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Expression of Glycoprotein gIII-Human Decay-Accelerating Factor Chimera on the Bovine Herpesvirus 1 Virion via a Glycosyl Phosphatidylinositol-Based Membrane Anchor†

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Mutants of bovine herpesvirus 1 that express a truncated envelope glycoprotein gIII or a gIII-human decay-accelerating factor (hDAF) chimeric protein (gIII.hDAF) were employed to evaluate the function of the transmembrane and cytoplasmic domains of the gIII molecule. Truncated gIII (i.e., lacking the transmembrane and cytoplasmic regions) was readily released from infected cells and was not detected on mature virus particles. In contrast, replacement of the transmembrane and cytoplasmic domains with the carboxyl-terminal portion of hDAF restored the expression of gIII on the membranes of infected cells as well as on virion surfaces. The presence of the gIII.hDAF chimera on virus particles was also associated with normal gIII function, i.e., the mediation of virus attachment and penetration. The gIII-hDAF chimera, which is present on both infected cell surfaces and virions, could be cleaved by a phosphatidylinositol-specific phospholipase C, indicating that it was anchored in the membrane via glycosyl phosphatidylinositol. Our results from this study suggest that the transmembrane and cytoplasmic regions of the gIII molecule serve as a general membrane anchor, but they do not contain structural signals required for the specific assembly of envelope proteins into mature virions.

Bovine herpesvirus 1 (BHV-1) is a member of the *Alphaherpesvirinae* subfamily and is an economically important pathogen of cattle. The genome of BHV-1 encodes a set of at least seven viral envelope glycoproteins (20, 24, 31, 32). At present, four major glycoproteins have been characterized. They have been named gI, gII, gIII, and gIV on the basis of decreasing molecular weights, and gI, gIII, and gIV are homologs of herpes simplex virus (HSV) glycoproteins gB, gC, and gD, respectively (27, 35). Among the four major glycoproteins, gIII has been shown to be nonessential for virus growth in cultured cells (14).

gIII is a 95-kDa protein containing both N-linked and O-linked oligosaccharides (32), and it forms the most pronounced projections on the surface of the virion envelope (26a). One of the major functions of BHV-1 gIII is to facilitate virus entry into permissive cells, and in this respect it represents the dominant virus attachment protein (14, 25). It is now clear that gIII contains multiple heparin-binding sites, which interact with cellular heparin-like components to constitute the basis of gIII-mediated virus attachment (17, 26). As with other alphaherpesviruses, the initial BHV-1 attachment events are followed by penetration involving membrane fusion (9, 12, 30). An additional requirement for gIII in the virus penetration process was suggested by the observation that a gIII-negative BHV-1 mutant exhibited a slower penetration rate than its wild-type (wt) counterpart (15). However, the actual role played by gIII in virus penetration is not known at present. gIII also mediates functions other than those required for virus entry. For example, gIII-negative viruses always yield lower virus titers than wt virus in single-step growth experiments (14). For pseudorabies virus, another alphaherpesvirus, gIII has been shown to facilitate virus egress from infected cells and to maintain the thermostability of extracellular virus (28). The binding of complement C3 represents an additional gIII function (11). Finally, a BHV-1 gIII gene deletion mutant was found to have reduced replication efficiency in cattle, its natural host (16). Therefore, although gIII is not essential for virus replication in vitro, it does represent an important, multifunctional virus structural component and virulence factor.

The present study was carried out to further define the structure-function relationship of the BHV-1 gIII molecule, focusing on the membrane anchor and cytoplasmic domains. BHV-1 gIII, which is 521 amino acids long, contains two hydrophobic regions located between amino acids 1 and 21 and 467 and 500. The 34-amino-acid carboxyl-terminal hydrophobic segment, which has a predicted alpha-helical structure and is immediately followed by two positively charged arginine residues, has previously been postulated to be the transmembrane domain (7). Accordingly, the 21-amino-acid segment following the transmembrane sequence constitutes the cytoplasmic domain. We wished to examine (i) whether the putative transmembrane domain could indeed function as a membrane anchor for the presentation of gIII on the surfaces of infected cells and on the virion envelope and (ii) whether the transmembrane and cytoplasmic domains bear any specific signals for proper assembly of the viral envelope proteins into virus particles. To address these questions, we constructed a BHV-1 recombinant that expresses truncated gIII containing deleted transmembrane and cytoplasmic domains and a second recombinant that expresses mutated gIII in which the transmembrane and cytoplasmic domains were replaced with the carboxyl terminus of human decay-accelerating factor (hDAF). The hDAF segment contains a signal for the addition of a glycosyl phosphatidylinositol (GPI)-based membrane anchor

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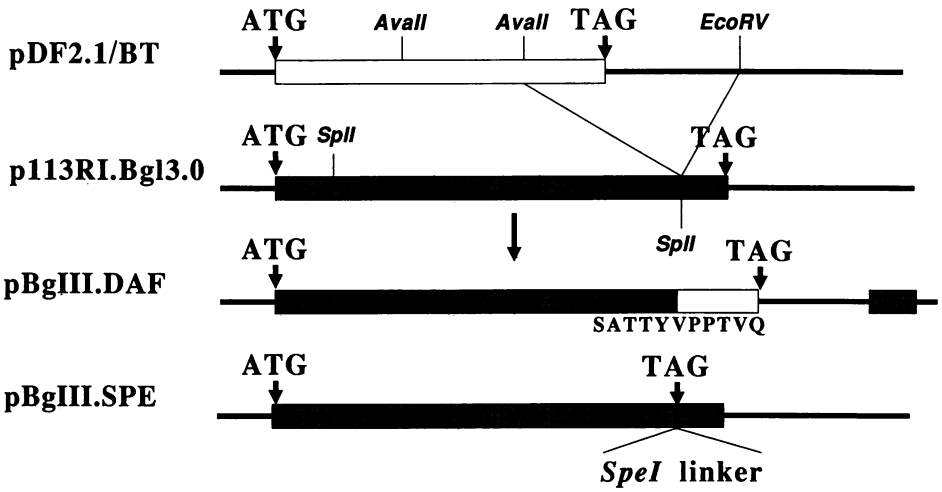

FIG. 1. Schematic representation of the transfer vectors used for generating the recombinant viruses. pDF2.1/BT is the plasmid which contains the hDAF gene; p113RI.Bgl3.0 is the plasmid which contains the gIII gene; pBgIII.DAF is the transfer vector used to make the gIII.hDAF chimera; and pBgIII.SPE is the transfer vector for truncated gIII. Coding sequences of hDAF and gIII are represented by open and shaded bars, respectively. The noncoding sequences of both genes are represented by solid lines. Presented under pBgIII.DAF is the deduced amino acid sequence at the junction between gIII and hDAF.

(23). We found that removal of the putative transmembrane and cytoplasmic regions of gIII resulted in efficient secretion of truncated gIII from infected cells, indicating that the deleted regions indeed contain a membrane anchor. The authentic gIII membrane anchor, however, could be effectively replaced with the GPI-based membrane anchor to facilitate expression of the viral protein on cell surfaces and virions. Therefore, the transmembrane and cytoplasmic domains of gIII do not appear to contain specific structures for the integration of this envelope protein into the maturing virion.

MATERIALS AND METHODS

Virus, cells, and reagents. The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratories, Ames, Iowa, and was propagated in Madin Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO). Plaque-purified virus stocks were used for all experiments. *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from the culture medium of a *Bacillus subtilis* strain that had been transformed with the gene for *B. thuringiensis* PI-PLC (6; provided by T. L. Rosenberry, Case Western Reserve University, Cleveland, Ohio).

Construction of transfer vectors and recombinant viruses. Figure 1 depicts a schematic representation of the transfer vectors used for producing the recombinant viruses. p113RI.Bgl3.0 was produced by subcloning the 3.0-kb *Eco*RI-*Bgl*II subfragment from the BHV-1 *Hin*dIII genomic clone pSD113 (21) into the *Eco*RI and *Bam*HI sites of pBR322 (7). This plasmid contains the 1,563-bp gIII coding sequence, approximately 640 bp of 5′ flanking sequence, and 800 bp of 3′ flanking sequence. p113RI.Bgl3.0 served as a parent for transfer vectors. In order to generate the truncated gIII transfer vector, p113RI.Bgl3.0 was partially digested with *Spl*I and treated with DNA polymerase I Klenow fragment to blunt the asymmetric ends. A *Spe*I linker, which contains translation termination codons in all possible reading frames (CTAGACTAGTCTAG; New England BioLabs, Mississauga, Ontario, Canada), was inserted within the gIII coding sequence at the second *Spl*I site. The resultant plasmid, pBgIII.SPE, contains the gIII gene coding sequence with a translation stop codon inserted 1,400 bp downstream from the gIII translation initiation codon. To generate a transfer vector required for the expression of the gIII.hDAF chimeric protein, plasmid pDF2.1/BT, which contains the hDAF gene (22), was first digested with *Ava*II and *Eco*RV and then treated with Klenow fragment. An approximately 0.75-kbp blunt-ended *Ava*II-*Eco*RV hDAF gene fragment was inserted into the second, Klenow fragment-treated *Spl*I site of the gIII gene coding sequence. A plasmid which contains the hDAF fragment with the appropriate orientation was isolated, and the junction between gIII and hDAF was confirmed by sequencing. This plasmid was named pBgIII.DAF.

Recombinant viruses were generated by cotransfecting MDBK cells with the corresponding transfer vector and naked BHV-1 genomic DNA by electroporation (4). In order to obtain recombinant virus expressing truncated gIII, cells were cotransfected with pBgIII.SPE and wt BHV-1 DNA. Recombinant virus was identified from the progeny of this transfection by its lack of gIII expression by using gIII-specific monoclonal antibodies (MAbs) in a black plaque assay (see below). To produce recombinant virus expressing the gIII.hDAF chimera, MDBK cells were cotransfected with pBglII.DAF and genomic DNA from a BHV-1 gIII gene deletion mutant (14). Recombinant virus was identified by expression of gIII on the surfaces of infected cells by the black plaque assay.

Black plaque assay. The black plaque assay was carried out by the method of Holland et al. (10). An agarose overlay of an MDBK cell monolayer with an appropriate number of viral plaques was marked for its position and transferred to an additional dish, and the cells were fixed with 0.25% glutaraldehyde (Sigma, St. Louis, Mo.) in phosphate-buffered saline (PBS), pH 7.2, for 3 min, washed three times with PBS, and blocked for 1 h with 1% bovine serum albumin

(BSA; Sigma) in PBS (BSA-PBS). An anti-gIII MAb mixture (33), diluted to 1:1,000 in BSA-PBS, was added to the plates. The infected monolayers were then incubated for 1 h at 24°C; incubation was followed by three washes of PBS. Affinity-purified, peroxidase-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim, Dorval, Quebec, Canada) at a dilution of 1:2,000 in BSA-PBS was then added. After an additional 1 h of incubation, the plates were again washed with PBS, and then 0.01% 4-chloro-1-naphthol (Sigma) and 0.0003% H_2O_2 (Sigma) in water were added. The plates were then incubated at 24°C until black plaques developed. Viral plaques with the desired phenotypes were isolated from the agarose overlay for further plaque purification.

Immunoprecipitation assays. Subconfluent monolayers of MDBK cells were infected with virus at a multiplicity of infection (MOI) of 5. After 90 min at 37°C, the virus inoculum was removed and cells were cultured in glucose-free MEM (GIBCO) supplemented with 10% dialyzed FBS for 5 h. This was followed by the addition of 100 μCi of [^{3}H]glucosamine (Amersham, Oakville, Ontario, Canada) per ml of medium. Eighteen hours postinfection, the cells and culture media were harvested separately. Immunoprecipitation assays were carried out as previously described with a mixture of gIII-specific MAbs (14). Antibody-precipitated samples were separated on sodium dodecyl sulfate–7.5% polyacrylamide gels under reducing conditions.

Flow cytometric analysis of surface expression of gIII. Confluent MDBK cells in 100-mm dishes were cooled at 4°C and then incubated with the designated viruses at an MOI of 5 at 4°C for 1 h and then at 37°C in MEM with 10% FBS. Nine hours postinfection, cells were harvested by trypsinization, washed three times with MEM containing 0.05% sodium azide, and suspended at 2×10^7 cells per ml of MEM with sodium azide. Approximately 10^7 cells were then incubated with 5 μg of PI-PLC per ml at 37°C for 1 h and washed twice with MEM with sodium azide. For surface staining, cells were first incubated with a mixture of gIII-specific MAbs at a dilution of 1:1,000 at 4°C for 30 min. After three washes, the cells were incubated with a fluorescein-labeled goat anti-mouse antibody (Beckton-Dickinson, Mountain View, Calif.) at 4°C for 1 h, washed and fixed in 2% formaldehyde, and stored at 4°C until analysis. Cells were analyzed with a Coulter Electronics Ltd. EPICS CS System flow cytometer.

Immunocytochemistry assay. Confluent MDBK cells grown in glass chamber slides (Miles Laboratory, Rexdale, Ontario, Canada) were infected with the designated virus at an MOI of 0.5. Twelve hours postinfection, the cells were fixed with 2% paraformaldehyde for 5 min at 4°C. For intracellular staining, the paraformaldehyde-fixed cells were further permeabilized with absolute methanol at −20°C for 15 min. Cells were then washed three times with PBS. After blocking with 3% normal horse serum for 30 min at room temperature, cells were incubated with a mixture of gIII MAbs at a dilution of 1:1,000 for 1 h; incubation was followed by three additional PBS washes. Subsequent steps were carried out with an avidin-biotin-based immunoperoxidase assay kit (Vector Laboratories, Burlingame, Calif.) according to the manufacturer's instructions. After substrate addition, the reaction was stopped after 5 min of incubation at room temperature by rinsing the slides with tap water.

Adsorption and penetration assays. For the adsorption assay, confluent MDBK cells grown in 6-well culture plates were first cooled at 4°C for 1 h. A total of 0.4 ml of virus inoculum, containing about 500 PFU/ml in MEM, was added to each well. After 60 min of incubation at 4°C, virus inocula were removed and the plates were washed once with MEM and overlaid with 0.8% agarose in MEM supplemented with 2% FBS. Plaques were counted 4 days later.

For the penetration assay, MDBK cells in 6-well culture plates at 4°C were infected as described above. After virus adsorption, the inocula were removed, the cells were washed once with MEM, 2 ml of precooled (4°C) MEM was added per well, and the cells were incubated at 37°C. At each specified time point, three wells of cells per plate were treated with 0.1 M glycine-HCl, pH 3.0, for 2 min; the duplicate wells were treated with MEM. After three washes with MEM, the cells were overlaid with agarose and incubated at 37°C. Viral plaques were counted 4 days later. The percentage of virus that penetrated into cells at each time point was calculated by the following equation: (the number of plaques formed in acid-treated wells/the number of plaques formed in MEM-treated wells) × 100.

Plaque reduction assay. Virus at approximately 500 PFU/ml in MEM was incubated at 37°C for 1 h with the specified antibodies at an appropriate dilution in the presence of complement. Reconstituted guinea pig serum (GIBCO) was used as the complement source at a final dilution of 1:20. Antibody-treated virus was then added to confluent MDBK cells grown in 6-well tissue culture plates and was incubated at 37°C for 90 min. Inocula were then removed, and the cells were washed once with MEM and overlaid with 0.8% agarose in MEM with 2% FBS. Plaques were counted 4 days later. Virus that was treated with complement alone was included as a control for normal plaque formation.

RESULTS

Construction of gIII mutants. According to protein sequence analysis, the predicted 34-amino-acid transmembrane domain of gIII is situated between residues 467 and 500 and which is followed by a 21-amino-acid cytoplasmic tail. In order to delete and replace the transmembrane and cytoplasmic domains of gIII, we took advantage of an *SplI* restriction endonuclease site which naturally occurs at the triplet encoding amino acid residue 465 within the gIII coding sequence. In order to obtain the simple truncated gIII, a *Spe*I linker containing a three-frame translation termination codon was directly inserted into this *SplI* site. The resultant truncated form of gIII consists of the first 465 amino acids of the gIII ectodomain (including the signal sequence), followed by Leu-Asp-*amber* introduced by the linker. In order to construct the gIII.hDAF chimeric gene, a 764-bp *Ava*II-*Eco*RV fragment that contains the 447-bp carboxyl-terminal coding sequence and 317 bp of 3′ untranslated sequence from the hDAF gene was inserted in the same *SplI* site. The chimeric protein produced by this construct consists of the 465-amino-acid ectodomain of gIII fused to the 149-amino-acid carboxyl terminus of hDAF (Fig. 1).

Mutant virus that expresses truncated gIII was produced by cotransfecting MDBK cells with wt BHV-1 genomic DNA and the transfer vector pBgIII.SPE and identified by a gIII MAb-based black plaque assay to detect the absence of gIII on the surface of infected cells. In order to establish that the selection was not biased toward only those recombinant viruses that efficiently secrete gIII, transfections with DNA from a gIII gene deletion mutant were also done. In this case, no gIII-positive plaques were detected on the basis of surface expression measured by the black plaque assay. Therefore, the lack of surface expression of truncated gIII as

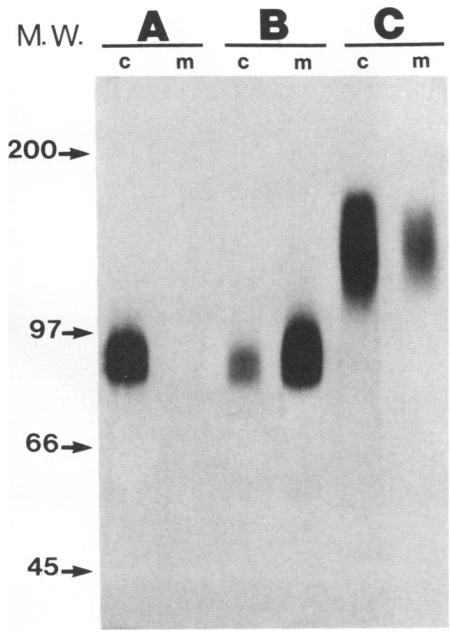

FIG. 2. Immunoprecipitation assay of mutated gIII produced in virus-infected cultures. MDBK cells were infected with wt BHV-1 or recombinant virus at an MOI of 5 and labeled with [^{3}H]glucosamine. After labeling, medium and cells were collected and precipitated with a mixture of anti-gIII MAbs. Samples were separated on sodium dodecyl sulfate–7.5% polyacrylamide gels under reducing conditions. (A) wt BHV-1; (B) mutant expressing truncated gIII; (C) mutant expressing the gIII.hDAF chimera. "m" and "c" indicate culture medium and cellular fraction, respectively. M.W., molecular weight markers.

determined by this screening method reflects the general property of efficient secretion caused by the deletion of the gIII transmembrane domain rather than a property peculiar to the mutant virus that we isolated and used in our studies.

A recombinant virus that expresses the gIII.hDAF chimera was produced by cotransfecting MDBK cells with genomic DNA from a virus in which the gIII gene had been deleted (14) and pBgIII.DAF. The recombinant virus was successfully identified by its ability to express gIII on the surfaces of infected cells as measured by the black plaque assay.

Characterization of the gIII molecules produced by BHV-1 recombinants. In order to evaluate the expression of truncated gIII and the gIII.hDAF chimera, both immunoprecipitation and immunocytochemical assays were carried out. Figure 2 shows the results from immunoprecipitations of gIII from virus-infected cell cultures. Under the conditions tested, gIII expressed by wt virus could be precipitated directly only from infected cells, while truncated gIII was predominantly found in the culture medium. Therefore, removal of the transmembrane and cytoplasmic domains caused efficient secretion of truncated gIII from the infected cells. In contrast, gIII.hDAF was detected in both the cellular and medium fractions. This indicates that gIII.hDAF but not truncated gIII can be associated with cells.

The fact that truncated gIII has an electrophoretic mobility comparable to that of wt gIII suggests that removal of the transmembrane and cytoplasmic domains did not alter post-translational modifications and processing of the secreted molecule. However, gIII.hDAF showed a significantly retarded mobility compared with that of wt gIII. The difference in apparent molecular weights between gIII.hDAF and wt gIII could not be accounted for by the difference in the number of amino acid residues making up the two proteins (521 amino acids for gIII versus 614 amino acids for gIII.hDAF). hDAF has previously been shown to be a highly glycosylated protein, decorated with large quantities of O-linked oligosaccharides (18); it is also well documented that the attachment sites for O-linked carbohydrate are concentrated at the carboxyl terminus of hDAF's ectodomain (22), which is present in the gIII.hDAF chimera. Indeed, when gIII.hDAF was treated with *O*-glycosidase, the molecular weight of gIII.hDAF was reduced to a predicted range (data not shown). Therefore, the higher-than-calculated molecular weight of gIII.hDAF most likely resulted from the addition of O-linked oligosaccharides to the hDAF subfragment.

Our immunocytochemistry assays (Fig. 3) demonstrated that wt gIII and gIII.hDAF are essentially indistinguishable with respect to their cellular distribution. Most significantly, both gIII.hDAF and wt gIII were present in large amounts on the surfaces of infected cells. This finding, in conjunction with the result from the immunoprecipitation assay, suggests that gIII.hDAF could be associated with the cell membrane. Truncated gIII was not detected on the surfaces of infected cells. It was found only in permeabilized cells localized at the perinuclear membrane region. The amounts of intracellular truncated gIII are much smaller than those of either cell-associated wt gIII or gIII.hDAF.

Susceptibility of gIII.hDAF to cleavage by PI-PLC. Having demonstrated that gIII.hDAF could be associated with the cell membrane, we attempted to ascertain whether the membrane association is mediated by GPI as it is for authentic hDAF. We anticipated that if gIII.hDAF is anchored by GPI, it should be susceptible to the cleavage by PI-PLC. The presence of gIII on infected cell surfaces was measured by flow cytometric analysis (Fig. 4). We found that treatment of wt BHV-1-infected cells with PI-PLC had no effect on the profiles of gIII surface expression, as predicted. In contrast, treatment of gIII.hDAF virus-infected cells with PI-PLC resulted in a significant reduction in the amount of gIII expressed on cell surfaces. Therefore, gIII.hDAF must be anchored on the cell membrane by GPI as well. It should be noted that the PI-PLC treatment did not reduce gIII.hDAF to background levels. The lack of complete removal of GPI-anchored protein by PI-PLC was also evident in a previous study involving the expression of HSV gD.DAF chimeric protein (1). It could be due to the variable action of PI-PLC. Alternatively, it could have resulted from the presence of intracellular gIII.hDAF, which we also observed in our immunocytochemical assays described above. Intracellular staining could have been brought about by virus infection causing cell membrane permeabilization.

Expression of gIII.hDAF but not truncated gIII on virus particles. The presence of the mutant forms of gIII on the surfaces of virions was determined by a gIII-monospecific-antibody-mediated virus neutralization assay. Table 1 shows the results from one of two identical experiments. We found that virus expressing truncated gIII responded to gIII-specific antibody neutralization, as did a gIII-negative virus, whereas the mutant expressing gIII.hDAF was neutralized to an extent similar to that of wt BHV-1. Therefore, we concluded that gIII.hDAF, but not truncated gIII, was present on the envelopes of virus particles.

Functional expression of gIII.hDAF on virus particles via a GPI-based membrane anchor. We next examined the effects

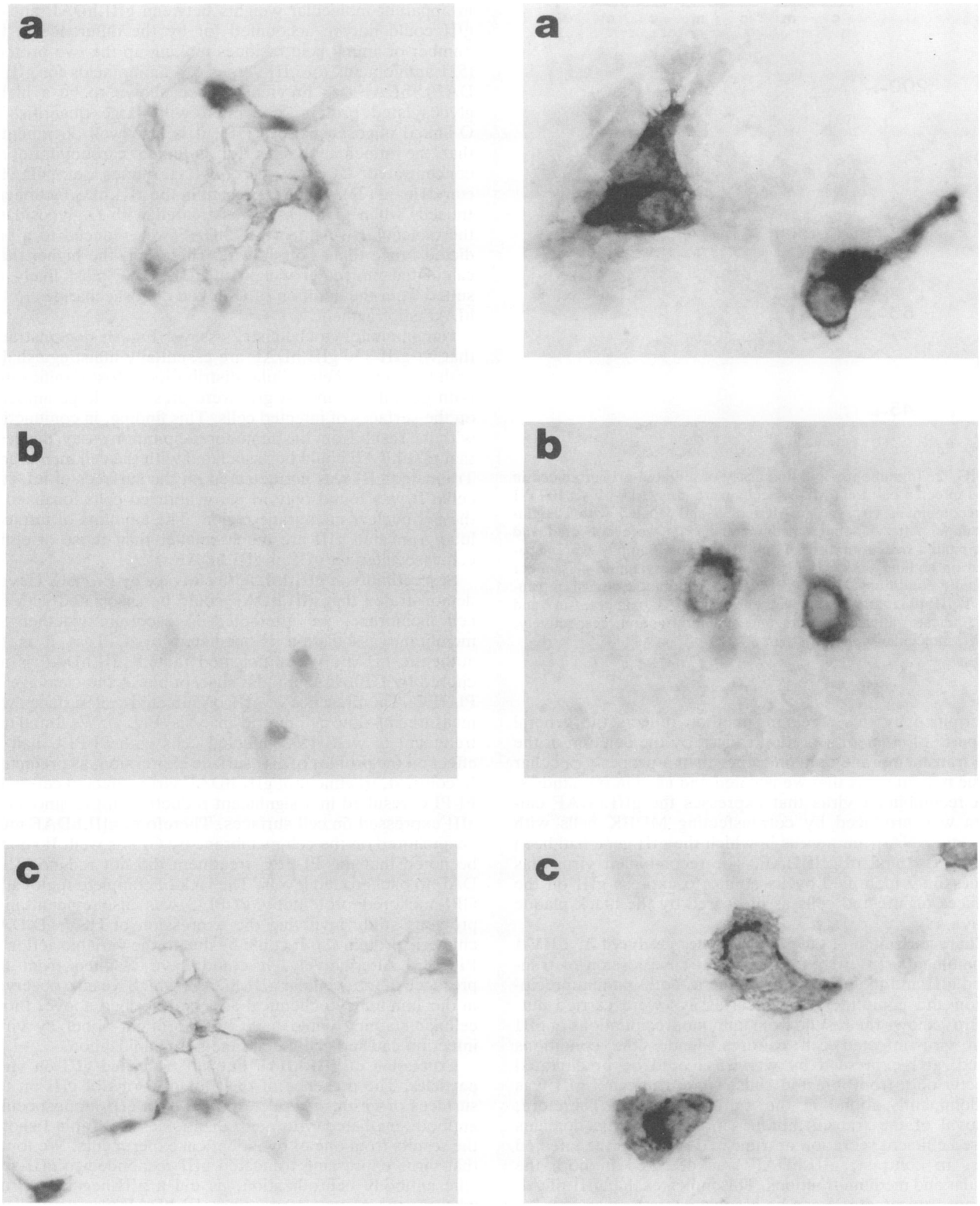

FIG. 3. Cellular distribution of mutant gIII molecules in virus-infected MDBK cells as determined by an immunocytochemical assay. Cells at 12 h postinfection were fixed with 2% paraformaldehyde only (left panels) or permeabilized with methanol (right panels), incubated with gIII-specific MAbs, and then processed with a horseradish peroxidase-based detection system (see Materials and Methods). (a) wt BHV-1-infected cells; (b) truncated gIII mutant-infected cells; (c) gIII.hDAF mutant-infected cells.

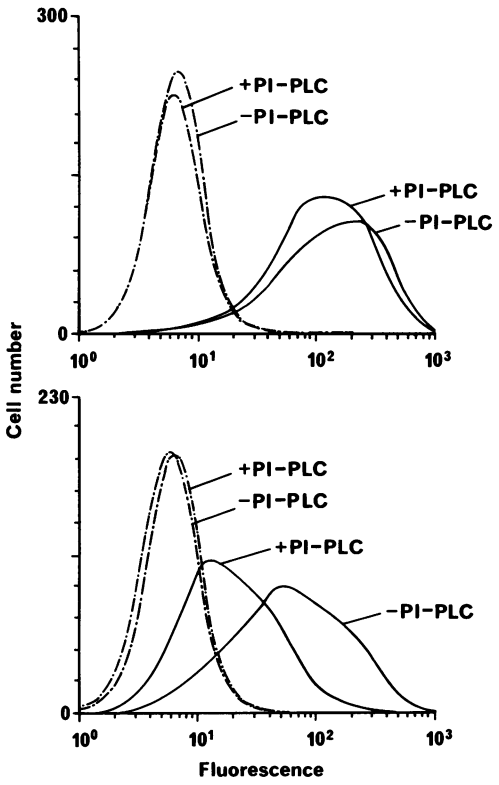

FIG. 4. Flow cytometric analysis of surface-expressed gIII in response to PI-PLC treatment. MDBK cells were infected with either wt BHV-1 (top) or the mutant expressing gIII.hDAF (bottom). Nine hours postinfection, cells were trypsinized, suspended in MEM containing 0.05% sodium azide, and treated with 5 μg of PI-PLC per ml as indicated. After washing, cells were reacted with gIII-specific MAbs; the reaction was followed by the addition of fluorescein-labeled goat anti-mouse antibody, and then the cells were subjected to flow cytometric analysis. Cell numbers and the intensity of surface staining are indicated on the vertical and horizontal axes, respectively. The cells reacted with the goat anti-mouse antibody alone were included as controls for background staining, which are indicated by the dashed lines.

of PI-PLC treatment on wt and mutant virus attachment to permissive cells. Since gIII is the dominant BHV-1 attachment protein, and gIII deletion is associated with a significant reduction of virus binding to host cells (14), we expected that, if gIII.hDAF still conducts gIII-specified functions and if it is anchored to virus particles via GPI, the mutant virus after PI-PLC treatment should exhibit a reduced ability to attach to permissive cells. The virus attachment experiment was carried out by first incubating the viruses to be tested with the cells at 4°C, a temperature at which virus penetration does not take place. After adsorption, free viruses were removed and cell monolayers were overlaid with agarose. Because the gIII glycoprotein affects only the rate of virus penetration (but is not essential for virus penetration, as indicated in Fig. 6), the attached viruses, either with or without gIII, would eventually penetrate and develop plaques. Therefore, this assay most likely reflects the capability for virus attachment. As shown in Fig. 5, pretreatment of wt virus or the gIII-negative mutant with PI-PLC had no effect on virus attachment. In contrast, attachment of the mutant virus to permissive cells was significantly reduced by PI-PLC pretreatment in a dose-dependent fashion. For example, a dose of 5 μg of PI-PLC per ml reduced attachment of the mutant virus by as much as 95%. Therefore, the results from this experiment indicate that (i) gIII.hDAF is expressed on virus particles via a GPI-based membrane anchor (ii) the chimeric molecule is functional with respect to mediating virus attachment.

TABLE 1. Plaque reduction assay[a]

Virus	Mean no. of plaques (% reduction[b]) with:	
	Control	Anti-gIII antibody
wt	172 (0)	24 (86)
gIII negative	179 (0)	146 (18)
vBgIII.SPE	281 (0)	221 (21)
vBgIII.DAF	186 (0)	55 (70)

[a] The assay was carried out in the presence of guinea pig serum complement. Control, complement only; anti-gIII antibody, 1:10 dilution of bovine gIII-monospecific antiserum (34). The results represent means of triplicate samples.

[b] Plaque reduction percentages were calculated by setting the number of plaques formed in the controls to 0% reduction.

Previously, we found that a gIII-negative virus was associated with a reduced penetration rate compared with that of wt virus (15). In order to further substantiate the proper function of gIII.hDAF, we compared the penetration activities of wt virus, a gIII-negative mutant, and the mutant expressing gIII.DAF (Fig. 6). Consistent with our previous finding, the gIII-negative mutant exhibited a slower penetration rate than the wt virus. However, the mutant that expresses gIII.hDAF exhibited penetration kinetics very similar to those of the wt virus. Therefore, GPI-anchored gIII is also functional in this aspect of the virus entry process.

DISCUSSION

Two salient findings were derived from the present study. First, the introduction of an *amber* mutation in front of the sequences encoding the putative transmembrane domain of BHV-1 gIII resulted in the efficient secretion of the normally membrane-associated viral envelope protein. Second, replacement of the gIII transmembrane and cytoplasmic domains with the carboxyl-terminal segment of hDAF restored the expression of gIII on the surfaces of both infected cells and mature virions via a GPI-based membrane anchor.

On the basis of protein sequence analysis, BHV-1 gIII appears to be a class I integral membrane protein that contains a putative transmembrane domain located between amino acids 467 and 500, which is followed by a 21-amino acid cytoplasmic tail (7). However, until the present study, the actual use of this putative transmembrane domain as a membrane anchor has not been demonstrated experimentally. Fitzpatrick et al. previously showed that a gIII carboxyl-terminal truncation (ending at residue 426) was not secreted when expressed in mouse L cells, nor was it expressed on the cell surface (8). In this study, we demonstrated that the removal of the carboxyl terminus from residue 466 led to the efficient secretion of gIII. This finding establishes that the putative transmembrane domain indeed represents the gIII membrane anchor. This conclusion is also consistent with findings derived with other gIII homologs, including HSV type 1 gC (10) and pseudorabies virus gIII (29). Two major factors may account for the differences between the previous observation made with the

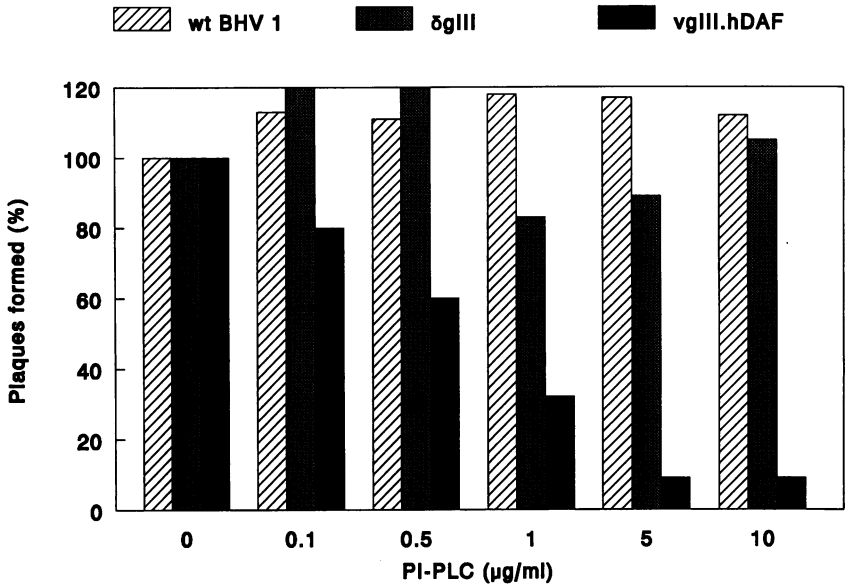

FIG. 5. Effects of PI-PLC treatment on virus attachment to MDBK cells. wt BHV-1 and the mutant expressing gIII.hDAF were treated with PI-PLC at the indicated concentrations for 1 h at 37°C, added to a precooled (4°C) monolayer of MDBK cells, and allowed to adsorb at 4°C for 90 min. After adsorption, the cells were washed with MEM and overlaid with agarose. Viral plaques were counted 4 days later. The number of plaques formed in the absence of PI-PLC treatment was set as 100% adsorption and used for calculating the percentage of adsorption of PI-PLC-treated viruses. Results are presented as means of triplicate samples. δgIII, gIII deletion mutant (14).

gIII-expressing cell line and our present results. First, the two truncated gIII molecules differ by 40 amino acids at their carboxyl termini. The removal of the additional 40 amino acids from the previous version of truncated gIII might have altered the tertiary or quaternary structure of the ectodomain of gIII, which might in turn affect the intracellular transportation of the mutated protein. Second, in the present study, truncated gIII was expressed as a result of virus infection. It is also possible that other viral components coexpressed with the mutated gIII may facilitate gIII transportation and subsequent secretion.

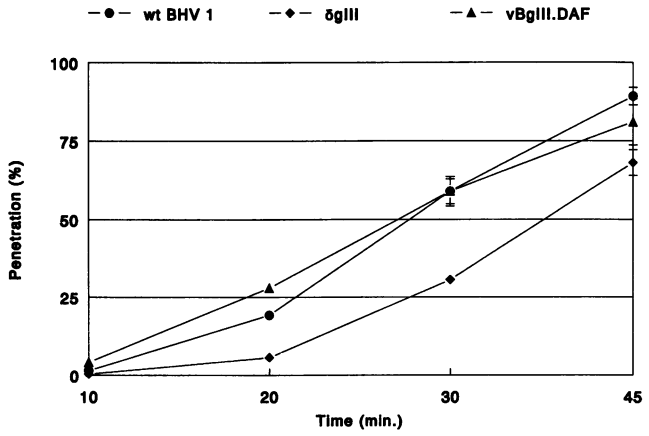

FIG. 6. Penetration kinetics of wt and mutant viruses in MDBK cells. Confluent MDBK cells in 6-well plates were cooled to 4°C and incubated with 0.4 ml of virus per well at 4°C for 1 h. After adsorption, virus inocula were removed and the cells were incubated with MEM at 37°C. At the indicated times, cells were treated with either an acid solution or MEM. After three washes with MEM, cells were overlaid with agarose. Viral plaques that developed were counted 4 days later. The number of plaques formed at each time point in the wells treated with MEM were taken as 100% penetration to calculate the percentage of virus which penetrated (number of plaques formed) in the acid-treated wells. Results are presented as means ± the standard deviations of triplicate samples. δgIII, gIII deletion mutant (14).

The replacement of the transmembrane and cytoplasmic domains of gIII with an hDAF sequence that provides a signal for the addition of GPI caused gIII to be expressed on the surfaces of both infected cells and virions. The utility of GPI as a membrane anchor for gIII.hDAF was demonstrated by its susceptibility to cleavage by PI-PLC. In virus-infected cell cultures, a portion of the chimeric protein, but not wt gIII, was found in the medium. The partial release of the chimeric protein into medium is somehow consistent with an observation previously made with authentic DAF expressed in the transfected cells (1). In the latter case, it was speculated, on the basis of pulse-chase experiments, that DAF that existed in the medium might have been derived from a membrane-associated form that resulted from phospholipase cleavage. The amount of gIII.hDAF present on the virus particles appears to be significant, although no direct quantitation was performed. In support of this conclusion, we found that (i) the mutant virus was as sensitive as wt BHV-1 to gIII-specific-antibody-mediated virus neutralization; (ii) the removal of gIII.hDAF from the mutant virus by PI-PLC treatment reduced virus attachment by as much as 95%; and (iii) the mutant virus had penetration kinetics identical to those of wt BHV-1.

Assembly of envelope proteins into the virion appears to be a highly organized event, since nonvirus, cellular membrane proteins are normally excluded from the mature virion. Although the mechanism that dictates this specificity is still poorly understood, one of the factors involved appears to be the specific recognition of the envelope proteins by the nucleocapsid during the virus budding process. For example, matrix protein 1 of influenza virus (13) and para-

influenza virus (3) has been shown to interact with the viral envelope proteins and with the nucleocapsid. Since GPI is associated only with the outer leaflet of the lipid bilayer of the cytoplasmic membrane (5, 19) and since it shares no structural identity with any of the typical transmembrane or cytoplasmic domains of viral envelope proteins, it is of interest that BHV-1 gIII with a GPI-based membrane anchor can be assembled into virus particles. This suggests that, at least for BHV-1 gIII, the transmembrane and cytoplasmic domains do not carry a signal required for the envelope assembly. Since BHV-1 encodes multiple envelope glycoproteins, it not unlikely, however, that one or more of these proteins could carry such a signal(s). This speculation remains to be investigated further.

Although expression of the HSV type 1 gD-DAF chimera on cell surfaces has previously been documented (2), we are not aware of any report which shows the expression of the GPI-anchored protein on virus virions. The observation that a viral protein can be expressed via a GPI-based membrane anchor and that it can be specifically removed from the virion by enzymatic digestion may suggest a novel approach to study the functions of viral envelope proteins. Alphaherpesviruses, for example, encode several envelope proteins which together provide the functional machinery for virus entry into permissive cells. Mutant viruses in which specific viral envelope proteins have been deleted have proved to be valuable tools for defining the role of each protein in the virus entry process. Conventionally, viable virus mutants with defects in essential proteins can be obtained only with complementary cell lines (cells expressing an unaltered version of the essential protein). By the strategy of linking these proteins to a GPI membrane anchor, it might be possible to obtain a recombinant virus expressing an essential protein from a noncomplementing cell line. The essential protein may then be selectively removed from the virion by PI-PLC treatment. The fact that the amount of the viral protein present on virus particles can be controlled by varying the conditions of PI-PLC treatment may represent an additional advantage of this approach. Furthermore, as discussed above, substitution of the normal transmembrane and cytoplasmic domains of a given envelope protein by GPI may provide insight into the roles played by these structures in various stages of the virus replication cycle.

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