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Bovine Herpesvirus 1 Attachment to Permissive Cells Is Mediated by Its Major Glycoproteins gI, gIII, and gIV†

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A bovine herpesvirus 1 (BHV-1) gIII deletion mutant (gIII$^-$) was produced by means of recombinant DNA that retained the ability to replicate in cell culture. However, the gIII$^-$ mutant was functionally defective, showing impaired attachment to permissive cells, a delay in virus replication, and reduced extracellular virus production. The attachment defect exhibited by the gIII$^-$ mutant is an indication of the role played by gIII in the normal infection process. This was shown by dramatically decreased binding of radiolabelled gIII$^-$ virus to permissive cells and a slower adsorption rate, as measured by plaque formation, than the wild-type (wt) virus. Furthermore, treatment of the gIII$^-$ virus with neomycin increased virus adsorption and plaque formation by severalfold, whereas neomycin treatment had no effect on the wt virus. This observation showed that the gIII$^-$ mutant was strictly defective in adsorption but fully competent to produce productive infections once induced to attach. The gIII$^-$ mutant showed greater sensitivities than did the wt virus to anti-gI and anti-gIV antibody-mediated neutralization. Analyses with panels of monoclonal antibodies to gI and gIV revealed that the epitopes gI-IV and gIV-III were the main targets for enhanced neutralization. This provided evidence that gI and gIV may also participate in virus attachment. Finally, when affinity-purified gI, gIII, and gIV were tested for their ability to inhibit virus adsorption, gIII had the most pronounced inhibitory effect, followed by gI and then gIV. gIII was able to completely inhibit wt virus adsorption, and at a high concentration, it also partially inhibited the gIII$^-$ mutant. gI and gIV inhibited wt and gIII$^-$ mutant adsorption to a comparable extent. Our results collectively indicate that gIII plays a predominant role in virus attachment, but gI and gIV also contribute to this process. In addition, a potential cooperative mechanism for virus attachment with these three proteins is presented.

Virus infections are initiated through specific interactions between viral attachment proteins (VAPs) and their receptors on the surfaces of permissive cells (17). For alphaherpesvirus, the initial interaction between the VAP and its cellular receptor is followed by virus penetration involving membrane fusion (24). These two steps constitute the virus entry process (24). At present, the nature of alphaherpesvirus VAPs still remains to be defined. For herpes simplex virus type 1 (HSV-1), studies involving antibody inhibition (7), virosomes (12), and cell membrane fractionation (13) have indicated that this virus uses envelope glycoproteins, including glycoprotein B (gB), gC, and gD, as the major VAPs. However, since gC is dispensible for virus growth in cell culture, the role played by this protein as a VAP has been questioned (11, 16). In addition, even though gB and gD are not dispensable, gB$^-$ and gD$^-$ mutants are still able to bind to permissive cells (3, 10), suggesting that they may not be required for attachment.

Recently, it was reported that the cellular receptor of HSV-1 contains a heparinlike component (32), and the interactions between HSV-1 but not HSV-2 and permissive cells could be interrupted by polycationic compounds, including neomycin (14–16). By making intertypic recombinants between HSV-1 and HSV-2, the gene(s) coding for the viral component(s) responsible for the neomycin effect was mapped to a region of the HSV-1 genome that encoded the gC gene (16). However, since an HSV-1 gC$^-$ mutant was still sensitive to neomycin, and since no other glycoprotein gene is located near the gC gene, the authors suggested that a nonglycosylated protein encoded by a gene near the gC locus may be the HSV-1 VAP (16).

Studies of a pseudorabies virus (PRV) gIII$^-$ mutant suggested that gIII (the homolog to HSV-1 gC) plays a predominant role in virus attachment to host cells (25, 34). In support of this, antibodies against PRV gI, gII, or gp50 could not inhibit attachment of the PRV gIII$^-$ mutant, and in addition, wild-type (wt) PRV failed to compete with the gIII$^-$ virus for binding to permissive cells. As a result, it was postulated that under normal circumstances, PRV uses gIII for virus-specific attachment and that the gIII$^-$ mutant initiates infection through a nonspecific mechanism (25, 34).

Four major bovine herpesvirus 1 (BHV-1) glycoproteins have thus far been characterized, namely gI, gII, gIII, and gIV (19, 21, 29). BHV-1 gI, gII, gIII, and gIV are homologs of HSV gB, gE, gC, and gD and of PRV gII, gI, gIII, and gp50, respectively (22, 33). Based on the results from antibody inhibition experiments, we previously suggested that gI, gIII, and gIV are important for virus attachment, while gI and gIV might also participate in penetration (6, 8, 23a). The putative function of each glycoprotein has also been correlated to corresponding epitopic structures by mapping antigenic domains recognized by virus-neutralizing monoclonal antibodies (MAbs).

The present study was carried out to further explore the roles of these glycoproteins in virus attachment. We first produced a gIII$^-$ mutant virus, with which we showed that gIII of BHV-1 was dispensable for virus growth in cell

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culture. Using the gIII$^-$ mutant as a tool, we then took three different approaches to elucidate the function of gIII as well as gI and gIV by (i) comparing the attachment efficiency of the wt virus and the gIII$^-$ mutant, (ii) identifying compensatory functions provided to the gIII$^-$ mutant by gI and gIV, and (iii) testing the inhibitory effects of purified gI, gIII, and gIV on virus adsorption. The results from these studies provided evidence that while gI, gIII, and gIV all contribute to virus attachment, gIII appears to play a predominant role in this process.

MATERIALS AND METHODS

Virus and cells. 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.

Purification of virus and viral DNA. MDBK cells were infected at a multiplicity of infection (MOI) of 1. After all cells expressed cytopathology, cell supernatants containing virus were clarified by low-speed centrifugation at 1,000 × *g*. Virus was harvested from clarified supernatants by pelleting through 30% sucrose in phosphate-buffered saline (PBS) at 100,000 × *g* for 60 min. The virus pellet was then resuspended in 0.05 M Tris-HCl–0.15 M NaCl–10 mM EDTA (pH 8.0) and applied to 20 to 50% potassium-sodium tartrate discontinuous gradients and centrifuged at 100,000 × *g* for 90 min. Following centrifugation, the virus band was collected, diluted in PBS, and pelleted at 75,000 × *g* for 60 min (2). Purified virus was stored at −70°C.

Viral DNA was purified by the method of Summers and Smith (27). Briefly, purified virus was suspended in 0.1 M Tris-HCl–0.15 M NaCl–0.1 M EDTA–0.1 M KCl, pH 7.5, containing 45 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml and incubated at 50°C for 1 h. Sarkosyl was added to a final concentration of 1%, and the incubation was continued at 50°C for an additional 1 h. The sample was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. The DNA pellet was resuspended in 0.05 M Tris-HCl–10 mM EDTA, pH 8.0, divided into portions, and stored at −70°C.

Purification of viral glycoproteins. Glycoproteins gI, gIII and gIV were purified from a virus-infected cell lysate as previously described (1, 28). Briefly, virus-infected cells were harvested and centrifuged at 1,000 rpm to obtain infected-cell pellets. Subsequently, the cells were resuspended in 1% Nonidet P-40 and 1% sodium deoxycholate in 0.1 M Tris-HCl–0.15 M NaCl, pH 7.5, to produce the cell lysate.

Immunoadsorbent MAb columns with specificities for gI, gIII, and gIV were coupled in tandem and equilibrated at 4°C. After passage of the cell lysate over the columns, they were washed with 3 volumes of wash buffer (WB; 0.1 M Tris-HCl, 0.5 M NaCl, pH 7.5) with 0.1% Nonidet P-40, followed by another 3 volumes of WB. Subsequently the columns were uncoupled and the respective glycoproteins were eluted from the columns with 50 mM diethylamine, pH 11.5. During collection the glycoproteins were neutralized with 1 M Tris-HCl, pH 7. The purified glycoproteins were concentrated by ultrafiltration with an Amicon YM30 membrane and dialyzed extensively against PBS. Protein concentration was determined with the Bio-Rad (Missisauga, Ontario, Canada) protein determination kit.

Plasmid construction. *Escherichia coli* MC1000 (18) was used for propagation of all plasmids. Plasmids were constructed by standard recombinant DNA techniques (18).

p113RI-Bgl3.0 was derived by subcloning the 3.0-kb *Eco*RI-*Bgl*II fragment of the BHV-1 *Hin*dIII genomic clone pSD113 (20) into the *Eco*RI and *Bam*HI sites of pBR322. The 3.0-kb *Eco*RI-*Bgl*II BHV-1 fragment is located between 0.118 and 0.141 map units and contains the 1,563-bp gIII coding sequence, approximately 640 bp of 5′-flanking sequence, and 800 bp of 3′-flanking sequence (5). Construction of pBHVΔgIII is shown in Fig. 1. p113RI-Bgl3.0 was partially digested with *Bgl*I, repaired with T4 DNA polymerase to blunt the single-stranded ends, and digested to completion with *Sph*I. A 700-bp fragment which contained the gIII gene 5′-flanking sequence was isolated and ligated to the *Sma*I and *Sph*I sites of polink26 (31), resulting in polink5′ gIII. Next, p113RI-Bgl3.0 was digested with *Sal*I, treated with Klenow fragment to fill in single-stranded ends, and then digested with *Eco*RI. The 1.0-kb fragment which contained the gIII gene 3′-flanking sequence was isolated and inserted into the *Hpa*I and *Eco*RI sites of polink5′ gIII. This resulted in pBHVΔgIII, which possesses both 5′ and 3′ gIII-flanking sequences, and at the region corresponding to the gIII coding sequence, there are an 81-bp sequence from the 5′ end of gIII, a 29-bp sequence derived from polink26 (which contains *Bam*HI, *Kpn*I, *Hin*dIII, and *Cla*I restriction endonuclease sites), and a 234-bp sequence from the 3′ end of gIII, which is out of frame relative to the gIII initiation codon (verified by sequencing).

Recombinant virus production. To generate recombinant viruses, MDBK cells were cotransfected with pBHVΔgIII (linearized with *Eco*RI) plus naked BHV-1 genomic DNA by electroporation (4). MDBK cells at approximately 70% confluency were trypsinized, resuspended in ice-cold HEPES-buffered saline (HeBS; 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 137 mM NaCl, 5 mM KCl, 0.7 mM $NaHPO_4$, 5 mM D-glucose, pH 7.1), and washed three times in HeBS at 4°C. After the final wash, cells were resuspended to approximately 3 × 10^6/ml in HeBS and transferred to an electroporation cuvette (Bio-Rad Laboratories, Richmond, Calif.) to which 10 μg of linearized plasmid DNA and 20 μg of viral DNA were added, followed by incubation on ice for 10 min. Electroporation was performed at 500 μF and 200 V with a Bio-Rad Gene Pulser. After the electroporation and an additional 10-min incubation on ice, the cells were transferred to a 100-mm culture dish containing 15 ml of MEM with 10% FBS and incubated at 37°C. Five hours later, when the cells were completely attached, the culture medium was replaced with a 1% agarose (in MEM containing 2% FBS) overlay. The overlaid cultures were then incubated at 37°C for 4 to 5 days. Following incubation, the agarose overlay was removed, and the cell monolayer was screened for gIII-negative virus plaques by an antibody-based black-plaque assay (see below). Where necessary, virus extracted from the agarose overlays was also screened for the presence of recombinants.

The black-plaque assay was carried out by the method of Johnson et al. (11). MDBK cell monolayers with an appropriate number of viral plaques were fixed with 0.25% glutaraldehyde (Sigma) in 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 (30), 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, followed by three washes with PBS. Affinity-

purified, peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; 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, followed by addition of 0.01% 4-chloronaphthol (Sigma) and 0.0003% H_2O_2 (Sigma) in water. The plates were then incubated at 24°C until black plaques developed. Putative gIII-negative white plaques were identified and picked for further plaque purification.

^{35}S labeling of virus and immunoprecipitation assays. Subconfluent monolayers of MDBK cells were infected with virus at an MOI of 5. After 90 min at 37°C, the virus inoculum was removed and cells were cultured in methionine-free MEM (GIBCO) supplemented with 10% dialyzed FBS for 5 h. This was followed by adding 50 μCi of L-[^{35}S]methionine (Amersham, Oakville, Ontario, Canada) per ml of medium. Twenty-four hours later, the culture medium was harvested and virus was purified as described above.

Immunoprecipitation assays were carried out as previously described with either monospecific rabbit antisera or mixtures of MAbs (28, 30). Antibody-precipitated samples were separated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels under reducing conditions.

Single-step growth curve. MDBK cells grown in 60-mm culture dishes to approximately 90% confluency were infected with either the wt or the gIII$^-$ BHV-1 virus at an MOI of 1. At various times postinfection, culture media were collected and stored at −70°C until titrated by plaque assays. The plates containing the cell monolayers were frozen immediately at −70°C for subsequent quantitation of cell-associated virus. To harvest cell-associated virus, the cells were thawed, scraped from the plates, and suspended in 2 ml of MEM with 10% FBS per plate. The cell suspensions were frozen and thawed twice and then sonicated for 20 s at a setting of 10 with a horn probe sonicator (VibraCell, Sonics and Materials Inc., Danbury, Conn.) prior to titration.

Adsorption and adsorption inhibition assays. Virus binding experiments involving [^{35}S]methionine-labeled virus were carried out in 24-well plates containing confluent MDBK cells. Plates were cooled at 4°C for 1 h and blocked with BSA-PBS for 30 min. ^{35}S-labeled, tartrate gradient-purified virus, either wt or mutant, was diluted to 2×10^6 cpm/ml in BSA-PBS. Then 0.1 ml of virus was added to each well and incubated at 4°C. At the indicated time points, plates were washed with PBS and cells were lysed by addition of 0.2 ml of 1% Triton X-100 and 1% SDS in PBS per well. Cell-associated radioactive counts were determined by liquid scintillation counting of the total cell lysates from each well.

For adsorption studies with plaque assays, MDBK cells grown to confluency in six-well culture plates were cooled at 4°C for 1 h. Then 0.4 ml of virus inoculum, containing about 500 PFU/ml in MEM, was added to each well and incubated at 4°C. At different times, the viral inocula were removed, and the plates were washed once with MEM and overlaid with MEM-supplemented agarose. Plaques were counted 4 days later. The number of plaques formed after 180 min of incubation at 4°C was defined as 100% adsorption and used to calculate the percentages of virus adsorbed at the other time points.

For adsorption inhibition with purified viral glycoproteins, MDBK cells grown to confluency in 24-well plates were cooled at 4°C for 1 h. Viral glycoproteins were diluted in MEM to specified concentrations and added to the cells in volumes of 50 μl per well. The plates were incubated for 1 h at 4°C, followed by the addition of the virus inoculum,

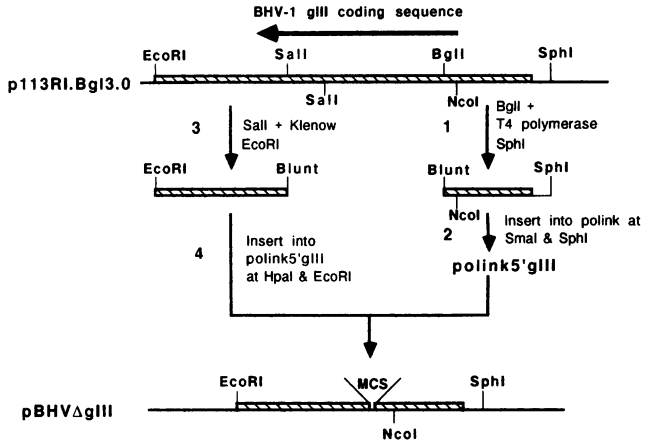

FIG. 1. Construction of transfer vector pBHVΔgIII. Stippled bars represent gIII gene fragment; solid lines represent pBR322 backbone in p113RI.Bg13.0. MCS, Multicloning site, containing *Bam*HI, *Hin*dIII, *Kpn*I, and *Cla*I sites in order from 5′ to 3′.

approximately 150 PFU per well in 50-μl volumes, and incubated at 4°C for an additional 1 h. The plates were then washed three times with MEM and overlaid with 1% agarose (supplemented with MEM plus 2% FBS). Plaques were counted 4 days later.

Plaque reduction and neutralization assays. Virus at approximately 500 PFU/ml in MEM was incubated with the specified antibodies at an appropriate dilution at 37°C for 1 h in either the presence or absence of complement. Reconstituted guinea pig serum (GIBCO) was used as the complement source at a final dilution of 1:30. The antibody-treated virus was then added to confluent MDBK cells grown in six-well tissue culture plates and incubated at 37°C for 90 min. Inocula were then removed, and the cells were washed once with MEM and overlaid with 1% agarose in MEM with 2% FBS. Plaques were counted 4 days later. Virus treated with irrelevant antibody was included in each experiment as controls. Neutralization assays were carried out in microtiter plates as described previously (2).

RESULTS

Construction of a BHV-1 gIII gene-deleted virus. gIII gene deletion mutants were isolated from virus progeny produced by MDBK cells cotransfected with BHV-1 naked genomic DNA and linearized pBHVΔgIII plasmid DNA. From the structure of pBHVΔIII (Fig. 1) and the nucleotide sequence of the gIII gene (5), the mutants resulting from reciprocal recombination events between the linearized plasmid and the viral genomic DNA should carry a 1.25-kbp deletion in the gIII gene coding sequence, except for the putative gIII signal peptide and an additional six amino acids thereafter.

After cotransfection, the virus pools produced were screened for the presence of gIII-negative virus by a black-plaque assay with a cocktail of anti-gIII MAbs. The gIII-negative virus constituted approximately 0.3% of the total virus population. Isolated gIII$^-$ mutants were subjected to further plaque purification, and their lack of gIII production was verified by black-plaque assays with monospecific polyclonal anti-gIII antiserum (data not shown). One mutant was selected for further analyses.

The genomic structure of the gIII$^-$ mutant was analyzed by restriction endonuclease and Southern blot analyses.

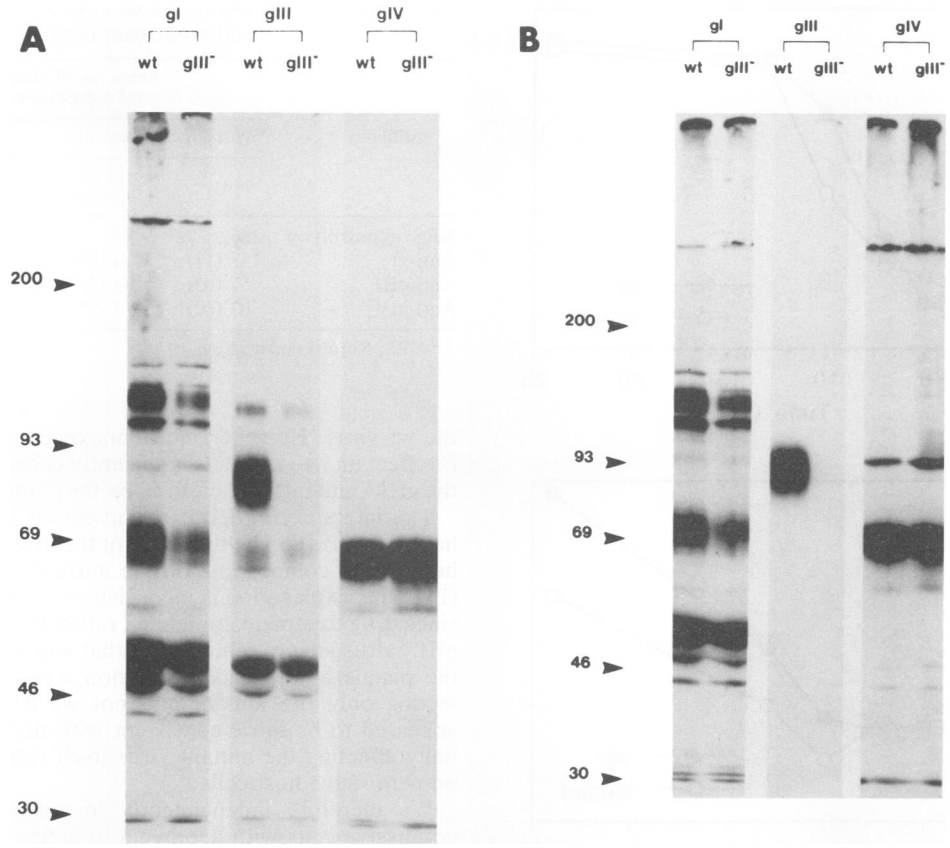

FIG. 2. Detection of gI, gIII, and gIV from BHV-1 wt- and gIII$^-$ mutant-infected MDBK cells by immunoprecipitation. MDBK cells were infected with either the wt virus or the gIII$^-$ mutant at an MOI of 5 and labeled with 50 μCi of [^{35}S]methionine per ml. After 18 h of labeling, cells were lysed and immunoprecipitated for individual glycoproteins with either rabbit monospecific antisera (A) or MAbs (B). Samples were separated on an SDS–7.5 polyacrylamide gel under reducing conditions. On the left side of each panel are molecular mass standards (sizes shown in kilodaltons) which were run in parallel with the samples on the same gel.

These studies confirmed the deletion of the gIII coding sequence as predicted above (data not shown).

To confirm that we had in fact produced a gIII-negative mutant as well as to detect any possible, though unlikely, alterations that might have occurred to other major glycoproteins during the production of the recombinant virus, immunoprecipitation assays were carried out. Both monospecific polyclonal antisera (Fig. 2A) and panels of MAbs which recognize separate and distinct epitopes on gI, gIII, and gIV (Fig. 2B) were used in these analyses. Anti-gIII antibodies precipitated the prominent 91-kDa gIII protein in wt virus-infected cells (Fig. 2A, lane 3, and Fig. 2B, lane 3). As expected, the 91-kDa protein was not detected in cells infected with the gIII gene-deleted virus (Fig. 2A, lane 4, and Fig. 2B, lane 4). In addition, no truncated forms of the gIII protein were found in mutant virus-infected cells with high-titer monospecific polyclonal anti-gIII antiserum (28), indicating that no portion of a functional gIII protein was present. The products visualized by antibodies to gI and gIV from the cells infected either with wt virus or with the gIII$^-$ mutant showed identical patterns and comparable molecular masses. Thus, expression of gI and gIV by the mutant virus was not qualitatively altered compared with wt virus.

Single-step growth curves. One apparent feature of the gIII$^-$ mutant in cell culture was its slower growth rate, as evidenced by its delay in producing cytopathology. To assess the growth efficiency of the gIII$^-$ virus, a single-step growth curve was generated for the mutant and compared with that of the wt virus. Two major differences were noticed (Fig. 3). First, the gIII$^-$ mutant virus showed a delay in the production of cell-associated virus (Fig. 3A). At 10 h postinfection, a 10-fold-higher virus titer was produced by wt virus-infected cells than by mutant virus-infected cells. However, by the time of maximal virus production (13 h postinfection), both the wt and the gIII$^-$ viruses produced comparable levels of intracellular virus. In contrast, the wt virus produced a 10-fold-higher extracellular virus titer than the gIII$^-$ mutant (Fig. 3B).

gIII$^-$ mutant attachment to permissive cells. In this communication, the term attachment is used to indicate the simple process of virus binding to permissive cells, only a fraction of these events leading to productive infections, and the term adsorption is used to specify virus binding which is followed by cell penetration and virus replication. By distinguishing between attachment and adsorption, we wish to emphasize productive attachment, which is measured by plaque assay and is relevant to the virus infectious process. To elucidate the role played by gIII in the initial stages of infection, the gIII$^-$ mutant was compared with the wt virus for its relative efficiency in attachment and adsorption. Two types of comparisons were made: total virus attachment to cells was measured with radiolabeled virus, and the rate of virus adsorption to cells was measured by plaque assays (Fig. 4).

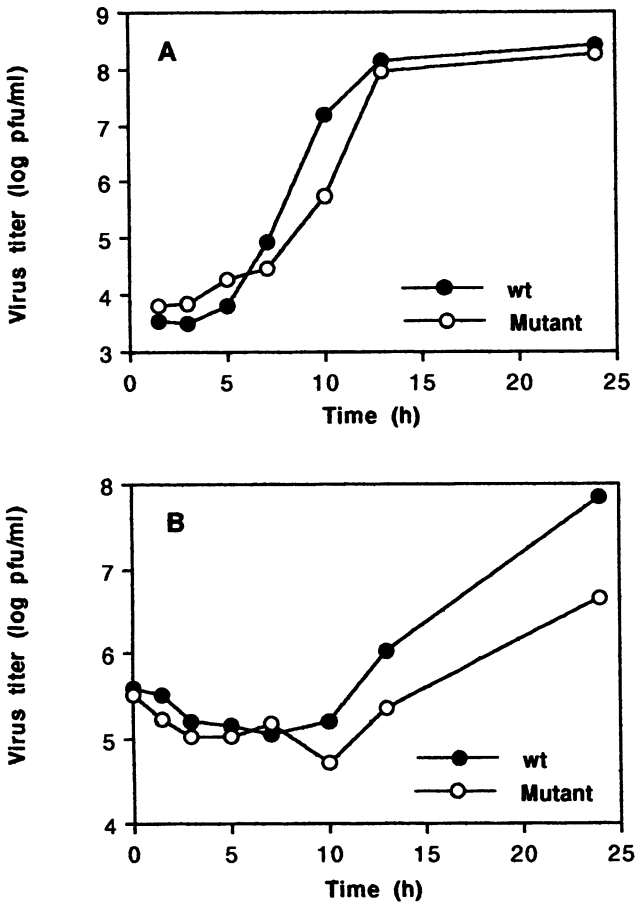

FIG. 3. Single-step growth curves of the wt virus and the gIII$^-$ mutant virus. MDBK cells were infected with virus at an MOI of 1 and incubated at 37°C. At 1.5, 3, 5, 7, 10, 13, and 24 h postinfection, culture media and cells were harvested separately, and virus titers were determined by plaque assay. (A) Cell-associated virus titers; (B) virus titers in medium.

To focus on the initial attachment process, experiments were carried out at 4°C, a temperature at which the energy-dependent process of penetration cannot occur. For the experiment with radiolabeled virus, the specific activities of labeling were calculated from the DNA contents of the virus (23), 6.13×10^{-6} cpm per particle for the mutant virus and 7.03×10^{-6} cpm per particle for the wt virus. Virus attachment was determined at two time points, 30 and 90 min. Total gIII$^-$ mutant bound at 30 and 90 min were 1,450 ± 400 cpm and 2,850 ± 270 cpm, and total wt virus bound at 30 and 90 min were 9,780 ± 780 cpm and 21,350 ± 1,640 cpm, respectively. Therefore, the gIII$^-$ mutant showed significantly less binding to permissive cells than did the wt virus. A comparison of the adsorption rates of the wt and the gIII$^-$ viruses demonstrated more rapid adsorption of the wt virus (Fig. 4).

Effect of neomycin on adsorption. Neomycin has been previously shown to inhibit HSV-1 infection by interacting with host cell receptors, thereby preventing their recognition by the virus (14–16). Therefore, during the characterization of the BHV-1 gIII$^-$ mutant, we tested the effect of neomycin on adsorption for both the wt and gIII$^-$ viruses, anticipating that the drug might have a preferential inhibitory effect on the wt virus. However, quite unexpectedly, neomycin had no effect on wt virus but significantly enhanced adsorption of the gIII$^-$ mutant, as measured by the plaque assay (Fig. 5A).

The inhibitory effect of neomycin on HSV-1 attachment has been shown to be the result of the drug's interaction with heparinlike components on the surface of permissive cells (14). This explanation is inconsistent with our observations, since drug treatment stimulated rather than inhibited BHV-1 gIII$^-$ virus adsorption, at least that which was measured by the plaque assay, and in addition was discriminatory, affecting only the mutant and not wt BHV-1. Our results appeared to be more consistent with neomycin's preferentially affecting the mutant virus itself rather than receptors on permissive host cells.

We therefore independently incubated either virus or permissive cells with neomycin to define the target of drug action. Treatment of permissive cells with neomycin prior to the addition of virus had no effect on subsequent gIII$^-$ virus adsorption, whereas treating the gIII$^-$ mutant virus with neomycin before adding it to the cells increased adsorption (Fig. 5B). These results suggest that neomycin's action was on the virus and not on the cell and its effect was specific to

TABLE 1. Sensitivities of wt virus and gIII$^-$ mutant to antiserum neutralization

Antibody	Mean no. of plaques formed (% reduction vs control)			
	Without complement		With complement	
	wt	gIII$^-$ mutant	wt	gIII$^-$ mutant
NRS[a] (control)	166	84	167	99
Anti-gI	148 (11)	4 (95)	63 (62)	0 (100)
Anti-gIII	57 (63)	84 (0)	8 (95)	85 (14)
Anti-gIV	63 (62)	4 (95)	1 (99)	0 (100)

[a] NRS, Normal rabbit serum.

TABLE 2. MAbs to which the gIII$^-$ mutant showed enhanced sensitivity[a]

MAb	Epitope specificity	Activity[b] (titer)
gI specific		
1B10	gI-I	0
3F3	gI-II	2
1E11[c]	gI-III	4
1F8	gI-IVa	4
3G11	gI-IVb	8
6G11	gI-IVc	32
5G11	gI-IVc	32
1F10[c]	gI-V	4
gIV specific		
pB136[c]	gIV-Ia	0
9D6[c]	gIV-Ib	0
3E7	gIV-II	0
10C2	gIV-IIIa	8
4C1	gIV-IIIb	2
2C8	gIV-IIIc	4

[a] MAbs were developed and characterized by van Drunen Littel-van den Hurk et al. (28) and Hughes et al. (8). Data for epitope specificity are derived from Fitzpatrick et al. (6) and Hughes et al. (8).

[b] Expressed as the difference in neutralization titer between the wt virus and the mutant.

[c] These MAbs also show postadsorption neutralization activity (7, 8).

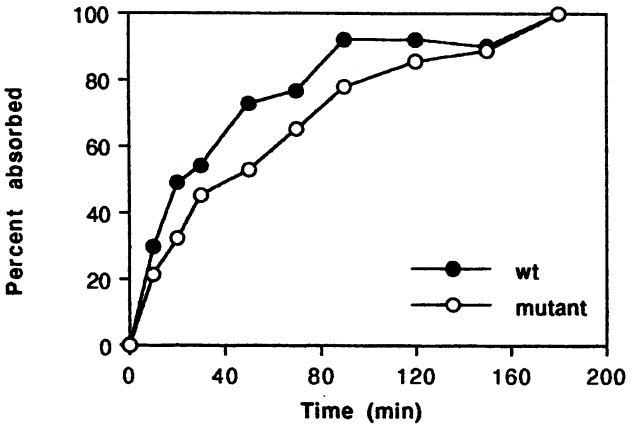

FIG. 4. Adsorption of the wt virus and the gIII$^-$ mutant virus to MDBK cells. MDBK cells in six-well culture plates were cooled at 4°C for 1 h, and 0.4 ml of virus, approximately 1,000 PFU/ml in MEM, was added to each well, followed by incubation at 4°C. At indicated time intervals, the viral inoculum was removed, and plates were washed once with MEM and overlaid with agarose. Plaques were counted 4 days later. The number of viral plaques formed following a 180-min incubation at 4°C was defined as 100% adsorption. Data represent means of triplicates.

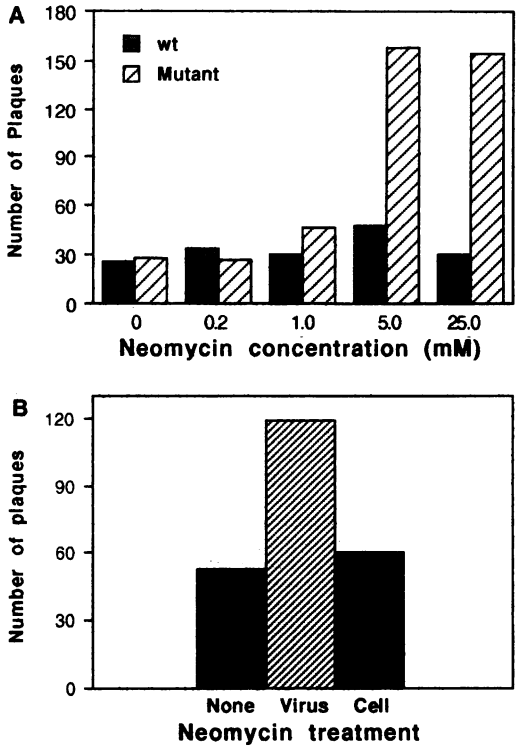

FIG. 5. Effects of neomycin on wt virus and gIII$^-$ mutant virus adsorption. (A) Neomycin dose-response curve. MDBK cells at confluency in six-well plates were cooled at 4°C for 1 h; 0.2 ml of virus, approximately, 250 PFU/ml in MEM, and 0.2 ml of neomycin, at different concentrations in MEM, were added to each well, followed by incubation at 4°C for 90 min. After incubation, the cells were washed once with MEM and overlaid with agarose. Plaques were counted 4 days later. Indicated neomycin concentrations represent final concentrations. (B) Effects of neomycin on cells and on virus. For treating the virus with neomycin, the virus at approximately 2,500 PFU/ml was incubated with 5 mM neomycin at 4°C for 30 min and diluted at 1:20 in MEM, and 0.4 ml of the neomycin-treated virus was then added to each well of MDBK cell monolayers in six-well culture plates, followed by the procedures described for panel A. For testing cells, MDBK cells in six-well culture plates were cooled at 4°C for 1 h and incubated with 0.4 ml of 5 mM neomycin in MEM for 30 min, followed by three washes with MEM. Virus at 2,500 PFU/ml was diluted 1:20 in MEM, and 0.4 ml of virus was added to each well, followed by plaque assay as described above.

the mutant virus. In order to confirm that the drug effect was a result of the loss of the gIII gene product, we made a BHV-1 gIII-positive recombinant virus derived from the gIII$^-$ mutant by marker rescue. The rescued virus responded in a manner identical to the original wt virus (data not shown).

Sensitivity of the gIII$^-$ mutant to antibody neutralization. The relative sensitivities of the wt and the gIII$^-$ mutant viruses to antibody neutralization were determined and served as an additional measure of gIII function. Initially, a plaque reduction experiment was performed with monospecific antiserum against gI, gIII, or gIV (Table 1). As expected, the anti-gIII antiserum neutralized the wt virus but not the gIII$^-$ mutant. In the presence of complement, anti-gI and anti-gIV antisera showed more profound virus-neutralizing capacities for both the wt and the mutant virus (complement alone had no effect on either virus). However, antibody neutralization differentiated between the two viruses, since the mutant virus was completely neutralized by either anti-gI or anti-gIV antibody but the wt virus was only partially neutralized (Table 1). It should be noted that although the gIII$^-$ mutant showed enhanced sensitivity to both anti-gI and anti-gIV antibodies, the augmentation of neutralization was more pronounced for anti-gI antibody.

In view of the increased sensitivity of the gIII$^-$ mutant virus to anti-gI and anti-gIV antibodies and since the gIII$^-$ mutant was defective in attachment, we investigated whether these increased sensitivities could be related to given domains previously suggested to be involved in attachment (6, 8, 23a). Plaque reduction experiments with panels of MAbs which recognize separate antigenic domains on gI and gIV were therefore carried out. Results are presented as enhanced neutralization activities for individual MAbs, comparing the gIII$^-$ mutant with the wt virus (Table 2). In agreement with the results from the studies with monospecific polyclonal antisera, the gIII$^-$ mutant showed increased sensitivity to MAb neutralization, with enhanced sensitivities being more dramatic with anti-gI MAbs than with anti-gIV MAbs. All anti-gI MAbs, with the exception of 1B10, showed higher neutralization titers for the gIII$^-$ mutant than for the wt virus. MAbs specific to domain gI-IV (1F8, 3G11, 6G11, and 5G11), particularly to domain gI-IVc (6G11 and 5G11), had the most profound effect. The pattern of enhanced antibody sensitivity associated with a single domain was also observed for anti-gIV MAbs. In this case, only the MAbs directed at domain gIV-III (10C2, 4C1, and 2C8) showed enhanced activities for the gIII$^-$ mutant. Previously, it has been shown that domains gI-IV and gIV-III have attachment functions, while domains gI-III, gI-V, and gIV-1 are involved in penetration (6, 8, 23a). Therefore, the supersensitivity of the gIII$^-$ mutant to anti-gI and anti-gIV antibody-mediated neutralization is associated with the putative attachment but not penetration domains on gI and gIV.

Effects of purified viral glycoproteins on virus adsorption. If a specific viral protein is indeed responsible for virus attach-

ment to permissive cells through a specific interaction with a cellular receptor, then treating cells with that viral protein should block virus infection. With this rationale, purified gI, gIII, and gIV were tested for their inhibitory effects on virus adsorption by plaque assays. Concentrations of gIII up to 50 μg/ml showed a significant inhibitory effect on wt virus adsorption but had no or minimal effects on the gIII$^-$ mutant (Fig. 6). Partial inhibition of the gIII$^-$ mutant occurred at higher concentrations of gIII. In contrast, purified gI and gIV inhibited both the wt viruses to comparable extents at all doses tested (Fig. 6). The observed differential inhibitory effects of gI, gIII, and gIV and the differential sensitivities of the gIII$^-$ mutant and the wt viruses indicate that the inhibition was specific to the glycoproteins and was not caused by potential detergent contamination of the glycoprotein preparations.

DISCUSSION

Using a BHV-1 gIII gene deletion mutant, we demonstrated that gIII function is not required for growth of virus in cell culture. However, some impaired functions were noted: defective attachment (Fig. 4), a delay in replication (Fig. 3), and lower titers of extracellular virus (Fig. 3). The BHV-1 gIII$^-$ virus defects resemble those reported for PRV gIII (25, 34) and HSV-1 gC (16) homologous gene deletions. These corresponding results demonstrate that the proteins encoded by the gC family of genes from different alphaherpesviruses share some functional properties.

The most important finding of this study was the identification of BHV-1 gI, gIII, and gIV as viral attachment proteins. gIII appears to play a predominant role, followed by gI and gIV. These conclusions were derived from three lines of evidence.

First, a deletion specific for the BHV-1 gIII gene substantially inhibited virus attachment. For example, deletion of the gIII gene reduced total virus attachment by as much as 93%. The defect in productive attachment was made evident by the slower adsorption rate of the gIII$^-$ virus (Fig. 4) and in particular by the induction of mutant virus adsorption following neomycin treatment (Fig. 5). The fivefold increase in gIII$^-$ virus adsorption caused by the drug indicated that a substantial functional proportion of the gIII$^-$ mutant virus population could not be measured by normal plaque assays because they were defective in adsorption and thus could not initiate productive infections. However, if they were induced to attach (e.g., by the addition of neomycin), they were then fully functional and able to cause infection, indicating that the major defect of the gIII$^-$ virus was restricted to attachment.

Second, the gIII mutant showed an enhanced sensitivity to gI and gIV antibody-mediated neutralization (Table 1). This increased sensitivity was primarily caused by antibodies specific to antigenic domains gI-IV and gIV-III (Table 2), which are involved in virus attachment (6, 23a). In contrast, domains such as gI-III, gI-V, and gIV-I, which participate in virus penetration (6, 8), were not the targets of enhanced antibody inactivation for the gIII$^-$ virus. This specificity of enhanced neutralization associated only with those antibodies which recognize attachment domains suggests that the gIII$^-$ mutant virus uses gI and gIV to compensate for its attachment defect.

Third, purified gI, gIII, or gIV was able to inhibit virus adsorption, as measured by plaque assays (Fig. 6). This suggested that the glycoproteins can bind on the surface of permissive cells and this binding is required for the adsorp-

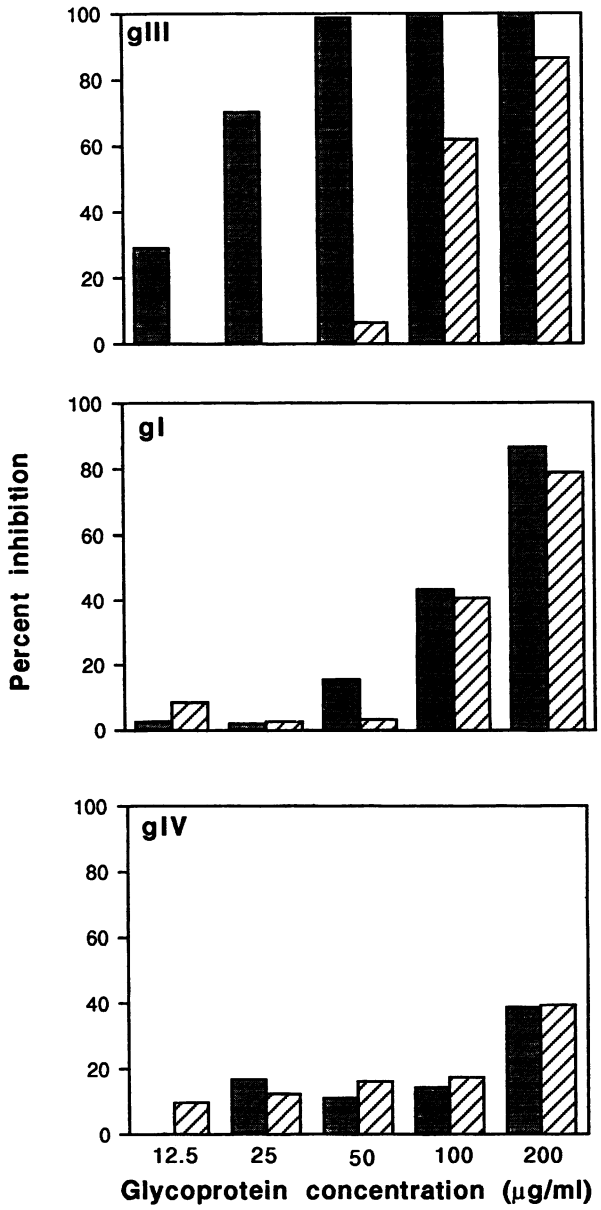

FIG. 6. Inhibition of virus adsorption by purified glycoproteins. Glycoproteins were diluted in MEM and cooled to 4°C. MDBK cells in six-well plates were cooled at 4°C for 1 h; 50 μl of each glycoprotein at the concentrations indicated was added and incubated at 4°C for 1 h. The virus was diluted in MEM to approximately 3,000 PFU/ml; 50 μl of the virus was then added to each well of cells in the presence of glycoproteins, followed by an additional 1 h of incubation at 4°C. After the incubation, the cells were washed three times with MEM and overlaid with agarose, and plaques were counted 4 days later. BSA at 200 μg/ml was used as an additional control in place of the glycoproteins. Mean numbers of plaques formed ± standard deviation for quadruplicate trials for the controls are: wt virus, 86.5 ± 3.2; wt virus with BSA, 87.5 ± 5.7; gIII$^-$ mutant, 95 ± 2.4; gIII$^-$ mutant with BSA, 77.5 ± 9.9. Data are presented as percent inhibition, where the number of plaques formed in the presence of BSA (200 μg/ml) was defined as 0% inhibition. Shaded bars, wt virus; hatched bars, gIII$^-$ mutant.

tion process. In this regard, purified gIII was the most effective glycoprotein in blocking virus adsorption to permissive cells. Addition of 12.5 μg of purified gIII per ml to MDBK cell cultures was clearly able to inhibit wt virus adsorption. In order to achieve the same degree of inhibition, gI and gIV were required at much higher concentrations (Fig. 6). Most significantly, gIII but not gI or gIV was able to completely block wt virus adsorption.

gI and gIV have previously been implicated in virus penetration (24). Although none of the present experiments could independently differentiate between virus attachment and penetration, we believe that taken together they indicate a role for gI and gIV in attachment. First, the MAbs used were directed at attachment domains rather than penetration domains. Second, one would expect that preincubation of cell monolayers with purified glycoproteins involved in attachment would saturate the binding sites of virus attachment. Subsequent incubation of these cells with virus should prevent attachment, and the virus would be removed after washing of the monolayer and not be available for development of plaques. If the glycoproteins were involved in preventing penetration and not attachment, one would expect that the attached virus would not be removed and eventually penetrate after glycoprotein receptor turnover occurred. Since this did not occur, we believe that these two experiments are highly suggestive of a role for gI and gIV in attachment.

Our finding that gI, gIII, and gIV are involved in the BHV-1 attachment is consistent with the reported results from HSV-1 studies involving antibody inhibition (7) and virosome binding (12), which suggested that gB, gC, and gD were involved in HSV-1 attachment. In addition, Kuhn et al. (13) recently demonstrated that HSV gB, gC, and gD complex with cellular components after initial virus adsorption. Formation of these virus-host cell complexes was blocked by the addition of antibodies to the glycoproteins.

Johnson et al. (9) recently reported that soluble HSV gD was able to inhibit virus infection, whereas soluble gB was ineffective. In our studies (Fig. 6), gI (a gB homolog) and gIII (a gC homolog) were much more effective in inhibiting virus attachment than gIV (a gD homolog). However, a major difference exists between our experimental conditions and those used in the HSV-1 studies. Our experiments were carried out at 4°C. Virus adsorbed at this temperature was fully susceptible to acid washes (e.g., with glycine-HCl buffer, pH 3.0). Therefore, in contrast to the HSV-1 study, our results may more likely reflect the initial viral attachment process than the subsequent penetration step. In fact, the report by Johnson et al. (9) showed that soluble gD inhibited penetration rather than attachment.

Neomycin restored, at least partially, the efficiency of gIII$^-$ virus attachment, while it had no effect on wt virus. The drug action was specific to the mutant virus and not to the virus-permissive cells. Therefore, it appeared that neomycin was somehow replacing the gIII function lost by the mutant virus. It must be noted that our data only suggest enhanced productive attachment (Fig. 5). The mechanism by which neomycin mediates this effect is under investigation.

The major paradox for the role of HSV-1 glycoproteins in virus attachment has come from observations that mutants lacking any one of the three glycoproteins were still capable of binding to permissive cells (3, 10, 16). This has often been used as evidence to reject the major glycoproteins as VAP candidates. However, since multiple glycoproteins are expressed by all herpesviruses, it is not unlikely that they each possess complementary mechanisms for attachment as a possible means of increasing virulence and broadening host range. The data from our present study support this notion.

The pattern of glycoprotein inhibition of virus adsorption is interesting and may suggest a potential mechanism for the virus attachment process. First, our data suggest that gI, gIII, and gIV recognize different binding sites on the surfaces of permissive cells. For example, purified gIII alone, but not gI or gIV, showed differential inhibitory effects on adsorption of the wt and gIII$^-$ viruses. gIII effectively blocked wt virus adsorption but was much less effective in altering adsorption of the mutant. If gI, gIII, and gIV were to occupy the same binding sites, addition of purified gIII should then inhibit the gIII deletion mutant to a greater extent than the wt virus, since in this case there would be fewer VAPs on the mutant virus (relative to the wt) competing for the same binding sites. We also observed that either gI or gIII alone was able to substantially inhibit wt virus absorption, and at high concentrations gIII also inhibited mutant virus adsorption to a limited extent. This last point suggests that occupying the gIII binding sites with purified protein affects the ability of a second glycoprotein to fulfill its attachment function. Such an explanation does not necessarily indicate antagonism between occupied binding sites. Instead, the interaction of different glycoproteins with their corresponding binding sites may be visualized as a sequential process. For example, the wt virus may use gIII to carry out the initial interactions with cellular receptors, which are then followed by interactions of gI and/or gIV with their receptors. This hypothesis may explain why purified gI and gIV inhibited both wt and gIII$^-$ virus attachment to a comparable extent. It is also consistent with the fact that the most extended projections present on the BHV-1 (23a) or HSV-1 (25) envelope are made up of gIII or gC molecules, respectively, and that gI and gIV are involved not only in attachment but in penetration as well.

In conclusion, we have been able to demonstrate that BHV-1 VAPs are composed of gI, gIII, and gIV, among which gIII is the predominant component. This finding should provide a basis for the identification of the BHV-1 attachment receptor(s) on permissive cells. From a practical point of view, the demonstration that a single glycoprotein, such as gIII, was capable of completely inhibiting virus attachment may also provide the basis for developing synthetic antagonists which prevent virus infection.

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