL49.5 C42S and C78S mutant virus-infected cells, MHC-I cell surface expression was not

**FIG 9** Analysis of UL49.5 incorporation in the virion envelope of a gMΔ mutant virus. Partially purified virions and infected cell lysates of BHV-1 wt and gMΔ viruses were separated by SDS-PAGE and immunoblotted with anti-gM- or anti-UL49.5-specific antibodies. Immunoblotting with anti-gC-specific antibody served as loading control.

**FIG 10** FACS analysis of MHC-I cell surface expression in wt and UL49.5 mutant virus-infected MDBK cells. Cells were infected with wt, C42S, and UL49.5 C78S mutant viruses. MHC-I cell surface expression was detected at 18 hpi with a monoclonal anti-MHC-I antibody in combination with an anti-mouse FITC antibody and determined by FACS analysis. (A) Representative graphs of MHC-I cell surface expression of virus-infected cells. (B) Mean of three independent experiments of MHC-I expression in virus-infected cells.
affected. For a better understanding of the results and the discussion below, the interactions of wt and mutant U49.5 with gM and VP22 and their effects on the U49.5/gM complex and/or their virion incorporation are shown in Fig. 11.

Previously, by using stable cell lines expressing gM or both U49.5 and gM, Lipinska et al. (12) reported that the U49.5/gM complex formation was required for gM maturation in the Golgi compartment. They also reported that in cells infected with a virus with a deletion of the U49.5 TM domain (BHV-1 U49.5ΔTM), gM was not processed in the Golgi compartment. Since the U49.5 TM domain contains one of the two cysteine residues of U49.5 (C78) and since U49.5/gM complex formation involves covalently linked disulfide bonds, they suggested that the U49.5 C78 residue is essential for U49.5/gM complex formation and gM processing. However, in that study, the status of the mutant U49.5 protein expressed by U49.5Δ TM virus was not analyzed. Recently, we reported that deletion of the U49.5 TM domain resulted in degradation of the mutant U49.5 protein and that gM expressed by the mutant U49.5Δ TM virus was not processed (15). Here, we have characterized the U49.5 C42S
and C78S mutant viruses for UL49.5/gM complex formation and gM processing. Our results demonstrate that the UL49.5 residue C42 and not C78 was required for the formation of the covalently linked UL49.5/gM complex and that in the absence of the covalently linked UL49.5/gM complex, the C42S mutant virus produced smaller plaques and replicated with reduced virus yield. Nevertheless, in agreement with Lipinska et al. (12), we found that gM processing in the Golgi compartment is dependent on the covalently linked UL49.5/gM complex formation. Therefore, in BHV-1, UL49.5 (gN homolog) is a dominant determinant of gM maturation in the Golgi compartment. However, the opposite is true for HSV and PRV because gM is required for transport and/or processing of gN in the Golgi compartment (10, 19, 20).

Our results also indicate the following in BHV-1: (i) that UL49.5 and gM incorporation into the virion may occur without a covalently linked UL49.5/gM complex and that the uncomplexed UL49.5 and gM virion incorporation require VP22; (ii) that in the absence of covalently linked UL49.5/gM complex, UL49.5 CT residues 80 to 96 are essential.

In alphaherpesviruses, the tegument protein VP22 is known to interact with multiple viral proteins and thereby regulate their cellular translocations (17, 18). In HSV-1 (17) and PRV (16, 18), VP22 binds to both gE and gM and bridges a complex between gE and gM. Hence, we predicted that VP22 also interacts (i) with UL49.5 in a gM-independent manner and (ii) with the immature gM. Therefore, VP22 may play a redundant role in UL49.5 and gM virion incorporation. Additionally, we hypothesized that UL49.5 CT residues most likely interact with VP22, and this interaction may be essential for gM-independent UL49.5 virion incorporation. We proved these possibilities in five different ways: (i) by showing that the levels of UL49.5 C42S/CT-null coimmunoprecipitated by an anti-VP22 antibody from the mutant virus-infected cell lysates is reduced; (ii) by showing that the level of UL49.5 C42S virion incorporation in a double UL49.5 C42S/VP22Δ mutant virus is vastly reduced; (iii) by showing that in the absence of gM (gMΔ virus) UL49.5 is incorporated in the virion; (iv) by showing that the immature gM expressed by the C42S and C42S/CT-null mutant viruses was incorporated into the virion but that the immature gM in the backbone of the C42S/VP22Δ virus was not; and (v) by determining that both the mature and immature gM proteins interacted with VP22 with similar efficiencies.

In summary, these results revealed a redundant role of VP22 in the virion incorporation of wt UL49.5/gM complex and a novel but essential role for both UL49.5 C42S and immature gM virion incorporation when they are not covalently linked.

Even though the covalently linked UL49.5/gM complex was not essential for UL49.5 or gM virion incorporation, the complex was essential for cell-to-cell spread of virus. Recently, El Kasimi and Lippe reported that UL49.5 regulates gM translocation and cell-to-cell fusion at the basolateral cell surface (19). In human herpesvirus 6 (HHV-6), gN was required for gM maturation, and the gN-gM complex interacted with v-SNARE protein vesicle-associated membrane protein 3 (VAMP3) in infected cells (21), which is known to facilitate membrane fusion (22). It is noteworthy that while UL49.5 down-regulates MHC-I cell surface expression during BHV-1 infection to evade cellular immune responses, it may also regulate the post-Golgi transport of gM and/or the UL49.5/gM complex to promote viral cell-to-cell spread and to avoid the circulating neutralizing antibodies. Therefore, in light of the above reports of gN/gM complex in HSV-1 and HHV-6, it could be interesting to determine whether the BHV-1 UL49.5/gM complex also interacts with v-SNARE, with or without VP22. In conclusion, our UL49.5 mutational study determined that the UL49.5/gM functional complex was necessary for efficient cell-to-cell spread of the virus but not for UL49.5 and gM virion incorporation and MHC-I downregulation. Importantly, the UL49.5 mutational study revealed a previously unidentified gM-independent novel, functional interaction of VP22 with UL49.5.

**MATERIALS AND METHODS**

**Cells and wt UL49.5-expressing cell line.** The MDBK cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 to 10% heat-inactivated fetal bovine serum (FBS). The MDBK cell line expressing wt UL49.5 was generated as described previously (15) and maintained in DMEM.
supplemented with FBS as above, but supplemented additionally with blasticidin as described earlier (15).

**Virus and bacterial strains.** The BHV-1 Cooper (Colorado-1) strain was obtained from the American Type Culture Collection (ATCC VR-864) and low-passage-number viral stocks were maintained. Reconstituted BHV-1 Cooper BAC-excised virus and BHV-1 UL 49.5 CT-null BAC-excised virus were generated previously (15, 23). BHV-1 virus with a deletion of UL49.5 was a kind gift from E. J. Wiertz (Leiden University, The Netherlands). Infectious BHV-1 wt and BHV-1 UL49.5 CT-null BAC clones were maintained in *Escherichia coli* strain DH10B. *E. coli* strain SW105 (kindly provided by N. G. Copeland) was used for Red recombination.

**Antibodies.** Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (Thermo), mouse anti-BHV-1 gC monoclonal antibody (MAb) F2 (24), HRP-conjugated goat anti-mouse IgG (Invitrogen), mouse anti-MHC-I Ab (H58A; Veterinary Medical Research and Development [VMRD]), and fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG (ebioscience) were purchased from commercial sources. The anti-BHV-1 gM-specific, anti-BHV-1 UL49.5-specific, and anti-BHV-1 gE ectodomain-specific rabbit polyclonal antibodies were produced previously (15, 25). Rabbit and goat anti-VP22 antibodies were produced commercially (Cocalico Biologicals) by using a cocktail of two peptides corresponding to predicted VP22 aa 22 to 34 ([H]-RENSLYDYESGSD-[OH]) and aa 244 to 258 ([H]-TSGGESRLRGERARP-[OH]) conjugated to polyethylene glycol.

Rabbit and goat anti-BHV-1 gE cytoplasmic tail-specific polyclonal antibody was generated (Cocalico Biologicals) using purified *E. coli*-expressed BHV-1 gE aa 451 to 564 as described earlier (26).

**Construction of mutant viruses.** Table 1 includes a list of viruses used in this study.

### Table 1: Viruses used and/or constructed in this study

| Virus Description | Virus |
|-------------------|-------|
| Wild type BHV-1   |      |
| C42S              | U1, 49.5 residue C42 replaced with a serine |
| C78S              | U1, 49.5 residue C78 replaced with a serine |
| C42S/C78S         | Dual U1, 49.5 C42S and C78S mutations |
| CT-null           | U1, 49.5 cytoplasmic tail residues truncated/deleted |
| C42S/CT-null      | Dual U1, 49.5 C42S and CT-null mutations |
| C78S/CT-null      | Dual U1, 49.5 C78S and CT-null mutations |
| C42S/C78S/CT-null | Triple U1, 49.5 C42S, C78S, and CT-null mutations |
| U1, 49.5 SA       | BHV-1 with U1, 49.5 (gN homolog) deletion |
| VP22Δ             | BHV-1 with tegument protein VP22 (U1, 49.5 gene) deletion |
| U1, 49.5 C42S/VP22Δ| U1, 49.5 C42S mutation in a VP22Δ virus |
| gMΔ               | BHV-1 with glycoprotein M (gM) deletion |

aReference 15.

bKind gift of E. J. Wiertz (Leiden University, The Netherlands).

supplemented with FBS as above, but supplemented additionally with blasticidin as described earlier (15).

**Virus and bacterial strains.** The BHV-1 Cooper (Colorado-1) strain was obtained from the American Type Culture Collection (ATCC VR-864) and low-passage-number viral stocks were maintained. Reconstituted BHV-1 Cooper BAC-excised virus and BHV-1 UL 49.5 CT-null BAC-excised virus were generated previously (15, 23). BHV-1 virus with a deletion of UL49.5 was a kind gift from E. J. Wiertz (Leiden University, The Netherlands). Infectious BHV-1 wt and BHV-1 UL49.5 CT-null BAC clones were maintained in *Escherichia coli* strain DH10B. *E. coli* strain SW105 (kindly provided by N. G. Copeland) was used for Red recombination.

**Antibodies.** Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (Thermo), mouse anti-BHV-1 gC monoclonal antibody (MAb) F2 (24), HRP-conjugated goat anti-mouse IgG (Invitrogen), mouse anti-MHC-I Ab (H58A; Veterinary Medical Research and Development [VMRD]), and fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG (ebioscience) were purchased from commercial sources. The anti-BHV-1 gM-specific, anti-BHV-1 UL49.5-specific, and anti-BHV-1 gE ectodomain-specific rabbit polyclonal antibodies were produced previously (15, 25). Rabbit and goat anti-VP22 antibodies were produced commercially (Cocalico Biologicals) by using a cocktail of two peptides corresponding to predicted VP22 aa 22 to 34 ([H]-RENSLYDYESGSD-[OH]) and aa 244 to 258 ([H]-TSGGESRLRGERARP-[OH]) conjugated to polyethylene glycol.

Rabbit and goat anti-BHV-1 gE cytoplasmic tail-specific polyclonal antibody was generated (Cocalico Biologicals) using purified *E. coli*-expressed BHV-1 gE aa 451 to 564 as described earlier (26).

**Construction of mutant viruses.** Table 1 includes a list of viruses used in this study.

### Table 2: PCR primers used for generation of BHV-1 U1, 49.5 cysteine residue mutants and for colony identification

| Primer function and name | Sequence (5’ to 3’)a |
|--------------------------|----------------------|
| Mutagenesis              |                      |
| C42S F                   | 5’-GATGCGGCAGAGGGGCAATGGACTTTTGAGCAGGTCgtTACGCGCGGGGTGCCGCTAGGGATGACGACGATAAGTAGGG-3’ |
| C42S R                   | 5’-GGGCTGCGGTCCTCGAGAGCGCACCAGCCGCGCTAGCGAGCGCGCTCAAAAGTCCAACCAATAAACCAATCTCAGTTAG-3’ |
| C78S F                   | 5’-CCCGGTAATGTTGCGCGGTCGCCCTGACTCGGTCAGGGGCTTCgtTATTGCTCATGGCCGTCAGGGAGATGACGACGATAAGTAGGG-3’ |
| C78S R                   | 5’-ACTCTTTTTTTATAGGGCCGCTGGCCGCACTGAGCGCTAAACgaAAGCCCGTACGCGTACAGGGCAACAAATATAACCAATTCTGATTAG-3’ |
| C78S CT-null F           | 5’-CCCGGTAATGTTGCGCGGTCGCCCTGACTCGGTCAGGGGCTTCgtTATTGCTCATGGCCGTCAGGGAGATGACGACGATAAGTAGGG-3’ |
| C78S CT-null R           | 5’-CCCCGCCGACCTTTTTTAGGGCCGCGTCTGGCCGCTTAAAACgaAAGCCCGTACGCGTACAGGGCAACAAATATAACCAATTCTGATTAG-3’ |

aBHV-1 U1, 49.5-specific sequences are shown in bold uppercase letters; the italicized sequences of forward (F) and reverse (R) primers, respectively, are complementary to each other in inverse orientation. Lowercase nucleotides code for serine instead of cysteine. Underlining indicates the pEPkan-S-specific sequences.

bPrimers used to PCR amplify the U1, 49.5 ORF and its downstream sequences for identification of BHV-1 U1, 49.5 BAC mutants from the selected kanamycin-sensitive colonies for sequencing.
(C42S/C78S) were synthesized (IDT). The first and second steps of Red-mediated mutagenesis were performed using SW105 competent cells harboring pBHV-1 BAC (wt) or pBHV-1 BAC UL49.5 CT-null infectious clones as described earlier (23). Reconstituted BAC-containing or BAC-excised mutant viruses were then generated as previously described (23). BAC-excised UL49.5 mutant viruses designated C42S, C78S, double C42S/CT-null, double C78S/CT-null, and triple C42S/C78S/CT-null mutant viruses (Table 1) were plaque purified and verified further by sequencing (Fig. 12) and immunoblotting.

Construction of VP22-deleted BHV-1 mutants. BHV-1 VP22 is encoded by the UL49 gene (GenBank accession number JX898220). To generate a VP22-deleted BHV-1 mutant (BHV-1 VP22Δ), a chimeric 2,666-bp-long DNA fragment was first synthesized and cloned into the EcoRI (5')/HindIII (3') sites of pUC57, resulting in a VP22 deletion plasmid (pBHV-1 VP22Δ) (Genscript USA, Inc., NJ, USA). The 2,666-bp EcoRI/HindIII fragment consists of the following (5' to 3'): an EcoRI site at the 5' end, followed by 1,189-bp nucleotide sequence comprising a partial UL50 ORF, the full UL49.5 ORF, and partial UL49 ORF sequence (nucleotides [nt] 8310 to 9498; GenBank accession JX898220). This sequence was followed by a 25-bp non-BHV-1 sequence consisting of two stop codons (in two different reading frames; in boldface in TAA TC) and the KpnI/BamHI restriction sites, fused to a 1,440-bp nucleotide sequence comprising the partial carboxy-terminal UL49 ORF and partial UL48 gene sequences (nt 9846 to 11285), followed by the restriction site HindIII at the 3' end (Fig. 13A and Table 3). In the resulting plasmid, pBHV-1 VP22Δ, nt 9499 to 9845 of the UL49 gene, which code for aa 34 to 149 of the VP22 protein, were deleted, and two stop codons were inserted immediately after the VP22 residue 33 (Fig. 13A and Table 3). The deletion of VP22 residues 34 to 149 and the insertion of the stop codons were verified by sequencing. Finally, an approximately 2-kb KpnI/BamHI fragment containing an enhanced green fluorescent protein (eGFP) expression cassette (26) was inserted in the corresponding KpnI/BamHI sites of pBHV-1 VP22Δ, resulting in pBHV-1 VP22Δ/eGFP. BHV-1 VP22Δ virus was generated by cotransfection/homologous recombination of pBHV-1 VP22Δ/eGFP with full-length BHV-1 wt virus DNA. A recombinant, plaque-purified BHV-1 VP22Δ virus, verified by sequencing and immunoblotting analyses, was selected for further study.
virus DNA constructed above (template) were used to amplify the 1,191-bp fragment with EcoRI and KpnI sites at the 5′ and 3′ ends, respectively (Table 4). The 1,191-bp EcoRI/KpnI fragment was then cloned into the corresponding EcoRI/KpnI sites of the pBHv-1 VP22Δ GFP plasmid construct (Fig. 13A) described above. In the resulting plasmid clone, pUL49.5 C42S/VP22Δ GFP, the UL49.5 C42S mutation (TGC to TCG) was incorporated, and the nucleotide sequences coding for VP22 aa 34 to 149 were deleted. The C42S mutation in the plasmid pUL49.5 C42S VP22Δ GFP was verified by amplifying the entire UL49.5 ORF by PCR using UL49.5 upstream and downstream sequence-specific forward and reverse primers (Table 4) and sequencing. Subsequently, a UL49.5 C42S/VP22Δ GFP virus was generated by homologous recombination of pUL49.5 C42S/VP22Δ GFP DNA with full-length BHV-1 wt virus DNA. A recombinant double UL49.5 C42S/VP22Δ GFP mutant virus was plaque purified two times and verified further by sequencing and immunoblotting.

**Construction of a gM-deleted BHV-1 mutant.** The U10 gene encoding the envelope glycoprotein gM is transcribed from the complementary strand of the BHV-1 genome and is flanked on the left by UL11 and UL12 (3′ end) and on the right (5′ end) by UL9 (Fig. 13B). To generate a gM-deleted BHV-1 mutant (BHV-1 gMΔ), a gM deletion vector (pBHv-1 gMΔ) was generated. Briefly, a chimeric 2,100-bp-long DNA fragment was synthesized (Genscript) to include the following (5′ to 3′): an EcoRI site followed by a 1,000-bp sequence comprising a partial UL12 ORF, the full UL11 ORF, the authentic stop codon of the gM ORF (nt 83819 to 84818; GenBank accession number JX898220), a HindIII site, six additional nucleotides (CCGCGC), and a KpnI site followed by a 1,099-bp sequence comprising partial UL10 and partial UL9 ORFs (nt 85188 to 86287) and a BamHI restriction site. This 2,100-bp EcoRI-BamHI fragment was cloned into the corresponding EcoRI/BamHI sites of pUC57 vector (GenScript). In the resulting plasmid, nt 84819 to 85187 coding for gM residues 289 to 411 were deleted and replaced with HindIII/KpnI sites, which allowed insertion of an approximately 2-kb HindIII/KpnI fragment containing an eGFP expression cassette (27), resulting in plasmid pBHv-1 gMΔ GFP (Fig. 13B). BHV-1 gMΔ virus was generated by homologous recombination of pBHv-1 gMΔ eGFP with full-length BHV-1 wt virus DNA. A recombinant BHV-1 gMΔ virus, verified by sequencing and immunoblotting analyses, was selected for further study.

**Viral growth kinetics and plaque size determination.** One-step growth curve assays were performed twice as described earlier (27). Briefly, for each virus and time point (see below), 20 T25 flasks containing 4 × 10⁶ MDBK cells/flask were seeded. The prechilled (4°C) cells were infected with various viruses at a multiplicity of infection (MOI) of 5 and adsorbed for 1 h at 4°C. Following adsorption and washing, 4 ml of medium was added to each flask, and one flask was frozen immediately for each virus sample (0 h) at −80°C. The remaining flasks were incubated further at 37°C in a CO₂ incubator, and samples were frozen as described above at 3, 6, 12, 18, 24, 30, 36, and 42 h postinfection (hpi). Virus titers at these time points were determined by standard plaque assay as described earlier (28).
| Plasmid | Upstream flanking sequence | Downstream flanking sequence |
|---------|---------------------------|-------------------------------|
| pBHV-1 VP22Δ | GAATTC (EcoRI) CAGACAAA...TCCGGCTCG (8310–9498) | TAACTGAGGTACC (Stop-Stop-KpnI) CCGC (BamHI) GGATCC...GTTTTCGGG (9846–11285) |
| pBHV-1 gMΔ | GAATTC (EcoRI) CGGTCCGTG...GTCTCCTTA (83819–84818) | AAGCTT (HindIII) CCGCGC GGTACC (KpnI) TGCCACCAG...CGCGTGACC (85188–86287) |

aNucleotide (nt) positions refer to GenBank accession number JX898220; VP22 open reading frame, nt 9400 to 10176; gM open reading frame, nt 84816 to 86051.

bStop codons are in boldface.
To determine the average plaque size of each mutant, two wells of a six-well plate containing confluent monolayers of MDBK cells or MDBK cells expressing wt U49.5 were infected with 80 to 100 PFU of mutant viruses and overlaid with 1.6% carboxymethyl cellulose (CMC) at 2 hpi. At 48 hpi, the cells were fixed (10% formaldehyde) and stained with crystal violet. Average plaque size of wt and mutant viruses was calculated by measuring approximately 50 randomly selected plaques of each virus under a microscope with a graduated ocular objective, as described previously (15).

Radiolabeling of mock- or virus-infected MDBK cell proteins, SDS-PAGE, and immunoprecipitation/immunoblotting analysis. The method for [35S]methionine-cysteine labeling of mock- or virus-infected MDBK cells and immunoprecipitation of virus-specific proteins using protein A-Sepharose/virus protein-specific antibody was described previously (29). For the analysis of gM, virus-infected cell lysates and immunoprecipitates were incubated at 60°C in reducing sample buffer containing 100 mM dithiothreitol (DTT) as described previously (15) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Unless otherwise mentioned in the figure legend, the SDS-PAGE was performed under reducing conditions. For SDS-PAGE under nonreducing conditions, sample buffer without DTT was used. For all other samples, cell lysates were prepared as described previously (29), and immunoprecipitates were boiled for 5 min in reducing sample buffer containing β-mercaptoethanol and separated by SDS-PAGE. Immunoprecipitated/SDS-PAGE-separated proteins were visualized by autoradiography or by immunoblotting as described earlier (15).

EndoH digestion. Endoglycosidase H (EndoH) digestion was performed as described previously (30). The digested samples were subjected to SDS-PAGE, and labeled proteins were visualized by autoradiography.

FACS analysis of MHC-I cell surface expression. MDBK cells either mock infected or infected with BHV-1 wt, U49.5 C42S, or C78S mutant virus were collected at 18 hpi, blocked with IgG-free bovine serum albumin (BSA), incubated with mouse anti-bovine MHC-I antibody (Ab), and subsequently stained with FITC-conjugated rat anti-mouse Ab and analyzed by flow cytometry as described previously (15). MDBK cells infected with the respective viruses were stained by FITC-conjugated mouse IgG2a and used as isotype controls.

Statistical analysis. Normality of distribution of the examined variables was evaluated by a D’Agostino-Pearson omnibus normality test. Statistical significance of plaque size variations between the mutant and wt viruses was determined using a one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test using Graph Pad Prism (GraphPad Software, La Jolla, CA, USA). A P value of ≤0.05 was considered statistically significant.

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TABLE 4 PCR primers for generation of BHV-1 U49.5 C42S/VP22Δ mutant

| Primer function and name (restriction site) | Sequence<sup>a</sup> | Position (nt)<sup>b</sup> |
|---------------------------------------------|----------------------|-------------------------|
| Cloning                                    |                      |                         |
| VP22/34-149 del F (EcoRI)                   | 5'-GGGAAATTCGAACAGCAATTGGCGAGGGC-3' | 8308–8331              |
| VP22/34-149 del R (KpnI)                    | 5'-GGGCTGGTACCTACGAGGACCGCATGTAATGACATTGAACGGCTG-3' | 9471–9498              |
| Verification<sup>c</sup>                    |                      |                         |
| U49.5 F                                    | 5'-AGGGTTCGGGCTCACAGACG-3' | 8917–8938              |
| U49.5 R                                    | 5'-AACCAGCCATGGCAAGGAGTC-3' | 9388–9410              |

<sup>a</sup>BHV-1-specific sequences are shown in boldface letters, italicized sequences are integrated restriction sites, and underlined sequences are integrated stop codons.

<sup>b</sup>Positions are based on GenBank accession number JX899822.

<sup>c</sup>Primers used for verification of the recombinant BHV-1 by sequencing.
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