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Interaction of Herpes Simplex Virus Glycoprotein gC with Mammalian Cell Surface Molecules

RUTH TAL-SINGER,[1,2] CHARLINE PENG,[1,2] MANUEL PONCE DE LEON,[1,2] WILLIAM R. ABRAMS,[2,3] BRUCE W. BANFIELD,[4] FRANK TUFARO,[4] GARY H. COHEN,[1,2] AND ROSELYN J. EISENBERG[2,5]*

Department of Microbiology,[1] Center for Oral Health Research,[2] Department of Anatomy and Histology, School of Dental Medicine,[3] and Department of Pathobiology, School of Veterinary Medicine,[5] University of Pennsylvania, Philadelphia, and the Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada[4]

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The entry of herpes simplex virus (HSV) into mammalian cells is a multistep process beginning with an attachment step involving glycoproteins gC and gB. A second step requires the interaction of glycoprotein gD with a cell surface molecule. We explored the interaction between gC and the cell surface by using purified proteins in the absence of detergent. Truncated forms of gC and gD, gC1(457t), gC2(426t), and gD1(306t), lacking the transmembrane and carboxyl regions were expressed in the baculovirus system. We studied the ability of these proteins to bind to mammalian cells, to bind to immobilized heparin, to block HSV type 1 (HSV-1) attachment to cells, and to inhibit plaque formation by HSV-1. Each of these gC proteins bound to conformation-dependent monoclonal antibodies and to human complement component C3b, indicating that they maintained the same conformation of gC proteins expressed in mammalian cells. Biotinylated gC1(457t) and gC2(426t) each bind to several cell lines. Binding was inhibited by an excess of unlabeled gC but not by gD, indicating specificity. The attachment of gC to cells involves primarily heparan sulfate proteoglycans, since heparitinase treatment of cells reduced gC binding by 50% but had no effect on gD binding. Moreover, binding of gC to two heparan sulfate-deficient L-cell lines, gro2C and sog9, both of which are mostly resistant to HSV infection, was markedly reduced. Purified gD1(306t), however, bound equally well to the two mutant cell lines. In contrast, saturating amounts of gC1(457t) interfered with HSV-1 attachment to cells but failed to block plaque formation, suggesting a role for gC in attachment but not penetration. A mutant form of gC lacking residues 33 to 123, gC1(Δ33-123t), expressed in the baculovirus system, bound significantly less well to cells than did gC1(457t) and competed poorly with biotinylated gC1(457t) for binding. These results suggest that residues 33 to 123 are important for gC attachment to cells. In contrast, both the mutant and wild-type forms of gC bound to immobilized heparin, indicating that binding of these proteins to the cell surface involves more than a simple interaction with heparin. To determine that the contribution of the N-terminal region of gC is important for HSV attachment, we compared several properties of a mutant HSV-1 which contains gC lacking amino acids 33 to 123 to those of its parental virus, which contains full-length gC. The mutant bound less well to cells than the parental virus but exhibited normal growth properties. While we cannot rule out the possibility that other regions of gC contribute to its function in attachment, our studies show that the N terminus of gC is important for efficient attachment to cells.

The entry of herpes simplex virus (HSV) into host cells involves a cascade of interactions between several virion envelope components and host cell surface molecules (16, 31, 59). Three approaches have been used to show that gC mediates the initial interaction between virus and cells (86). First, antibodies directed against gC inhibit HSV adsorption to cells (33). Second, isolated HSV type 1 (HSV-1) gC can bind to cells (38, 90). Third, HSV mutants lacking gC adsorb to cells inefficiently (40, 77). In the absence of gC, gB mediates this step (39).

Other studies have implicated heparan sulfate moieties of cell surface proteoglycans as the receptors for gC. First, heparin inhibits the binding of HSV to cells (64, 98), and HSV attaches poorly to cells lacking heparan sulfate (35, 80, 98). Second, heparitinase treatment of cells significantly reduces virus binding (98). Third, gC and gB isolated from HSV-1-infected cells bind to immobilized heparin (40). No studies to date have examined the heparin binding properties of HSV-2 gC (gC2). However, there is evidence that HSV-2 attachment involves binding to a heparin-like molecule (80, 98). Several studies suggest that gC may also interact with a specific receptor other than HSPG, possibly CR1 (76, 77).

A subsequent step in virus entry is the interaction of gD with a second cell surface molecule (8, 49, 50, 54), possibly the mannose-6-phosphate receptor (5). Virus penetration occurs by pH-independent fusion of the virion envelope with the cell plasma membrane (32, 34, 97). At least four viral glycoproteins are required for viral penetration, designated gB, gD, gH, and gL (5, 7, 18, 27, 32, 46, 57, 71, 74, 77). However, the precise role of each glycoprotein in penetration is currently not defined.

The object of this study is to characterize of the role of gC in virus attachment by investigating the interaction of purified gC with components of the host cell surface. Three truncated forms of gC, namely, gC1(457t), gC2(426t), and a deletion mutant, gC1(Δ33-123t), were expressed in the baculovirus system. This system allows us to obtain large amounts of protein

* Corresponding author. Phone: (215) 898-6552. Fax: (215) 898-8385.

(81). The proteins lack the transmembrane region (TMR) and the carboxyl-terminal region, are secreted from infected insect cells, and are easily purified from supernatants by immunoaffinity chromatography. The lack of the TMR eliminates the need to use detergents to maintain the proteins in soluble form, thereby facilitating their use in in vitro assays with intact cells. We used these proteins in four types of experiments: binding to immobilized heparin, binding to cells by using an enzyme-linked immunosorbent assay (ELISA) for detection, blocking of HSV attachment to cells, and blocking of HSV plaque formation. For many of these experiments we used a soluble truncated form of gD, gD1(306t), as a control (81).

Our results show that purified gC1(457t) and gC2(426t) bind specifically to immobilized heparin and to the cell surface. In addition, gC1(457t) binds with reduced efficiency to two HSPG-deficient L-cell lines which are resistant to HSV infection (35). Saturating amounts of gC inhibited HSV-1 attachment to cells but failed to block plaque formation, suggesting that gC functions during HSV attachment but not during penetration.

A mutant form of gC, lacking amino acids 33 to 123 [gC1(Δ33-123t)], bound significantly less well to cells than did gC1(457t) and competed poorly with biotinylated gC1(457t) for binding. This suggests that residues 33 to 123 are important for gC attachment to heparan sulfate proteoglycans (HSPG). We used an HSV-1 mutant, vSH216, lacking the N-terminal region of gC to show that a region between residues 33 to 123 is important for efficient HSV-1 attachment to cells.

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MATERIALS AND METHODS

Cell culture and virus strains. Vero cells were propagated in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37°C. Mouse L cells (clone 1D line of Lmtk$^-$ murine fibroblasts) and the L-cell derivatives gro2C (35) and sog9 (3) were grown in DMEM supplemented with 10% FBS. BHK cells were grown in Eagle's minimum essential medium containing 5% FBS. HSV-1 strain NS, a low-passage clinical isolate (29), was propagated, and the titer in BHK cells was determined (22). HSV KOS gC$^-$39, a gC null virus (41); vSH214, which expresses wild-type gC; and vSH216, a virus mutant lacking amino acids 33 to 123 at the N-terminal end of gC (44), were propagated, and the titers in Vero cells were determined. Both vSH214 and vSH216 were derived from gC$^-$39.

Construction, production, and purification of baculovirus recombinants. Plasmids pCD14 (gC1) (79), pSH216 (gC1-Δ33-123) (44), and pSH141 (gC2) (45) were used as PCR templates to generate DNA fragments containing the gC gene as previously described (81). The 5′ primers used were GAAGATCTCGAAAC TGAAACTGCCTCCACC for gC1-derived constructs and ACGGGATCCCAA TGCCTCCCCC for the gC2 construct, and the 3′ primer used was GGGGTAC CTTAATGATGGTGGTGCTCTAGAACGGG for all constructs. These primers were designed to produce gC1 truncated at amino acid 457 and gC2 at amino acid 426, 18 amino acids prior to the hydrophobic TMR. In addition, these primers were designed to add three histidine residues to the two histidines already present at the carboxyl-terminal end of gC. The tail with five histidine residues provides a binding site for Ni plus 2-nitriloacetic acid agarose resin (Qiagen) for possible use in purification. The vector pVT-Bac (94) and the gC2 PCR fragment were digested with *Bam*HI and *Kpn*I. The gC1 PCR fragments were digested with *Bgl*II and *Kpn*I. Each fragment was ligated into pVT-Bac for 15 h at 15°C, by using T4 DNA Ligase (New England Biolabs). The ligated plasmids were used to transform *Escherichia coli* XL-1-Blue (Stratagene) competent cells. Plasmids from ampicillin-resistant colonies were screened by restriction enzyme analysis. Plasmids [pCP247 (gC1t), pCP248 (gC1-Δ33-123t), and pCP249 (gC2t)], each containing a truncated form of the gC gene, were recombined into baculovirus (*Autographa californica* nuclear polyhedrosis virus) by using Baculogold (Pharmingen) as the source of baculovirus DNA (81). Plaques were picked and amplified, and infected cell supernatants were screened for gC expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) by using polyclonal anti-gC1 serum as a probe. Baculovirus recombinants were subjected to two additional rounds of plaque purification, as previously described (81). The recombinant baculoviruses were designated bac-gC1(457t), bac-gC1(Δ33-123t), and bac-gC2(426t). The recombinant proteins were designated gC1(457t), gC1(Δ33-123t), and gC2(426t). The cloning and expression of bac-gD1(306t), which expresses truncated gD1, were described elsewhere (81).

Purification of gC1(457t), gC1(Δ33-123t), and gD1(306t). Truncated proteins were purified from culture supernatants of baculovirus-infected cells by immunoaffinity chromatography (22, 81). Briefly, Sf9 insect cells (Gibco-BRL) were grown in suspension in serum-free medium Sf900II (Gibco-BRL), infected with recombinant viruses at a multiplicity of infection of 4, and cultured for 72 additional h at 27°C in a New Brunswick Celligen Bioreactor. Cells were removed by centrifugation, and the clarified medium was passed over a 1C8 immunosorbent column [for gC1(457t) and gC1(Δ33-123t)] (22). A DL6 immunosorbent was used for gD1(306t) purification (22, 81). Each column was washed with 0.01 M Tris–0.5 M NaCl buffer (pH 7.5) and eluted with 0.1 M ethanolamine. The proteins were concentrated by using a PM10 membrane (Amicon) and dialyzed against phosphate-buffered saline (PBS). Purified proteins were analyzed by SDS-PAGE and visualized by Coomassie stain (81).

Purification of gC2(426t). We were unable to purify gC2(426t) by immunoaffinity chromatography because the protein bound irreversibly to monoclonal antibodies (MAbs). A similar problem was encountered with full-length gC2 (22). We were able to obtain small amounts of protein by using a polyclonal anti-gC (R46) immunosorbent. In preliminary small-scale purification experiments, we were able to use nickel-affinity chromatography for that purpose. However, we found heparin chromatography to be more efficient for large-scale purification. Clarified medium was passed over a heparin column (Pharmacia) and washed with PBS. The protein was eluted in 0.6 M NaCl, concentrated by using an Amicon PM10 membrane, and dialyzed against PBS.

Western blot analysis. SDS-PAGE under reducing conditions was performed in SDS–10 or 4 to 12% gradient (Schleicher & Schuell) polyacrylamide gels. Proteins were transferred to nitrocellulose, probed with either an anti-gC1 serum (R46) (22), an anti-gC2 serum (R81) (45), or an anti-gD1 serum (R7) (47). For antigenic analysis of gC, electrophoresis was carried out on native gels (14) and Western blots were probed with conformation-dependent MAbs against gC. We used gC1 MAb 1C8 or C15 (antigenic site I) (28, 45, 58) and MAb C13, C17, 27S, 31S, or 5S (antigenic site II) (58) to analyze gC1 constructs. For antigenic analysis of gC2, we used MAb MP1 or MP2 (antigenic site III) or MP5 (antigenic site IV) (19, 78). Blots were then probed with ^{125}I-protein A (ICN) (13).

Binding of purified gC to complement component C3. Purified C3 was prepared as described previously (23). The binding of purified gC to C3 was tested by ELISA (23, 44). Briefly, 50 μl of purified C3 (8 μg/ml) diluted in PBS was allowed to bind to wells of microtiter plates overnight at 4°C. Under these conditions of binding, C3 is converted to iC3 with binding properties similar to those of C3b (2). The wells were then saturated with 1% bovine serum albumin (BSA) (fraction V; Boehringer Mannheim) and 1% chicken ovalbumin (Sigma) in PBS (blocking buffer) for 30 min at room temperature (RT). gC proteins were serially diluted in blocking buffer, and 50 μl of each dilution was added to duplicate wells. After 1 h of incubation, plates were washed three times with 0.1% Tween 20 in PBS (washing buffer). Corresponding negative controls consisted of blocking buffer alone. To detect bound gC, polyclonal anti-gC1 (R46) or anti-gC2 (R81) antibodies were added to each well, followed by peroxidase-labeled protein A (Boehringer Mannheim). The plates were incubated for 30 min at RT, washed three times, and then washed with 20 mM citrate buffer (pH 4.5). A substrate of 2,2′-azino-di(3-ethylbenzthiozoline-6-sulfonic acid) (ABTS) (1.46 mmol liter^{-1}) in citrate buffer (pH 4) (Moss, Inc.) was added, and the reaction was allowed to proceed at RT until the optimal color change had been achieved, approximately 1 optical density unit in the darkest well. Absorbance was assessed with a Dynatech plate reader, using a 405-nm filter blanked against a negative-control well.

Binding of purified glycoproteins to immobilized heparin. Purified protein (250 μg) in PBS was applied to a 1-ml heparin column (Bio-Rad Affi-Prep matrix) and washed thoroughly with PBS. Fractions were eluted first with 2 mg of heparin (Sigma) per ml and then with a linear NaCl gradient (0.15 to 1.5 M). In other experiments, fractions were eluted with a linear NaCl gradient in the absence of heparin. Fractions were analyzed by immunodot blot (11). Equal volumes of each fraction were dotted onto nitrocellulose, washed with blocking buffer (13), and incubated with rabbit antiserum R46 (22), R81 (45), or R7 (47) and then with iodinated protein A (ICN Biochemicals). Blots were dried and exposed to X-ray film (Kodak XAR-5). The films were scanned with a densitometer (Molecular Dynamics), and each dot was integrated to determine the relative density for each fraction.

Biotinylation of purified glycoproteins. One milligram of each purified protein was incubated for 1 h at RT with a 50-fold molar excess of ε-caproylamidobiotin-*N*-hydroxysuccinimide ester (Gibco-BRL) and 0.05 M sodium carbonate (pH 9.0) in PBS. The reaction was stopped with 0.1 M NH_4Cl, and the protein was desalted on a Sephadex G-25 column (Pharmacia) and dialyzed against PBS. The number of accessible biotins per molecule of protein was determined by using an avidin–2-(4′-hydroxyazobenzene)benzoic acid solution, according to the manufacturer's instructions (Protein Biotinylation System; Gibco-BRL).

Cell fixation. A previous report indicated that pseudorabies virus (PRV) attaches to fixed cells as well as to untreated cells (52). We found that using fixed

cells in our ELISA experiments prevented cell loss and did not affect protein binding assays. Confluent cell monolayers in flat-bottom 96-well culture plates (Falcon) were fixed with 3% paraformaldehyde in PBS for 45 min. Unfixed cells were treated with trypsin and counted. Cells in the remaining wells were washed three times with washing buffer. To block nonspecific binding sites, cells were then incubated with blocking buffer for 30 min (32).

Binding of purified glycoproteins to cells. Proteins were serially diluted in blocking buffer, and 50 μl of each dilution was added, in duplicate, to fixed cells. We used three cell types for these experiments: L, Vero, and BHK. Corresponding negative controls consisted of blocking buffer alone. Plates were incubated at RT for 1 h and washed three times with washing buffer and once with peroxidase-conjugated avidin (Boehringer Mannheim) diluted in blocking buffer. The plates were incubated for 30 min at RT, washed three times with washing buffer, and washed once with 20 mM citrate buffer (pH 4.5) prior to the addition of the substrate. The binding of unlabeled gC to cells was determined by ELISA using polyclonal anti-gC1 serum (R46), followed by peroxidase-labeled protein A (Boehringer Mannheim) and substrate.

Competition ELISA. For competition assays, 3 μg of biotinylated gC or gD per ml was mixed with different amounts of proteins, or heparan sulfate, and then added to the cells as described above. The A_{405} obtained in the absence of competitor was considered to be 100% gC binding. The percentage of gC binding was calculated as follows: (A_{405} of gC binding in the presence of competitor/A_{405} of gC binding without competitor) × 100.

Enzyme digestion and analysis. Purified glycoproteins were digested with endoglycosidase F/*N*-glycosidase F (Endo F) or *O*-glycanase (Boehringer Mannheim), as previously described (83). Examination of enzyme-treated and untreated proteins by SDS-PAGE under reducing conditions was followed by silver stain with the use of the Pharmacia PhastSystem.

Enzymatic digestion of cells. Cells in 96-well plates were incubated with serial twofold dilutions of heparitinase (Sigma) in PBS for 1 h at 37°C. Following digestion, cells were washed twice with PBS and fixed with paraformaldehyde. To evaluate the effect of endoglycosidase treatment of fixed cells on the binding of gC, Endo F was added and the plates were incubated for 1 h at 37°C.

Virus labeling and purification. Purified virions were used for all experiments. Briefly, Vero cells were inoculated with vSH214, vSH216, or gC$^-$39 at 1 PFU per cell. After an adsorption period of 1 h, cells were overlayed with DMEM supplemented with 5% FBS and incubated at 37°C for 5 h. The medium was removed, and the cells were washed once in Hanks' balanced salt solution and then incubated with [^{35}S]methionine (1 mCi/ml) (ICN) for 40 min at 37°C. The cells were overlayed with medium and incubated at 37°C for 18 h. Extracellular virus was harvested and purified from the clarified medium by using a modification of several methods (15, 20, 85, 99). Briefly, virus was pelleted from clarified medium by centrifugation through a 5% sucrose cushion at 100,000 × *g* for 1 h. Pellets were resuspended in PBS, layered onto a stepwise sucrose gradient (10, 30, and 60% [wt/vol]), and centrifuged at 30,000 × *g* for 5 h. The light-scattering virus band at the 30 to 60% interface was collected, aliquoted, and stored at −80°C. Titers were determined by plaque assay on Vero cells and ranged from 10^8 to 10^9 PFU/ml. Radioactivity was determined by scintillation counting. The specific infectivity of each virion preparation was 0.02 cpm/PFU, or 20 VP5 units per PFU.

Characterization of [^{35}S]methionine-labeled virus. Purified virions were solubilized in disrupting buffer and boiled, and the proteins were electrophoresed on SDS–4 to 12% gradient polyacrylamide gels. Gels were fixed in 40% methanol and 7% acetic acid, incubated in Amplify (Amersham), dried, and exposed to XAR-5 film (Kodak). The relative amount of VP5 (major capsid) protein in each preparation of purified virions was quantitated by densitometry of the autoradiogram (40). Western blots of duplicate gels were probed with anti-gC1 serum R46 (22) to confirm the phenotype of each virus preparation.

Binding of radiolabeled virus to cells. The assays were modified from those described previously (35, 40, 98). Confluent monolayers of unfixed Vero, BHK, or L cells in 96-well plates were first incubated for 30 min at 4°C with PBS containing 1% BSA (PBS-BSA), in order to block nonspecific virus adsorption. The cells were then incubated with 2,000 VP5 units (approximately 100 PFU per cell) of radiolabeled virus in PBS-BSA for 1 h at 4°C with gentle rocking. The cells were washed three times with cold PBS and lysed with 1% SDS–1% Triton X-100 (lysis buffer), and the amount of radioactivity was determined by scintillation spectroscopy. The percentage of virus bound to cells was determined as follows: (cpm in cell lysate/cpm added) × 100.

For competition experiments, cells were incubated with serial twofold dilutions of a specific purified glycoprotein for 1.5 h at 4°C. Virus was then added for an additional 1.5 h at 4°C. The amount of virus bound was determined as described above.

One-step viral growth curve. The assay was performed as described previously (84). Briefly, Vero cells in 12-well plates were infected with vSH214, vSH216, or gC$^-$39 at a multiplicity of infection of 2 in DMEM containing 5% FBS. After 1 h, the virus inoculum was removed. The cells were washed, overlayed with medium, and incubated at 37°C. At various times thereafter, the medium containing extracellular virus was removed, and the intracellular virus was harvested. Virus titers were determined on Vero cells in an immunoperoxidase assay (42, 45, 63) using rabbit anti-gB serum (R69) (22).

Inhibition of HSV-1 plaque formation by purified glycoproteins. The HSV plaque inhibition assay was done essentially as described by Johnson et al. (49). Briefly, BHK cell monolayers in 48-well plates were treated with gD1(306t), gC1(457t), or BSA diluted in 5% DMEM for 1.5 h at 4°C. HSV-1 strain NS was added (50 PFU per well) for 1.5 h at 4°C. The cells were overlayed with 5% DMEM containing the competing proteins at the appropriate concentrations. After 21 h at 37°C, the medium was removed. The cells were fixed with methanol-acetone solution (2:1 ratio) for 15 min at −20°C and air dried. Virus titers were determined by an immunoperoxidase assay.

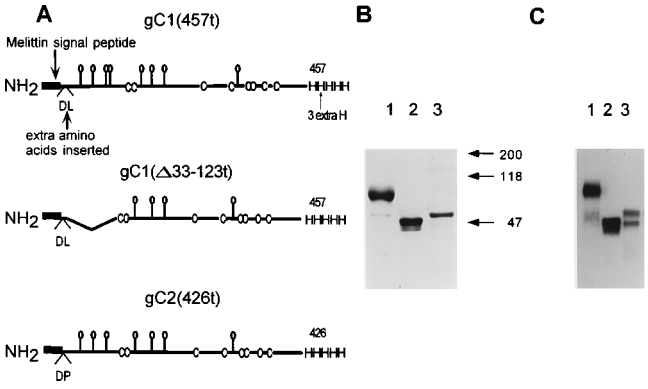

FIG. 1. gC constructs used in this study. (A) Schematic representation of truncated gC1(457t), gC1(Δ33-123t), and gC2(426t). Constructs were cloned into pVT-Bac and recombined into baculovirus as indicated in the text. In each case, the normal amino-terminal signal peptide of gC (30, 91) has been replaced with that of the honeybee melittin signal. As a result of cloning the genes into pVT-Bac, cleavage of the melittin signal leaves two additional amino acids on the N terminus of gC, aspartic acid (D) and leucine (L) in gC1 constructs, and aspartic acid and proline (P) in gC2, as shown in the diagram. The predicted sites for the addition of N-linked carbohydrates are shown as balloons. (B) Analysis of gC1(457t), gC2(426t), and gC1(Δ33-123t). Purified glycoproteins were electrophoresed on an SDS–10% polyacrylamide gel. The gel was stained with Coomassie brilliant blue. (C) Western blot analysis of purified truncated glycoproteins. Equal amounts of purified glycoproteins were electrophoresed on a 10% denaturing gel, transferred to nitrocellulose, probed with polyclonal antiserum directed against gC1 (R46) followed by ^{125}I-protein A. The sizes (in kilodaltons) of the glycoproteins are indicated next to the arrows. Lanes: 1, gC1(457t); 2, gC1(Δ33-123t); 3, gC2(426t).

RESULTS

Purification and characterization of gC1(457t), gC1(Δ33-123t), and gC2(426t). Our first goal was to obtain large quantities of purified glycoproteins. Previously, we described the expression and characterization of truncated HSV gD glycoproteins in the baculovirus-insect cell system (81). Truncated forms of gC1 and gC2 were expressed by using the baculovirus system (Fig. 1A). Each protein was secreted into the growth medium of infected insect cells and purified. Immunoaffinity chromatography proved to be most practical for purifying gC1(457t) and gC1(Δ33-123t). Heparin chromatography was used to purify gC2(426t). These purification protocols yielded 10 to 15 mg of purified protein per liter of infected insect cell medium. After purification, each protein exhibited a major Coomassie blue-stained band on SDS-polyacrylamide gels (Fig. 1B) and was detected by Western analysis with polyclonal anti-gC1 serum (Fig. 1C). Each protein reacted with conformation-dependent MAbs representing different antigenic sites on gC1 and gC2 (Table 1). These data indicate that baculovirus-expressed proteins are similar in conformation to full-length gC isolated from HSV-infected cells (12, 22, 28, 79).

gC1(457t), gC1(Δ33-123t), and gC2(426t) bind to C3. We previously showed that binding of gC1 and gC2 to human complement component C3b is highly dependent on gC conformation (44, 45, 78). Four separate regions of gC1 and three regions of gC2 are important for this activity. Thus, binding to C3b is a good indicator that gC retains its proper conformation. An ELISA was used to assess the binding of baculovirus-

TABLE 1. Antigenic properties of baculovirus-expressed gC[a]

Antibody	Site	Reaction with[b]:		
		gC1(457t)	gC1(Δ33-123t)	gC2(426t)
gC1-specific MAb				
1C8[b]	I	+	+	−
C15[c]	I	+	+	−
C13[c]	IIb	+	+	−
27S[c]	IIc	+	+	ND
31S[c]	IIc	+	+	ND
5S[c]	IIc	+	+	−
C17[c]	IIc	+	+	ND
gC2-specific MAb[d]				
MP1	III	−	−	+
MP2	III	−	−	+
MP5	IV	−	−	+
Polyclonal				
R46 (gC1)[e]		+	+	+
R81 (gC2)[b]		+	+	+

[a] 1C8, C13, C15, C17, R46, and R81 were analyzed by Western blot and ELISA; MP1, MP2, and MP5 were analyzed by Western blot and dot blot; and 27S, 31S, and 5S were analyzed by ELISA. −, no reaction; ND, not done.
[b] References 25 and 42.
[c] Groupings for gC1: site I (307 to 373) and site II (129 to 247) (55).
[d] References 17, 42, and 74.
[e] Reference 20.

expressed gC proteins to purified complement component C3, which had been fixed to microtiter wells. Under these conditions, the conformation of C3 is altered and resembles that of C3b (called iC3) (2). gC1(457t), gC1(Δ33-123t) (Fig. 2A), and gC2(426t) (Fig. 2B) bound iC3 in a dose-dependent manner. As shown for gC proteins expressed in mammalian cells (22, 44, 79), gC1(Δ33-123t) bound to iC3 better than did gC1(457t). Thus, the antigenic conformation and C3b binding properties of baculovirus expressed gC constructs are similar to those of the full-length proteins expressed in mammalian cells (44). We next utilized these proteins to study the direct interaction between gC and immobilized heparin.

gC1(457t), gC1(Δ33-123t), and gC2(426t) bind to immobilized heparin. The initial interaction between HSV and susceptible cells involves adsorption to HSPG (98). This interaction was mimicked by using a heparin-Sepharose column to show that full-length gB and gC, in detergent extracts obtained from HSV-infected cells, bind specifically to the column and are eluted with heparin (40). In preliminary experiments, we examined the binding of several immunoaffinity-purified full-length HSV glycoproteins (gC, gB, gD, and gH) to immobilized heparin, using various conditions for binding and elution. When the glycoproteins were added in 0.1% Nonidet P-40, they each bound efficiently to heparin by an ionic mechanism and each one eluted efficiently with salt. However, when the glycoproteins were added in the presence of higher concentrations of Nonidet P-40 (1%), as well as the anionic detergent sodium deoxycholate, only 10% of gC or gB, but little or no gD or gH, bound. The bound gC and gB were eluted with heparin in agreement with the findings of Herold et al. (40). We conclude that sodium deoxycholate was competing with the anionic heparin column for binding of the glycoproteins. If truncated forms of gC bind to heparin in the absence of detergents, it would eliminate any of the artifacts that complicated the analysis of full-length proteins.

All of gC1(457t) bound to the heparin column, and 95% of it eluted with soluble heparin (Fig. 3A). The small amount which was retained on the column eluted with salt. Similar profiles were obtained with gC2(426t) (Fig. 3B) and with the deletion mutant gC1(Δ33-123t) (Fig. 3C). In contrast, truncated gD did not bind to immobilized heparin since 95% of it eluted in the PBS wash (Fig. 3D, fractions 7 to 20). These results indicate that baculovirus-expressed gC1 and gC2 bind specifically to immobilized heparin, and that the N-terminal region of gC1 is not required for this interaction. It is possible that gC1(457t) and gC1(Δ33-123t) bound to the column with different affinities. To test this, we eluted the bound proteins using a linear salt gradient. No different in elution profiles was detected (data not shown). Next, we studied the interaction of these truncated proteins with the cell surface.

Specific binding of gC1(457t) to cells. Although several studies implicate gC in attachment (86), direct experiments characterizing the interaction of gC with cell surface molecules have been lacking. We developed an ELISA to examine the binding of purified gC to L, Vero, and BHK cells. Binding was performed on paraformaldehyde-fixed cells to minimize the effects of internalization and to prevent cell loss. We compared the binding of gC to fixed and unfixed cells and found no difference in binding (data not shown). Each of the three forms of truncated gC was biotinylated and then incubated with cells for 1 h at RT. For gC1(457t), the experiment was done with BHK, Vero, and L cells, with essentially the same results (Fig. 4A). The protein bound to cells in a dose-dependent manner with saturation occurring at 12.5 μg/ml (0.21 μM). gC2(426t) bound to BHK cells with similar kinetics and saturation at 12.5 μg/ml (0.24 μM) (Fig. 4B). In separate experiments, we obtained similar results for gC2(426t) using L and Vero cells (data not shown). Notably, the deletion mutant bound 25% as efficiently as gC1(457t) to all cell types (Fig. 4C).

A competition assay was used to determine whether binding of gC1(457t) to all three cell types is specific. A fixed amount of biotinylated gC1(457t) (3 μg/ml) was incubated with cells in the presence of various amounts of unlabeled gC1(457t) or gC2(426t). We used gD1(306t) protein as a control competitor. Binding of biotinylated gC1(457t) to BHK cells was inhibited up to 60% by unlabeled gC1(457t) or gC2(426t) (Fig. 5A). In contrast, binding of gC1(457t) to BHK, Vero, or L cells was not inhibited by 50 μg of gD1(306t) per ml (Fig. 5). In other experiments, concentrations of up to 100 μg of unlabeled gD1(306t) per ml had no effect on gC1(457t) binding, whereas similar concentrations of unlabeled gC1(457t) or gC2(426t) blocked binding by 90% (data not shown). This indicates that

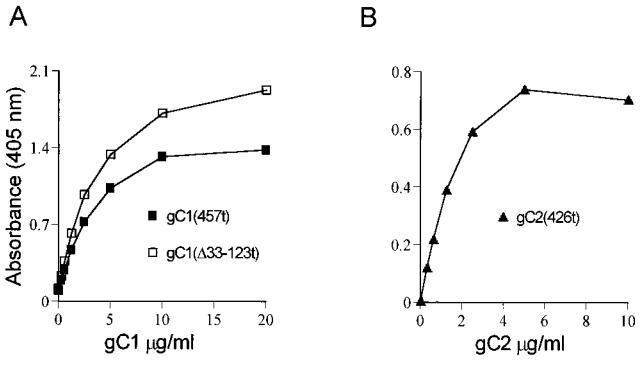

FIG. 2. Binding of purified gC proteins to purified C3. The proteins used were gC1(457t) and gC1(Δ33-123t) (A) and gC2(426t) (B). Fifty microliters of different dilutions of gC was applied to ELISA plates precoated with purified C3. The bound gC was detected with polyclonal antibodies against gC1 (R46) and gC2 (R81), followed by protein A conjugated with horseradish peroxidase and substrate.

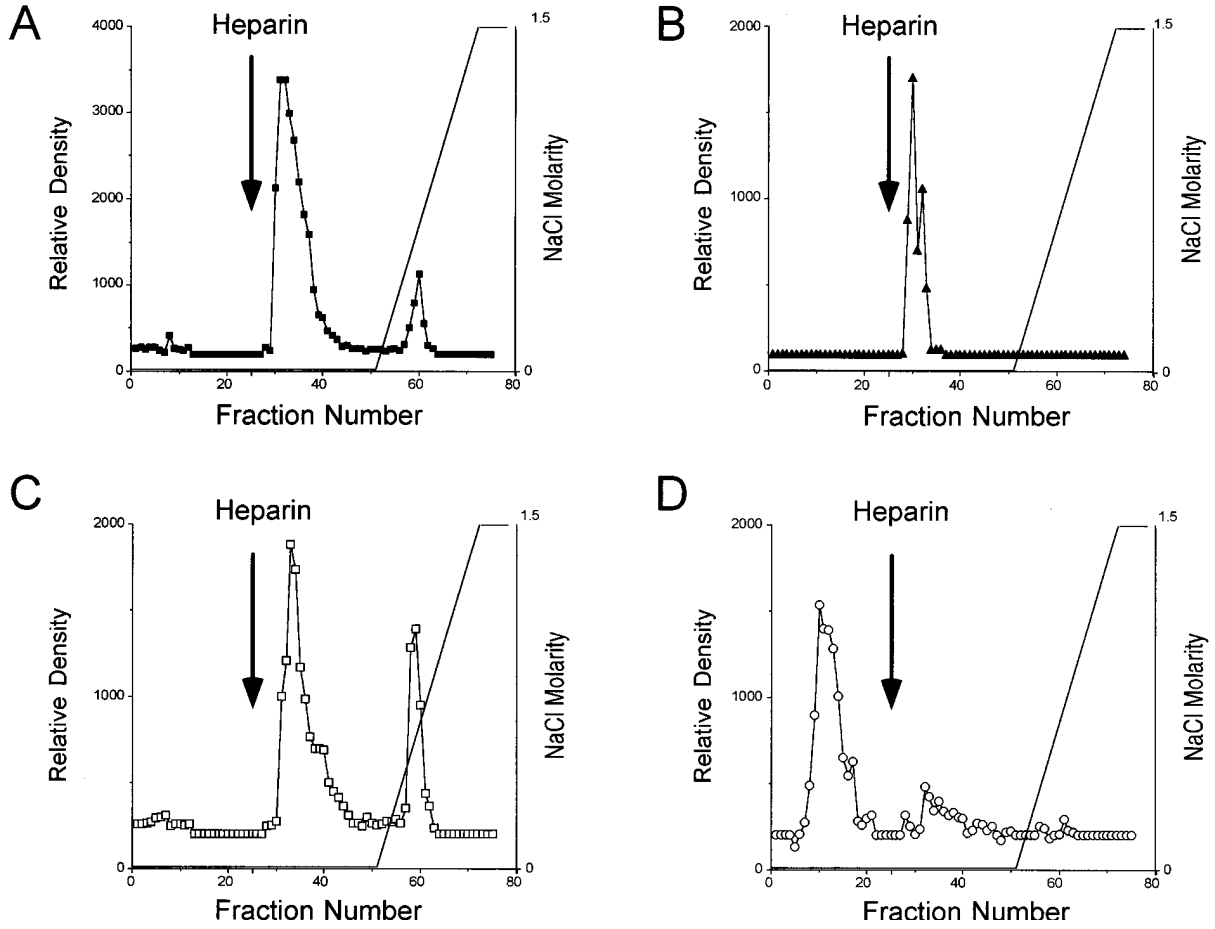

FIG. 3. Binding to heparin columns. Proteins (250 μg) were applied to a heparin column in PBS, and the column was washed with PBS (fractions 1 to 25). The column was then treated with 2 mg of heparin per ml in PBS. Fractions 25 to 45 represent the material eluted by heparin. A 0.15 to 1.5 M NaCl linear gradient was then used to elute bound proteins. Each fraction (50 μl) was spotted onto nitrocellulose and reacted with anti-gC or anti-gD antiserum, and then with ^{125}I-labeled protein A. The amount of label bound was determined by densitometry and expressed as the relative density per fraction. (A) gC1(457t); (B) gC2(426t); (C) gC1(Δ33-123t); (D) gD1(306t).

most of the binding we observe is specific. The deletion mutant gC1(Δ33-123t), however, did not block gC1(457t) binding to cells (Fig. 4C and 5B). These data suggest that the N terminus of gC is important for its interaction with the cell surface.

As another approach to determine the specificity of gC1(457t) binding to cells, we examined the binding of various amounts (0.01 to 5 μg/ml) of biotinylated gC to BHK cells in the presence of 100-fold excess of unlabeled gC1(457t) (Fig. 6)

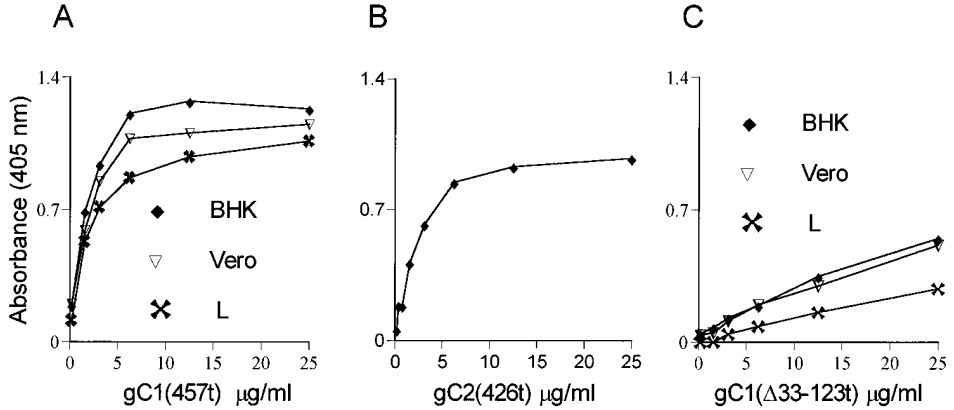

FIG. 4. Binding of biotinylated gC to cells. Biotinylated gC was serially diluted in blocking buffer, starting at a concentration of 25 μg/ml [or 0.43 μM gC1(457t)]. Biotinylated proteins were added to paraformaldehyde-fixed BHK, Vero, or L cells in 96-well plates and incubated for 1 h at RT. The cells were washed and incubated with peroxidase-labeled avidin followed by substrate. The amount of bound gC was determined by measuring the A_{405} in an ELISA plate reader. The results represent the averages of duplicate wells.

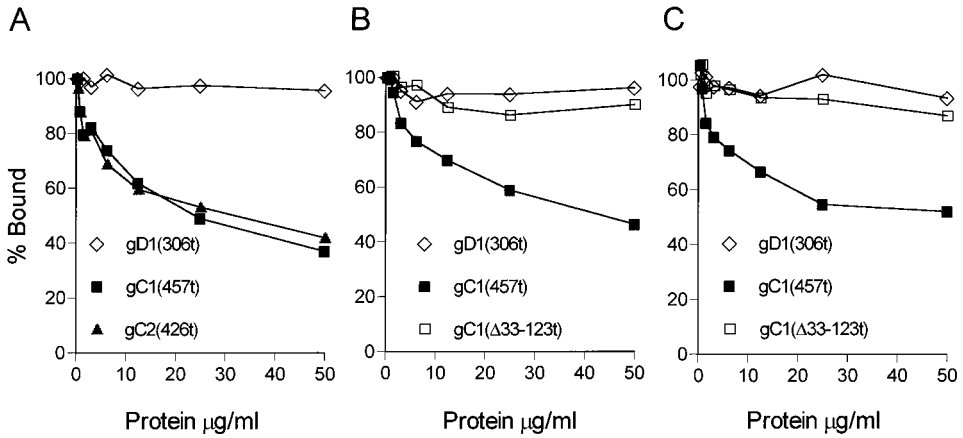

FIG. 5. Inhibition of gC1(457t) binding to cells. Biotinylated gC1(457t) at a concentration of 3 μg/ml was incubated for 1 h at RT in duplicate wells with fixed BHK (A), Vero (B), or L (C) cells in the presence of various amounts (0.2 to 50 μg/ml) of competitor protein: gD1(306t), gC1(457t), gC2(426t), or gC1(Δ33-123t). The cells were washed and incubated for 30 min at RT with peroxidase-labeled avidin followed by substrate. The percentage of gC binding was calculated as follows: (A_{405} of gC binding in the presence of competitor/A_{405} of gC binding without competitor) × 100. In this experiment, maximum absorbance (A_{405}) was 1.2.

(6). For example, 2 μg of biotinylated gC1(457t) per ml was mixed with 200 μg of unlabeled gC per ml, and 4 μg/ml was mixed with 400 μg/ml. Under these conditions, binding of biotinylated gC to BHK cells was completely inhibited (Fig. 6). These results provide further evidence that at low concentrations, gC binding is specific.

Purified gC binds to cell surface heparan sulfate. The conclusion that HSV interacts with HSPG at the cell surface has been based on two principal lines of evidence. First, up to 80% of HSV attachment is inhibited by soluble heparin or heparan sulfate (37, 64, 80, 98). Second, enzymatic treatment of cells with heparitinase reduces HSV plaque formation by up to 80% (98). We used the same methods to determine whether gC1(457t) binds to cell surface HSPG. First, cells were exposed to heparitinase, which selectively cleaves glycosidic linkages in HSPG. The binding of gC1(457t) to L cells was reduced by up to 50% (Fig. 7A). Similar results were obtained with Vero cells. In a separate experiment, the binding of gC1(Δ33-123t) to heparitinase-treated L cells was reduced by 10 to 20% (results not shown). However, since the initial level of binding was so low, it is difficult to extrapolate from this result. As a second approach, gC was added to L cells in the presence of various concentrations of soluble heparan sulfate (0 to 50 μg/ml). The binding of gC1(457t) and gC2(426t) was inhibited by up to 80% (Fig. 7B). As expected (49), heparitinase treatment of cells (Fig. 7A), or competition with heparan sulfate (Fig. 7B), had no effect on the binding of gD1(306t) to cells. Similar results were obtained with Vero, and BHK cells (data not shown). Taken together, these results indicate that gC binds to cell surface HSPG and corroborate earlier findings (9, 38, 40, 80, 98).

gC binds poorly to heparan sulfate-deficient cell mutants. Cell mutants defective in various aspects of glycosaminoglycan synthesis have been used to show that heparan sulfate at the cell surface is required for efficient HSV adsorption (35, 80). In this study, we examined the ability of gC to bind to two mutant L-cell lines which were selected for partial resistance to HSV-1 infection. gro2C cells are 85% resistant to HSV infection, lack heparan sulfate, but contain other proteoglycans (35). sog9 cells do not produce heparan sulfate, dermatan sulfate, or chondroitin sulfate and are only 1% susceptible to HSV infection (3). Biotinylated gC1(457t) bound with 55%-reduced efficiency to both gro2C and sog9 cells (Fig. 8A). These results

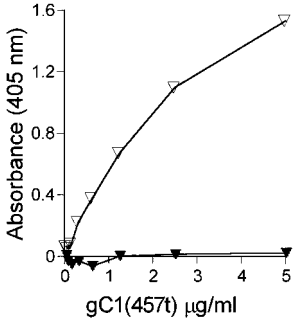

FIG. 6. Specific binding of gC1(457t) to cells. Biotinylated gC1(457t) was incubated for 1 h at RT with fixed BHK cells in the presence (▼) or absence (▽) of 100-fold excess of unlabeled gC1(457t). The cells were washed and incubated for 30 min at RT with peroxidase-labeled avidin followed by substrate.

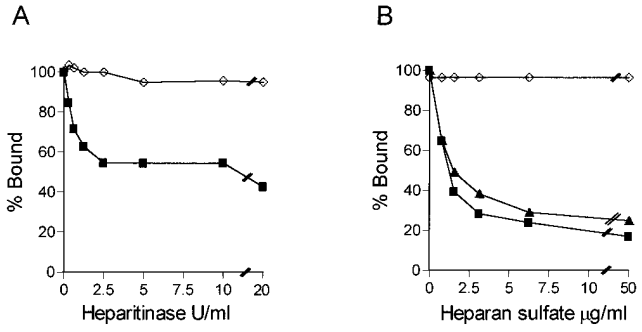

FIG. 7. Heparitinase treatment of cells or soluble heparan sulfate inhibit the binding of gC to cells. (A) L cells were treated with various amounts of heparitinase in PBS for 1 h at 37°C. As a control, cells were mock treated with PBS alone and incubated at 37°C. Cells were washed, fixed, and incubated with 3 μg of biotinylated gC1(457t) (■) or gD1(306t) (◇) per ml. After 1 h, cells were washed, and the addition of peroxidase-labeled avidin was followed by substrate. The maximum binding of gC (A_{405} of 0.8) was determined as 100% binding in untreated cells. (B) Biotinylated gC1(457t) (■), gC2(426t) (▲), and gD1(306t) (◇), each at a concentration of 3 μg/ml, were incubated for 1 h at RT with fixed L cells in the presence of various concentrations of heparan sulfate. Cells were washed and incubated with peroxidase-labeled avidin, followed by substrate. The maximum binding of each glycoprotein in the absence of heparan sulfate was considered to be 100%. The results reflect the averages of duplicate wells. The experiment was repeated twice with similar results.

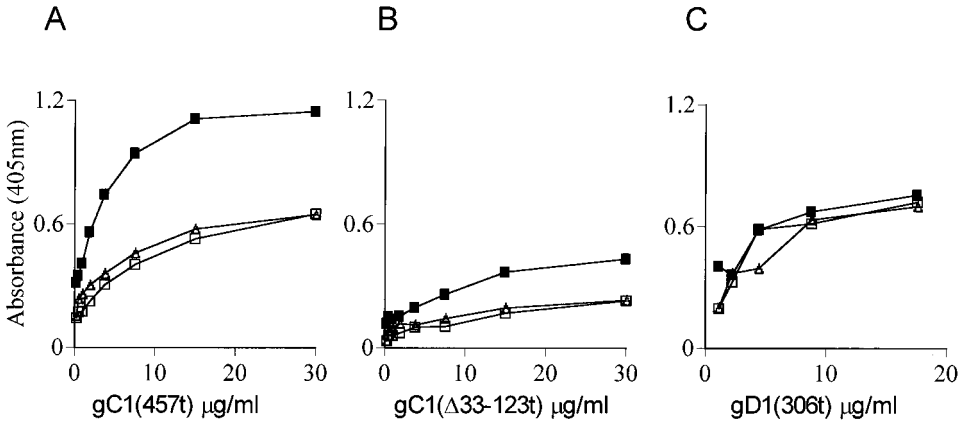

FIG. 8. Binding of gC to mutant L cells. Biotinylated gC1(457t) (A), gC1(Δ33-123t) (B), or gD1(306t) (C) at 3 μg/ml was incubated for 1 h at RT with L (■), gro2C (△), or sog9 (□) cells at a density of 10^5 cells per well. The cells were washed and incubated with peroxidase-labeled avidin, followed by substrate. The results represent the averages of duplicate wells. Similar results were obtained in seven separate experiments.

further suggest that the principal cell surface molecule that interacts with gC is heparan sulfate. The residual binding may be to a different molecule. The N-terminal deletion mutant gC1(Δ33-123t) bound poorly to all three cell types but reproducibly better to L cells than to gro2C or sog9 cells (Fig. 8B). Thus, some gC1(Δ33-123t) binding may also be to HSPG. These results suggest that the heparin binding domains in gC involve but are not limited to the N-terminal region.

As a control, we found that biotinylated gD1(306t) bound equally well to all three cell lines (Fig. 8C). This, and the fact that gD does not block the binding of gC to cells, indicates that gC and gD bind to two different molecules on the cell surface.

Role of carbohydrates in gC binding. What is it about the N terminus of gC1 that is important for binding to cells? The first 100 amino acids of gC1 and gC2 contain both N- and O-linked carbohydrates (N-CHO and O-CHO, respectively) (17, 66, 86). The deletion in gC1(Δ33-123t) removed the predicted O-CHO cluster, in addition to five N-CHO (Fig. 1). It has been shown that glycoproteins synthesized in baculovirus-infected cells contain N-CHO and O-CHO which are not modified to the same extent as their mammalian counterparts (95). To test whether carbohydrates contribute to the binding of gC1(457t) to cells, we treated the protein with glycosidases. As shown in Fig. 9A, both enzymes increased the mobility of gC1(457t), indicating that carbohydrates were removed by the enzymes. These results confirm the observation that gC-1 expressed in baculovirus-infected cells contains both N-CHO and O-CHO (72).

O-glycanase treatment of gC1(457t) had no effect on its binding to BHK cells (Fig. 9B). However, removal of N-CHO with Endo F reduced gC1(457t) binding by 40 to 60%. In this experiment, gC1(457t) binding was measured indirectly by using an anti-gC1 antibody. Similar results were obtained by using biotinylated gC1(457t) (data not shown). Endo F treatment also reduced gC1(457t) binding to L cells (results not shown). Pretreatment of cells with Endo F had no effect on subsequent gC1(457t) binding (Fig. 9C). These results indicate that N-CHO in gC is involved in binding of the glycoprotein to cells, either directly or indirectly by altering gC structural conformation (67, 82, 96).

Attachment of radiolabeled HSV-1 mutants to cells. Previous studies have shown that HSV mutants which lack gC in the virion envelope bind to cells with approximately 60%-reduced efficiency (39, 40). Here, we have shown that the N-terminal region of soluble gC is important for efficient binding of the purified proteins to cells. We therefore investigated the possibility that the N-terminal region of HSV gC is important for virion adsorption to cells. To do this, we prepared purified [^{35}S]methionine-labeled virions obtained from supernatant fluid of cells infected with three different HSV-1 strains: gC$^-$39 (a strain lacking the gC gene) (41), vSH214 (a strain in which wild-type gC1 was recombined back into gC$^-$39), or vSH216 (a virus mutant in which gC lacking amino acids 33 to 123 was recombined into gC$^-$39) (44). Strains vSH214 and vSH216 were originally developed in order to investigate the interaction of gC with C3b and the alternative complement pathway (44).

We first standardized the relative number of virion particles by examining the amount of VP5 (the major capsid protein) in each purified virus preparation (Fig. 10A). Densitometry indicated that similar amounts of radiolabeled VP5 were present in

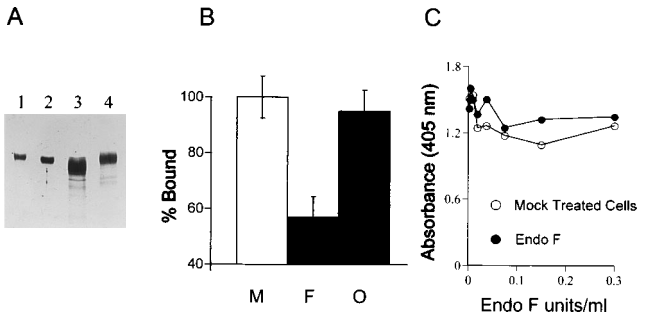

FIG. 9. Effect of carbohydrate-modifying enzymes on binding of gC1(457t) to cells. Purified gC1(457t) (10 μg) was treated with Endo F or *O*-glycanase or left untreated for 2.5 h at 37°C. (A) Samples were then electrophoresed under denaturing conditions on an SDS–12.5% polyacrylamide gel and silver-stained. Lanes: 1, mock-treated sample; 2, sample treated with *O*-glycanase; 3, sample treated with Endo-F; 4, mock-treated sample. (B) Different amounts (0.15 to 20 μg/ml in duplicate wells) of mock (M)-, Endo F (F)-, or *O*-glycanase (O)-digested gC1(457t) were incubated with BHK cells for 1 h at RT. Cells were washed and incubated with a polyclonal antibody against gC-1 (R46) followed by protein A conjugated with horseradish peroxidase and substrate. gC bound to the cells was detected by measuring the A_{405} in an ELISA plate reader. The results represent the averages of 16 wells; error bars indicate standard errors. Similar results were obtained with Vero and L cells. (C) Fixed BHK cells were treated with various amounts of Endo F. The cells were washed and incubated with 10 μg of untreated gC1(457t) per ml for 1 h at RT. In this experiment, gC binding was detected by using polyclonal anti-gC1 serum, followed by protein A conjugated with horseradish peroxidase and substrate. Similar results were obtained by using biotinylated gC1(457t).

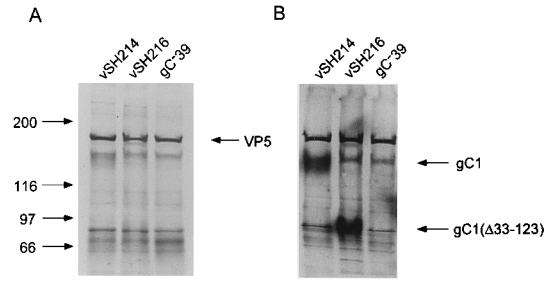

FIG. 10. Characterization of [^{35}S]methionine-labeled virions of vSH214, vSH216, and gC$^-$39. Labeled virions were purified from the extracellular fluid of HSV-infected Vero cells. Samples of each virion preparation were boiled in disrupting buffer and electrophoresed on SDS–4 to 12% polyacrylamide gels. (A) Gel was fixed with 40% methanol and 7% acetic acid, incubated in Amplify, dried, and exposed to film. The density of VP5 bands was quantitated by densitometry. The numbers obtained were defined as VP5 units. (B) The presence of gC-1 in purified ^{35}S-labeled virions was determined by Western blot analysis. Blots were probed with polyclonal anti gC-1 serum, followed by incubation with ^{125}I-labeled protein A. The metabolically labeled protein bands are the same as those in panel A. The additional bands correspond to gC1. The expected positions of gC1 and gC1(Δ33-123) on a gradient gel are indicated on the right.

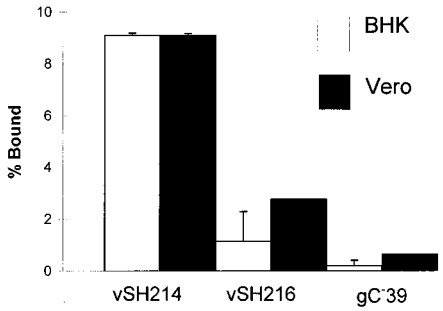

FIG. 11. Attachment of purified vSH214, vSH216, and gC$^-$39 to BHK or Vero cells. Radiolabeled virus (2,000 VP5 units, ~100 PFU per cell) was added to confluent monolayers of BHK or Vero cells in 96-well plates for 1 h at 4°C. Unbound virus was removed, and the cells were washed with cold PBS and treated with lysis buffer. The radioactivity in cell lysates was determined by scintillation analysis. The percentage bound virus was determined as follows: (cpm in cell lysate/cpm added) × 100. Each bar represents the mean of quadruplicate wells; error bars indicate standard errors. Similar results were obtained in six separate experiments. The specific infectivity of each strain was similar (0.02 cpm/PFU, or 20 VP5 units per PFU).

similar numbers of PFU (20 VP5 units per PFU) (Fig. 10B). It was previously shown that this method yields results similar to the particle count results obtained by electron microscopy (40).

We used Western blot analysis with anti-gC1 serum to confirm that each virus preparation used for adsorption studies had the proper gC phenotype. Purified [^{35}S]methionine-labeled virions were solubilized, electrophoresed on SDS-denaturing gels, transferred to nitrocellulose, and probed with anti-gC1 serum. The amount of virus was adjusted to yield the same amount of VP5 per lane (Fig. 10B). Full-length gC1 was found in vSH214, and truncated gC1(Δ33-123) was found in vSH216 (Fig. 10B). As expected, gC was not observed in the gC$^-$39 preparation. This confirms that the gC$^-$39 virus is a true gC null mutant (41, 44).

We found that the counts per minute/PFU or VP5/PFU ratios of the virus preparations on Vero cells were similar, indicating that these preparations had similar specific infectivities. This was different from the finding reported by Herold et al. (40), who found reduced specific infectivity of a mutant virus gC$^-$3 which produces a truncated secreted form of gC. It is possible that the discrepancy in our results is due to differences in virus strains.

To quantitate the adsorption of virus to cells, we incubated monolayers of unfixed BHK or Vero cells with 2,000 VP5 units of radiolabeled virus (100 PFU per cell). Cells were incubated with virus for 90 min at 4°C, washed to remove unbound virus, and lysed. The amount of [^{35}S]methionine which bound to the cells was considered to be directly proportional to the amount of bound virus (Fig. 11). Approximately 10% of input wild-type virus vSH214 (10^4 cpm) bound to both BHK and Vero cells. In contrast, 3% of vSH216 and 1 to 2% of gC$^-$39 bound. Similar results were obtained by using fixed cells (results not shown). Thus, the deletion in gC1(Δ33-123) caused a 66% reduction in attachment efficiency. The results with gC$^-$39 are similar to those reported by Herold et al. (40), using a different gC-negative mutant. We conclude that the N-terminal region of gC is important for HSV-1 attachment to cells.

gC inhibits HSV-1 attachment to cells. We next investigated whether soluble forms of gC could inhibit virus adsorption. This was done to determine whether the baculovirus-expressed glycoproteins bind to the same cellular receptors as the glycoproteins in the virions. Monolayers of unfixed Vero or BHK cells were incubated with serial dilutions of baculovirus-derived gC1(457t), gC1(Δ33-123t), or gD1(306t) for 90 min at 4°C. Wild-type virus vSH214 was added for a further 90 min at 4°C. The glycoproteins and unbound virus were removed, the cells were lysed for quantitation of bound virus, and samples were analyzed for ^{35}S by liquid scintillation spectroscopy.

As expected (49), gD1(306t) did not inhibit virus attachment to BHK or Vero cells even when the glycoprotein was present at high concentrations (Fig. 12A). However, gC1(457t) blocked virus attachment to BHK cells by as much as 95% (Fig. 12A) but was somewhat less efficient at inhibiting virus attachment to Vero cells (70%) (Fig. 12B). gC2(426t) also blocked HSV-1 attachment to Vero cells (Fig. 10B). In contrast, gC1(Δ33-123t) did not inhibit viral attachment to Vero cells (Fig. 12B).

These results provide direct evidence that the interaction of soluble gC with the cell blocks a step in HSV-1 attachment. Thus, the data indicate that truncated gC binds to the same receptor as HSV-1 gC. Second, the observation that gC2 blocks HSV-1 attachment suggests that gC1 and gC2 bind to the same or to similar receptors. Third, the failure of the deletion mu-

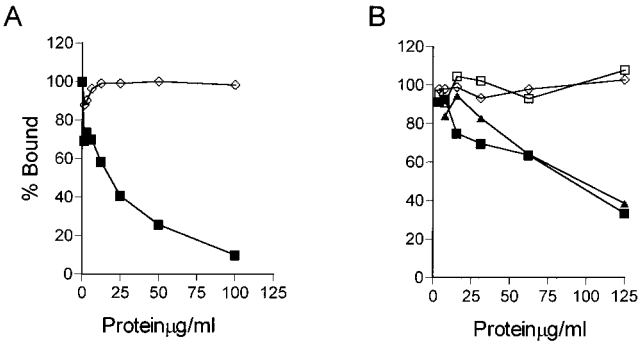

FIG. 12. Effect of purified glycoproteins on vSH214 adsorption to BHK or Vero cells. Monolayers of BHK (A) or Vero (B) cells in 96-well plates were incubated with different amounts of purified glycoproteins: gC1(457t) (■), gD1(306t) (◇), gC1(Δ33-123t) (□), or gC2(426t) (▲) for 90 min at 4°C. Two thousand VP5 units of vSH214 virus were added to each monolayer and the virus-cell mixture was incubated for 90 min at 4°C. Unbound virus was removed, and cells were washed, and treated with lysis buffer. Cell lysate from each well was placed in scintillation vials, and radioactivity was determined. 100% binding was defined as cpm obtained in the absence of competitor (11,000 cpm). Similar results were obtained in four separate experiments.

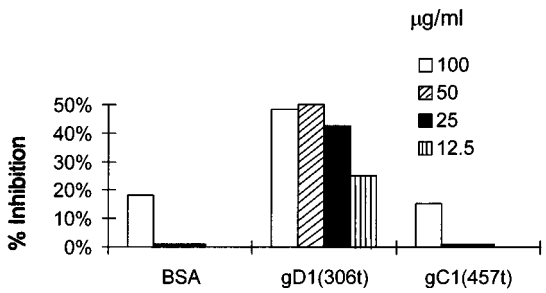

FIG. 13. Soluble gD1(306t) but not gC1(457t) inhibits HSV-1 plaque production. BHK cells growing in 48-well dishes were treated with either gD1(306t), gC1(457t), or BSA at increasing concentrations of 12.5 to 100 μg/ml for 90 min at 4°C. Virus at 50 PFU per well was added and allowed to adsorb for 90 min at 4°C followed by an overlay of 5% FBS in DMEM containing either gD1(306t), gC1(457t), or BSA at the indicated concentrations. Plates were incubated at 37°C for 21 h. The number of plaques was determined by black plaque assay. Percent inhibition was determined as follows: (number of plaques in the presence of protein/number of plaques in the absence of protein) × 100. Results represent the averages of duplicate wells.

tant to block attachment is further evidence that the N terminus of virion gC is important for HSV attachment.

gC does not inhibit HSV plaque production. To determine whether gC blocks HSV plaque formation, we used the plaque inhibition assay described by Johnson et al. (49). BHK monolayers were incubated with soluble gC1(457t), gD1(306t), or BSA. Virus was added, and an overlay containing the proteins at the designated concentrations was then added (Fig. 13). As reported for a truncated form of gD expressed in mammalian cells (31, 49), gD1(306t) inhibited plaque formation by as much as 50% at 50 μg/ml and by 25% at concentrations as low as 12.5 μg/ml. In contrast, gC1(457t) was not inhibitory at concentrations of up to 50 μg/ml. At 100 μg/ml, gC was inhibitory, but at these concentrations BSA also inhibited plaque formation. Similar data were obtained when the assay was done with Vero cells. We conclude that soluble gC has no detectable effect on viral penetration or cell-to-cell spread. In addition, our results indicate that baculovirus-expressed gD is functionally similar to truncated gD expressed in mammalian cells (31, 49).

One-step growth curve of HSV-1 vSH214, vSH216, and gC$^-$39. Does the impaired ability of vSH216 to attach to cells affect its capacity to replicate in those cells? We compared the growth of vSH214, vSH216, and gC$^-$39, using a one-step growth curve. In one experiment (Fig. 14A), there was a 1-log difference in the amount of intracellular vSH216 at 24 h postinfection, although the amounts of extracellular virus were similar at this time (Fig. 14B). However, in a separate experiment (Fig. 14C), there were no differences in the amount of intracellular virus. Therefore, we conclude that all three virus strains had the same one-step growth kinetics, and there were no differences in either the rate or the amount of virus produced in Vero cells. Taken together, the results of this study show that gC1 and gC2 enhance the ability of HSV to attach to cells by interacting with HSPG. Binding of gC to cells is largely associated with the N-terminal region of the glycoprotein. However, as shown by many other studies, gC is not essential for the viral life cycle in cell culture.

DISCUSSION

Previous studies have demonstrated that initial attachment of several alphaherpesviruses is mediated primarily by the binding of gC to HSPG at the cell surface. These results were obtained in part by using null viruses lacking gC (39, 52, 55, 62, 65, 86). Our object was to extend these studies and to advance the understanding of the role of gC in HSV attachment. To do this, we chose to examine the interaction of purified HSV gC with mammalian cell surface molecules. We produced large quantities of conformationally correct truncated forms of gC, gC1(457t), gC2(426t), and gC1(Δ33-123t), in the baculovirus system. Each protein lacks the TMR and carboxy terminal region, thus permitting us to carry out binding and functional studies in the absence of detergent. We used these proteins in several functional assays: (i) binding to complement component C3, (ii) binding to immobilized heparin, (iii) binding to uninfected cells, (iv) blocking of HSV attachment to cells, and (v) blocking of HSV infection. The second and third assays appear to be good ways to map the regions on gC which may be necessary for attachment. However, we found that the use of immobilized heparin does not distinguish differences in mu-

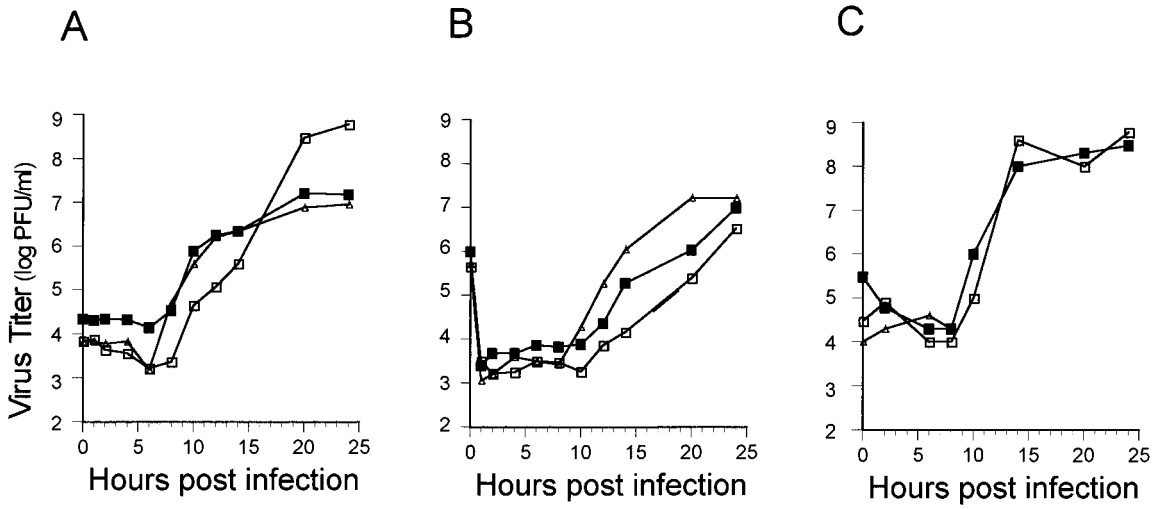

FIG. 14. One-step viral growth curve of vSH214, vSH216, and gC$^-$39 in Vero cells. Vero cells were infected with vSH214 (■), vSH216 (□), or gC$^-$39 (△) at a multiplicity of infection of 2 PFU per cell. At various times postinfection, the supernatant was removed and the intracellular virus was collected by scraping the cells into medium containing 5% FBS. Intracellular virus was released by a freeze-thaw cycle followed by sonication. Virus titrations were done on Vero cell monolayers in duplicate. (A) Intracellular virus; (B) extracellular virus. (C) The experiment was repeated, and intracellular virus titers were determined.

tant and wild-type forms of gC that were readily apparent by other means. Thus, the N-terminal deletion mutant gC1(Δ33-123t), which bound inefficiently to cells and did not block the binding of gC1(457t) to cells, was able to bind efficiently to immobilized heparin. It therefore appears that the use of heparin columns may not offer a good model system for testing the biological properties of mutant forms of gC and predicting their interaction with the cell surface.

gC1(457t) and gC2(426t) bind specifically to cells. A previous study indicated that truncated gC-1 binds to glutaraldehyde-fixed Green monkey kidney cells (90), but this interaction has not been fully characterized. We found that truncated gC1 and gC2 bind to several cell types, namely, L, Vero and BHK, in a dose responsive and saturable manner. The interaction is specific since labeled gC1(457t) is inhibited by itself and by gC2(426t) (60%) but not by gD1(306t). Furthermore, the histidine tail on the truncated forms of gC does not appear to contribute to its binding since gD1(306t) also contains six histidine residues at the carboxyl terminus. Our competition data also suggest that virion-associated gC1 and gC2 use the same receptor. WuDunn and Spear (98) reported that radiolabeled attachment of purified HSV-1 virions to cells can be inhibited up to 60% by unlabeled HSV-1 or HSV-2 virions. The two sets of studies suggest that gC of both serotypes makes initial contact with the same cell surface molecule. Both gC1(457t) and gC2(426t) bind saturably to BHK cells at 0.2 μM (12.5 μg of gC1 per ml). It has been reported that the dissociation constant may be determined as the concentration of ligand at half saturation (93). On this basis, we estimate the dissociation constant for both gC1(457t) and gC2(426t) to be approximately 10^{-7} M.

Truncated gC binds to cell surface HSPG. We demonstrated that truncated gC binds specifically to HSPG, employing three methods used previously to study the interaction of HSV virions with the cell surface. First, HSV attachment can be inhibited by soluble heparin or heparan sulfate (35, 37, 64, 69, 80, 98). We found that binding of gC1(457t) to cells was blocked by heparan sulfate. Second, removal of heparan sulfate from cells with enzymes reduces HSV binding by up to 80% (98). We found that heparitinase treatment of cells reduced gC1(457t) binding by 50%. Third, the binding of HSV to heparan sulfate-deficient cells is severely impaired (35, 80). We found that gC1(457t) bound with markedly reduced efficiency to two heparan sulfate-deficient cell lines, gro2C and sog9. These three types of experiments show that much of gC binding to cells is via HSPG. We did not note any difference in the relative binding of gC1(457t) to the two mutant cell lines, even though sog9 cells are 10-fold more resistant to HSV infection than gro2C cells. This suggests two possibilities: (i) that virion components other than gC, perhaps gB, are responsible for the difference in infectivity (39) and (ii) that the residual binding of gC to the two mutant cell lines may involve molecules other than heparan sulfate, chondroitin sulfate, or dermatan sulfate (3, 35). It appears that the absence of heparan sulfate on cells or the presence of heparan sulfate as a competitor has a more profound effect on virion binding (35, 80, 98) than on the gC binding that we observe. It is possible that a heparin binding domain on gC is not accessible when the protein is present in the intact virion but becomes exposed in the isolated protein. In other studies, it was shown that wild-type HSV-1 attaches to either surface of polarized MDCK cells, whereas a gC null virus attaches only to the basolateral surface (77). These investigators concluded that since HSPG is concentrated on the basolateral surface, gC may interact with a non-HSPG receptor on the apical surface. A similar receptor may be present in the HSPG-deficient L-cell lines, thus explaining the residual binding by gC1(457t).

The N terminus of gC is involved in gC and HSV attachment to cells. The reduced ability of gC1(Δ33-123t) to bind to cells predicts that a virus containing this mutation should also attach poorly to cells. We tested this prediction, using the virus vSH216 in which the gC gene contains the same N-terminal deletion. We found that vSH216 attaches as poorly to BHK or Vero cells as a gC null mutant virus, gC$^-$39. It is unlikely that a general conformational defect in the mutated gC accounts for these results since the antigenic conformation and C3b binding properties of gC1(Δ33-123t) appear to be unaffected by the deletion. On the basis of sequence analysis, the N-terminal region of gC is the least conserved portion of the protein among gC homologs (1, 4, 24, 30, 53, 70). However, a common feature of this region of gC among all of the homologs is a cluster of basic residues which spans amino acids 90 to 147 in HSV-1 gC (30) and 75-111 in PRV gC (70). The first 100 amino acids of HSV-1 gC include seven lysines and nine arginines; in HSV-2 gC there are 6 lysines and 13 arginines (91). This basic region of gC has been proposed as a good candidate to be a heparin binding domain or HBD (25, 86, 88, 96).

Several studies have attempted to map the portion of gC which is involved in virus attachment by several herpesviruses. It has been noted that there are several heparin binding consensus sequences (HBDs) in different regions of gC of PRV, bovine herpesvirus, and HSV (10, 25, 26). Synthetic peptides corresponding to some of these putative HBDs bind to immobilized heparin (56, 75, 96). Deletion mutants were used to map the principal attachment domain in PRV gC to the first third of the protein, which contains three of seven possible HBDs (25, 26, 56). Heparin binding sites have also been mapped to the N-terminal half of bovine herpesvirus 1 gC (65). Several HSV-1 gC MAb-resistant (*mar*) mutants that are defective in attachment to BHK cells have been mapped to antigenic site II (amino acids 129 to 247). Our studies indicate that residues 33 to 123 of HSV-1 gC may include an important attachment domain. Moreover, the limited binding of gC1(Δ33-123t) to L cells was reduced even further with *gro*2C and *sog*9 cells, suggesting that other regions of gC may contain HBDs. Thus, the two sets of results for HSV-1 suggest that there may be two HBDs in the N-terminal region of the protein, in agreement with the data for PRV gC (25, 26).

In addition to the high number of basic residues at the N terminus, this region of gC has an unusual amino acid composition, in that more than 75% of the amino acids are proline, serine, threonine, and glycine. Moreover, most of the O-CHO are clustered in this region (17, 66, 68), and it is therefore possible that the N terminus of gC has a mucin-like structure. Mucins are characteristically rich in amino acids such as threonine, serine, alanine, glycine, and proline, and often the threonines and serines are modified with O-CHO. One major function of such clustered O-linked glycans is to force these proteins into an extended rod or fiber-like conformation (48). Electron microscopy studies suggest that gC in the virion envelope has an unusual thread or rod-like structure (89). Thus, the N-terminal attachment domain of gC may have an unusual mucin-like structure that aids in its interaction with HSPG.

N-CHO may play a role in gC attachment to cells. We wanted to determine whether the reduced binding of gC1(Δ33-123t) to cells could be accounted for by the loss of carbohydrates caused by the deletion. All of the O-CHO have been mapped to the N terminus of gC (17), and we have confirmed that gC1(457t) but not gC1(Δ33-123t) contains a significant amount of O-CHO (73). *O*-glycanase treatment of gC1(457t)

removed O-CHO, as judged by SDS-PAGE, but had no effect on its binding to cells. These results suggest that O-CHO do not play a role in gC attachment. However, treatment of gC1(457t) with Endo F had a significant effect on attachment, reducing binding by 60%. It is known that N-CHO help to maintain the antigenic stability of gC (66) and that the removal of N-CHO affects the conformation of antigenic site II (82). In preliminary experiments, we found that Endo F treatment of gC1(457t) had some effect on binding of MAb 1C8 (antigenic site I) and C13 (antigenic site II). However, we found that the conformation of antigenic sites I and II was unaffected by deletion of residues 33 to 123, even though five sites for the addition of N-CHO were removed by the deletion. Thus, the negative effects of Endo F on gC1(457t) binding to cells may be due to removal of N-CHO outside this domain. Alternatively, removal of N-CHO may adversely affect the overall conformation of the protein.

gC may have distinct functional domains. In previous studies, we showed there are four distinct C3b binding regions in gC1 located within the central portions of the molecules (residues 123 to 367 of gC1) (44, 45). Receptors for C3b and iC3b were also localized within the central portion of gC2 (45, 78, 92). The ability of gC1 to interfere with properdin binding to C3 was mapped to the N terminus of gC1 (44). Interestingly, the same region that is important for blocking of properdin binding is also important for gC attachment to cells. While our data do not rule out the possibility that other regions in gC contribute to its attachment function, we speculate that gC has several functional domains. C3b binding depends on regions in the central portion of gC. The ability of gC to attach to cells appears to be associated with the N terminus.

What is the role of gC in HSV entry? Although gC enhances the ability of HSV to attach to cells, it is not essential for in vitro replication of the virus (21, 41, 43, 51, 100). Indeed, we found that there were no differences in the growth curves of virus containing mutant gC or lacking gC altogether. Our results and those of others (49) indicate that soluble gD inhibits HSV plaque formation. In contrast, soluble gC did not inhibit plaque formation even though it seems to be important for attachment. The plaque inhibition assay would argue against a significant role for gC in the entry process. These results are puzzling, but there are several possible ways to explain them. The presence of soluble gC may be enough to block the interaction of virion gC with the cell at 4°C. However, it may not be sufficient to overcome the interaction of gB or other viral glycoproteins with other receptors, especially when these interactions occur at the higher temperature used in the plaque inhibition assay. It is possible that virion gC binds to HSPG with higher affinity than truncated gC. If so, then during the long incubation times required for plaque formation, truncated gC would be displaced by virus. Furthermore, attachment may be 90% dependent on the interaction of gC with HSPG, but the other 10% is enough to allow virus entry given sufficient time or when large quantities of virus are available. Several studies have indicated that HSV and PRV gC null mutants are attachment and penetration deficient in some cell types (25, 40, 60, 101). This is thought to result from inefficient attachment of virus. Since plaque inhibition measures the effect of proteins on attachment, penetration, and cell-to-cell spread, further studies are required to more precisely determine the role of gC in virus entry. It is worth noting that our data support the findings by Fuller and Spear (33) that MAbs directed against gC blocked HSV attachment but not penetration.

What is the role of gC in vivo? It has been argued that gC is not essential in vivo (87). However, we take the position that in addition to its function in attachment, gC is used by the virus to evade complement-mediated attack (28, 29, 44, 79, 92). This is most likely to occur during the initial stages of primary infection, and experiments to test this hypothesis in vivo are underway. In addition, it is possible that in vivo, gC functions are supplemented or enhanced by the function of other viral glycoproteins. It was recently shown that a double PRV mutant lacking gC and gE is avirulent in an animal model, whereas single mutants lacking either glycoprotein alone were not attenuated (61, 102).

Finally, it is also possible that in vivo, gC may provide the virus with a docking mechanism which mediates rapid adhesion to cells and then allows other glycoproteins such as gD to interact with their respective receptors. Haywood (36) emphasized that low-affinity binding to a highly abundant receptor allows the virus to "browse" over the cell surface by making and breaking weak bonds until it finds a second receptor. Thus, docking may be one of the roles played by HSV gC and may be of great importance to the in vivo survival of the virus.

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