Virology **294,** 324–332 (2002)
doi:10.1006/viro.2001.1326, available online at http://www.idealibrary.com on IDEAL®

Kinetic Analysis of Glycoprotein C of Herpes Simplex Virus Types 1 and 2 Binding to Heparin, Heparan Sulfate, and Complement Component C3b

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Received September 26, 2001; returned to author for revision November 10, 2001; accepted December 5, 2001

Glycoprotein C (gC) from herpes simplex virus (HSV) facilitates virus entry by attaching the virion to host cell-surface heparan sulfate (HS). Although gC from HSV-1 (gC1) and from HSV-2 (gC2) bind to heparin, gC2 is believed to play a less significant role than gC1 in attachment of virus to cells. This attachment step is followed by the binding of gD to one of several cellular receptors. gC also plays an important role in immune evasion by binding to the C3b fragment of the third component of the host complement system. Yet, although both gC1 and gC2 protect HSV against complement-mediated neutralization, only gC on HSV-1-infected cells acts as a receptor for C3b. We used optical biosensor technology to quantitate the affinities (K_D) and the stabilities (k_{off}) between both serotypes of gC with heparin, HS, and C3b to address three questions concerning gC interactions. First, can differences in affinity or stability account for differences between the contributions of HSV-1 and HSV-2 gC in attachment? Our data show that the gC2–HS complex is highly unstable ($k_{off} = 0.2\ s^{-1}$) compared to the gC1–HS complex ($k_{off} = 0.003\ s^{-1}$), suggesting why gC2 may not play an important role in attachment of virus to cells as does gC1. Second, does gC2 have a lower affinity for C3b than does gC1, thereby explaining the lack of C3b-receptor activity on HSV-2 infected cells? Surprisingly, gC2 had a 10-fold higher affinity for C3b compared to gC1, so this functional difference in serotypes cannot be accounted for by affinity. Third, do differences in gC–HS and gD-receptor affinities support a model of HSV entry in which the gC–HS interaction is of lower affinity than the gD-receptor interaction? Our biosensor results indicate that gC has a higher affinity for HS than gD does for cellular receptors HveA (HVEM) and HveC (nectin-1).

Key Words: glycoprotein C; heparan sulfate; C3b; HSV; biosensor.

INTRODUCTION

Herpes simplex virus (HSV) codes for at least 11 glycoproteins, most of which are found on the virion envelope as well as infected cell surfaces (Spear, 1993a). Although glycoprotein C (gC) is not essential for viral growth *in vitro* (Draper *et al.,* 1984; Holland *et al.,* 1984; Johnson *et al.,* 1986; Zezulak and Spear, 1984), it is rarely absent in clinical isolates (Arvin *et al.,* 1983; Friedman *et al.,* 1986; Hidaka *et al.,* 1990) and is associated with two important functions: entry of virus into cells via binding to cell-surface heparan sulfate (HS) (Gerber *et al.,* 1995; Herold *et al.,* 1991, 1995; Sears *et al.,* 1991; Tal-Singer *et al.,* 1995) and immune evasion by HSV via binding to the C3b fragment of the third component of complement (Eisenberg *et al.,* 1987; Friedman *et al.,* 1984, 1986, 1996; Harris *et al.,* 1986; Hung *et al.,* 1992; Lubinski *et al.,* 1999, 1998; Seidel-Dugan *et al.,* 1990), leading to down-regulation of the complement cascade (Fries *et al.,* 1986).

The entry of HSV into cells involves a series of interactions between several viral glycoproteins and host cell molecules (Spear, 1993a). Infection is initiated by the attachment of gC and/or gB to HS glycosoaminoglycan side chains of cell-surface proteoglycans (Herold *et al.,* 1991, 1994; WuDunn and Spear, 1989). In models of HSV entry, this is considered to be a low-affinity interaction which is then followed by the binding of gD to one of several cellular receptors (reviewed in Spear *et al.,* 2000), three of which have been identified as HveA (HVEM) (Montgomery *et al.,* 1996) and HveC (nectin-1) (Geraghty *et al.,* 1998) and HS modified by a 3-*O*-sulfotransferase (Shukla *et al.,* 1999). It is generally thought that this step is a higher affinity interaction than that between gC and heparan sulfate (Flint *et al.,* 2000; Roller and Rauch, 1998). Following the gD-receptor interaction, a pH-independent fusion of the viral envelope with the cellular membrane occurs (Wittels and Spear, 1990), facilitated by gD, gB, and the gH–gL complex (Spear, 1993b; Turner *et al.,* 1998).

Although both HSV-1 and HSV-2 target HS as an attachment molecule, there are type-specific differences in the way these viruses interact with host cells. Previous studies indicated that HSV-1 mutants lacking gC1 were

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0042-6822/02 $35.00

impaired in the ability to bind cells and had reduced specific infectivities (Herold *et al.,* 1991). In contrast, HSV-2 mutants lacking gC did not exhibit this phenotype (Gerber *et al.,* 1995; Laquerre *et al.,* 1998). It has been suggested that gB2 may play a more important role in HSV-2 binding to cells than does gC2 (Gerber *et al.,* 1995; Herold *et al.,* 1996). Both gC1 and gC2 bind directly to heparin Sepharose (Herold *et al.,* 1991; Tal-Singer *et al.,* 1995). We previously showed that the HS-binding domains of gC1 involve, but are not limited to, the N-terminal residues between 33 and 123 (Tal-Singer *et al.,* 1995). Another study indicated that residues between 143 and 150, as well as residue 247, were important for the gC1–HS interaction (Trybala *et al.,* 1994). Although gC2 binds to heparin, specific binding domains on gC2 have not been localized.

Glycoprotein C modulates the host immune response by interacting with proteins from the complement system. Both gC1 and gC2 protect HSV against complement-mediated neutralization (Friedman *et al.,* 1996, 2000; Gerber *et al.,* 1995; Harris *et al.,* 1990; Lubinski *et al.,* 1999; McNearney *et al.,* 1987). *In vivo*, gC binding to C3b is an important virulence factor (Lubinski *et al.,* 1999). Mutant HSV-1 strains that lack this ability produce disease comparable to wild-type virus in C3 knockout mice, but not in C3 intact mice. In the latter, 50- to 100-fold higher doses of mutant virus are required to produce comparable disease as wild-type virus. *In vitro*, gC from both serotypes binds C3b (Eisenberg *et al.,* 1987; Tal-Singer *et al.,* 1995) and cells transfected with the gene for either gC1 or gC2 bind C3b (Seidel-Dugan *et al.,* 1988, 1990). Interestingly, although HSV-1 infected cells express C3b receptor activity (Friedman *et al.,* 1984), this is not the case for HSV-2-infected cells (Friedman *et al.,* 1984, 1986). There are several possible explanations for the lack of C3b receptor activity on HSV-2 infected cells: (1) Host cell membrane components may block the receptor activity of gC2 on the infected cell surface; (2) viral glycoproteins expressed on the cell surface during HSV-2 infection may inhibit binding of C3b to gC2 on the infected cell surface; or (3) the affinity of gC2 for C3b may be lower than that of gC1.

In this study we used optical biosensor technology (Canziani *et al.,* 1999; Karlsson *et al.,* 1991; Myszka, 1999) to address three issues concerning the interactions between gC and heparin, heparan sulfate, and C3b. First, can differences in affinity and/or stability account for the apparent differences between gC from HSV-1 and HSV-2 in their contributions to virus binding to cells? Second, can the lack of C3b-receptor activity on HSV-2-infected cells be explained by a lower affinity of gC2 for C3b than of gC1 for C3b? Third, given that both gC and gD bind to cell-surface receptors, what are the relative affinities of these interactions; i.e., do the differences in affinities support the current model for HSV entry?

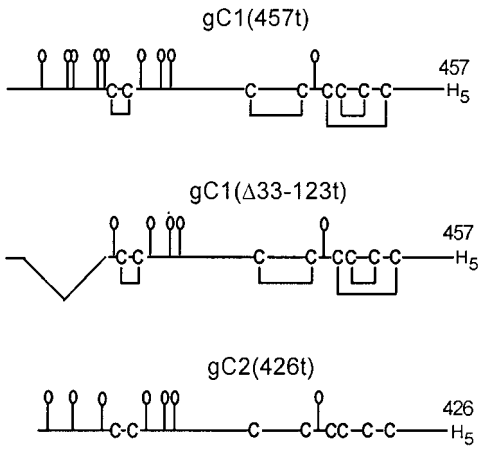

FIG. 1. Schematic diagrams of truncated forms of HSV gC1 and gC2 produced by baculovirus-infected insect cells. Both forms of gC1 were truncated at residue 457 and gC2 was truncated at residue 426. Additional histidine residues were added to aid in purification. The consensus sites for N-linked oligosaccharides are shown as balloons. In each case, the wild-type signal peptide of gC was replaced with that of the honeybee mellitin signal. As a result of cloning, cleavage at the mellitin signal leaves two additional amino acids on the N-terminus of gC, aspartic acid and leucine in gC1 constructs and aspartic acid and proline in gC2. The disulfide structure of gC1 was previously determined (Rux *et al.,* 1996). All three forms of gC have been previously shown to bind to heparin and C3b (Tal-Singer *et al.,* 1995; Kostavasili *et al.,* 1997).

RESULTS AND DISCUSSION

The three forms of soluble gC used for all experiments are shown in Fig. 1. The first set of experiments examined the binding of gC to heparin. Heparin is chemically related to HS (Kjellen and Lindahl, 1991) and is commonly used as a model for HS. However, while HS is found on cell surfaces, heparin is located intracellularly in mast cells. Also, heparin is more highly sulfated then HS and since the binding of heparin/HS to proteins is generally ionic, the difference in charge between the two may be significant. We quantitated the binding of the three forms of gC to immobilized heparin (Fig. 2). The solid lines represent the best global fits to a simple 1:1 binding model, using molecular masses obtained by mass spectrometry for the gC variants (Rux *et al.,* 1996). The 1:1 model means that one molecule of gC binds to one molecule of heparin to form one gC:heparin complex. gC1(457t) bound to heparin with an affinity (K_D) of 1.3×10^{-8} M (Table 1). Removal of residues 33–123 resulted in a 50-fold weaker affinity for heparin. This lower affinity was primarily due to a 100-fold slower on rate than that observed for the full ectodomain of gC.

The binding of gC2 to heparin (Fig. 2C) did not fit a 1:1 binding model; i.e., the interaction may be more complex than a simple bimolecular reaction, and thus kinetic rate constants cannot be calculated using this analysis. We estimated a maximum k_{off} of 0.02 s^{-1} (Table 1) (Karlsson *et al.,* 1991). These results indicate that the gC2–heparin

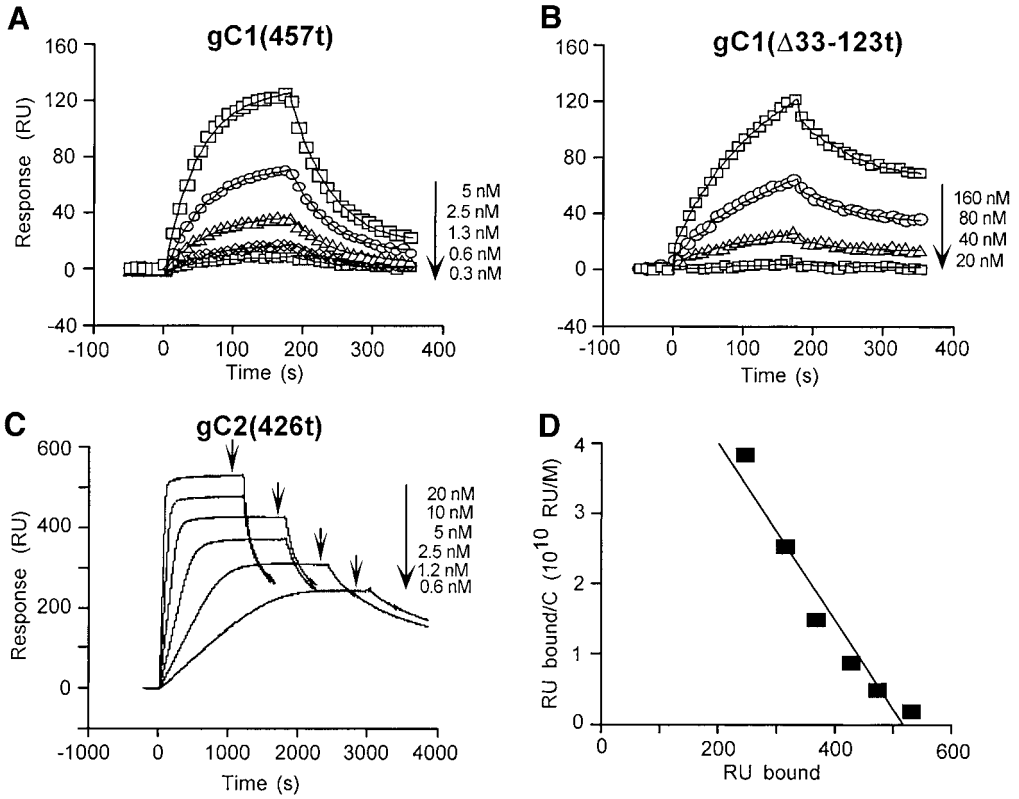

FIG. 2. Binding of decreasing concentrations of the indicated forms of gC to immobilized biotinylated heparin. In A and B, the flow was set to 30 μL/min and five data points per second were collected, but for clarity, selected points at every 10 s are shown as open symbols. The solid lines are the best fit to the simple bimolecular (1:1) model. In C the lines are the data for gC2(426t) where the flow was set to 2 μL/min so that the contact time could be varied from 20 min for the highest concentration to 50 min for the lowest concentration. The short arrows indicate the time points used for the Scatchard analysis. (D) Scatchard analysis. Equilibrium binding levels (RU bound) were obtained from sensorgrams in C. C is the molar concentration of gC2(426t). The negative inverse of the slope of the line gives a K_D of 8×10^{-10} M. The correlation coefficient for the linear fit is 0.94.

complex is 10 times less stable than the gC1–heparin complex. Because we could not determine an affinity constant from the on and off rates ($k_{off}/k_{on} = K_D$), we chose to determine the K_D under equilibrium conditions. The binding of gC2 to heparin was monitored until equilibrium was reached at each gC2 concentration. Scatchard analysis (Fig. 2D) gave a K_D of 8×10^{-10} M for the gC2–heparin complex. This is a 16-fold higher affinity than that observed for the full ectodomain of gC1. Unlike the interaction of gC2 with heparin, complex formation between either gC1(457t) or gC1(Δ33–123t) with heparin did not reach equilibrium (within 50 min); hence Scatchard analysis could not be carried out. As a control, gD1(306t) did not bind to heparin. We note that since there is some curvature in the Scatchard plot (Fig. 2D), the points can also fit a two component model. This resulted in two values for K_D which were not significantly different from the single component value; thus the single value was used to describe the interaction.

TABLE 1

Kinetic and Affinity Values for gCt Interactions with Heparin, Heparan Sulfate, and C3b

Immobilized ligand	Analyte	k_{on} (10^4 M^{-1} s^{-1})	k_{off} (10^{-3} s^{-1})	K_D (10^{-8} M) (k_{off}/k_{on})[a]
Heparin	gC1(457t)	130	1.6	1.3
	gC1(Δ33–123t)	1.4	0.78	55
	gC2(426t)	ND[b]	20[c]	0.08[d]
Heparan sulfate	gC1(457t)	3.0	3.0	10
	gC1(Δ33–123t)	0.40	3.0	75
	gC2(426t)	ND[b]	200[c]	2.0[d]
C3b	gC1(457t)	56	16	2.8
	gC1(Δ33–123t)	61	23	3.8
	gC2(426t)	400	11	0.28

[a] χ^2 values from the global fits were less than 3.

[b] ND, not determined.

[c] Estimated maximum k_{off} from $\ln(R_0/R_n) = k_{off}(t_n - t_0)$, where R_0 is the response at time zero (t_0) of dissociation and R_n is the response at time n (t_n) (Karlsson *et al.,* 1991).

[d] From Scatchard analysis.

In a competition assay, 200 nM soluble heparin completely inhibited the binding of 5 nM gC1(457t) to immobilized heparin (Fig. 3), suggesting that biotinylation probably did not alter the binding sites for gC. Furthermore, the complete inhibition of binding by soluble heparin suggested that the binding of gC to immobilized heparin was specific.

In an earlier report by Williams and Straus (1997),

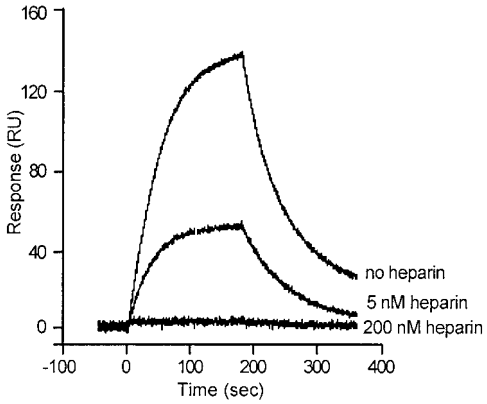

FIG. 3. Soluble heparin competes with immobilized, biotinylated heparin for binding to gC. In the top curve, 5 nM gC1(457t) alone was injected over the heparin surface. In the lower curves, soluble heparin at 5 or 200 nM was incubated with 5 nM gC1(457t) for 40 min and then injected. Soluble heparin alone (200 nM) did not bind to the heparin surface (not shown).

biosensor technology was used to investigate the interaction of a truncated form of gB2 binding to heparin. They determined a K_D of 7.7×10^{-7} M and a k_{off} of 4×10^{-4} s^{-1} for the gB2–heparin interaction. We determined a K_D value of 8.0×10^{-10} M and a k_{off} of 2×10^{-2} s^{-1} for gC2 binding to heparin. Thus, the gB2–heparin complex has a lower affinity but a higher stability than does the gC2–heparin complex. Since gB2 has been suggested (Gerber *et al.,* 1995; Herold *et al.,* 1996) to be more important than gC2 in HSV attachment to cells, the stability of the interaction may play a greater role than the overall affinity. Perhaps gC must bind to heparan sulfate long enough to allow gD to bind to its cell-surface receptor.

Next we examined the binding of gC1(457t), gC1(Δ33–123t), and gC2(426t) to immobilized biotinylated HS (Fig. 4). As expected, gD1(306t) did not bind to the HS surface. Removal of residues 33–123 of gC1 resulted in a weaker affinity for HS, again due to a slower on rate (Table 1). In this case, the affinity and on rate decreased about eightfold when these residues were removed. Previously (Tal-Singer *et al.,* 1995) we looked at the ability of all three forms of gC to bind to various cell lines (Vero, BHK, L cells). gC1(Δ33–123t) bound significantly less well to cells than gC1(457t) and competed poorly with biotinylated gC1(457t) for binding, suggesting that residues 33–123 are important for gC attachment to cells. The biosensor data confirm these observations and provide quantitative data.

gC2 bound to HS with very different kinetics when compared with gC1 (Fig. 4C). The increased rate at which the gC2(426t) sensorgrams reached a plateau compared with the sensorgrams of the gC1 complexes (Figs. 4A and 4B) indicates that the gC2–HS complex came to equilibrium faster than the other complexes. As

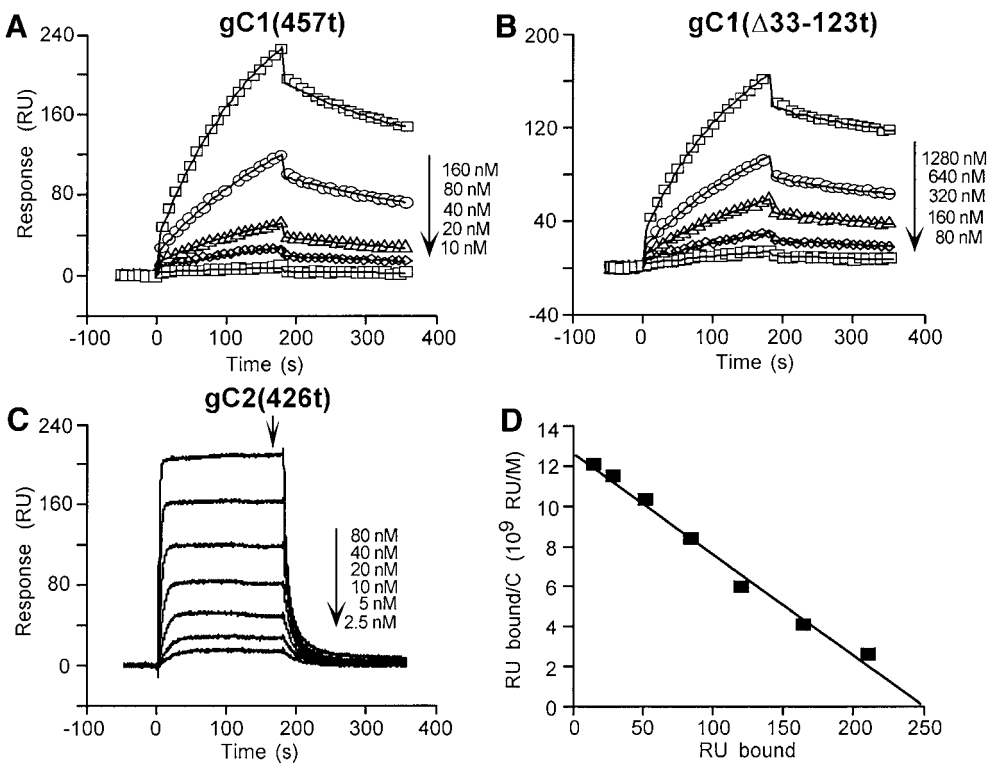

FIG. 4. Binding of decreasing concentrations of the indicated forms of gC to biotinylated heparan sulfate. Data for A–D are presented as described in Fig. 1, except that all data were collected at a flow of 30 μL/min. In D, the negative inverse of the slope of the line gives a K_D of 2.0×10^{-8} M. The correlation coefficient for the linear fit is 0.99.

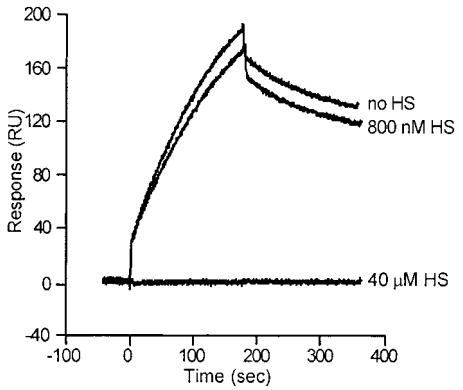

FIG. 5. Soluble heparan sulfate competes with immobilized, biotinylated heparan sulfate for binding to gC. In the top curve, 80 nM gC1(457t) alone was injected over the heparan sulfate surface. In the lower curves, heparan sulfate at 800 nM or 40 μM was incubated with 80 nM gC1(457t) for 40 min and then injected. Soluble heparan sulfate alone (40 μM) did not bind to the heparan sulfate surface (not shown).

was the case for heparin, the binding of gC2 to HS did not fit a 1:1 binding model. Scatchard analysis (Fig. 4D) yielded an equilibrium dissociation constant of 2×10^{-8} M for the gC2–HS complex. This is a fivefold higher affinity than that observed for the full ectodomain of gC1 (Table 1). In a competition assay, 40 μM soluble HS completely inhibited the binding of 80 nM gC1(457t) to immobilized, biotinylated HS (Fig. 5), suggesting that the binding of gC to immobilized HS was specific. It should be noted that we only examined one form of heparan sulfate. Clearly, cell and species differences in heparan sulfate could yield different affinities. This is a subject for further investigation.

The affinity values for gC1–HS and gC2–HS complexes are in good agreement with previous results (Tal-Singer *et al.,* 1995), which gave an estimated affinity of 10^{-7} M for the same forms of gC binding to BHK cells using ELISA. The estimated k_{off} for the gC2–HS complex was 0.2 s^{-1}, which is 2 logs faster than the k_{off} for the gC1–HS complex. These results indicate that the gC2–HS complex was unstable. We speculate that differences in stability of the respective gC–heparan sulfate complexes may help to explain why gC1 plays a more important role in virus attachment than gC2 (Gerber *et al.,* 1995).

It is of note that the calculated k_{on} for gC1 binding to HS was roughly 1 log slower than for gC1 binding to heparin, resulting in a lower affinity for the HS complex. Similar differences were seen in the affinity of gC2 for HS compared to heparin. These results suggest that some caution should be used when using heparin as a model to interpret binding to HS.

Last, we looked at the binding of gC to C3b. As previously described (Sarrias *et al.,* 2001), we attempted to mimic the *in vivo* orientation of C3b by biotinylating the protein site-specifically at the active thioester that participates in the anchoring of C3b to cells (Law and Dodds, 1997). The immobilization of biotinylated C3b on the surface of a biosensor streptavidin chip was intended to simulate the attachment of C3b onto the cell surface. This oriented C3b on the chip to present a homogenous ligand surface. Previously we used rosetting (Hung *et al.,* 1992), ELISA (Kostavasili *et al.,* 1997; Tal-Singer *et al.,* 1995), and C3 knockout mice (Lubinski *et al.,* 1999) to show that residues 33–123 of gC1 are not part of the C3b binding domain. The biosensor data confirm this observation and provide quantitative data (Fig. 6). Both gC1(457t) and gC1(Δ33–123t) bound to C3b with similar affinities and similar kinetics (Table 1). Surprisingly, gC2 bound to C3b with a 10-fold higher affinity than did gC1. This increased affinity is accounted for by

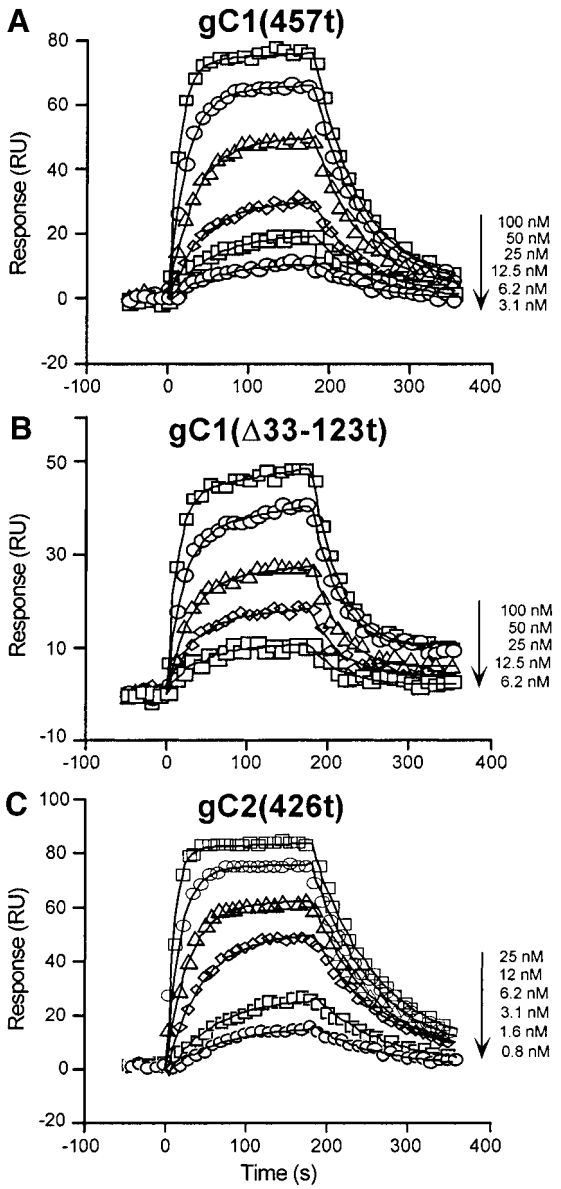

FIG. 6. Binding of decreasing concentrations of the indicated forms of gC to immobilized biotinylated C3b. Data are presented as described in the legend of Fig. 1.

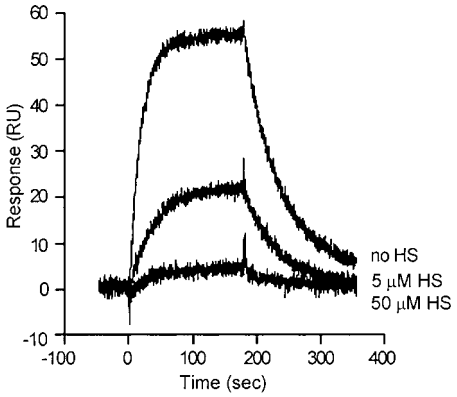

FIG. 7. Competitive inhibition of gC1(457t) binding to immobilized C3b by soluble heparan sulfate. In the top curve, 50 nM gC1(457t) alone was injected over the HS surface. In the lower curves, soluble HS at 5 or 50 μM was incubated with 50 nM gC1(457t) for 40 min and then injected. Soluble HS alone (50 μM) did not bind to the C3b surface (not shown).

a faster rate of complex formation, k_{on}. As a control, gD1(306t) did not bind to the C3b chip surface. The affinity of the gC1(457t)–C3b interaction, 2.8×10^{-8} M, is remarkably similar to that reported for the interaction between C3b and complement receptor 1 (CR1) (Becherer *et al.,* 1989), 2×10^{-8} M, while the affinity of the gC2(426t)–C3b complex is 10-fold higher.

gC from HSV-1 inhibits activation of the complement cascade by binding complement component C3b (Friedman *et al.,* 1984; Fries *et al.,* 1986; McNearney *et al.,* 1987) and by blocking the binding of properdin and C5 to C3b (Hung *et al.,* 1994; Kostavasili *et al.,* 1997). Our data show that the inability of gC2 on infected cells to bind to C3b is not an affinity issue, as gC2 has a higher affinity for C3b than does gC1. In addition, both the gC1–C3b and the gC2–C3b complexes have similar stabilities. Thus, the mechanism by which gC2 modulates complement-mediated neutralization remains unclear. One possibility is that gC2 on the virion binds C3b; this remains to be evaluated. In addition, it has been suggested (Eisenberg *et al.,* 1987) that other cell-surface molecules expressed during HSV-2 infection prevent C3b receptor activity.

To determine whether there is overlap between the binding sites for C3b and HS on gC, two concentrations of HS were incubated with 50 nM gC1(457t) for 40 min prior to injection over the C3b chip surface (Fig. 7). HS at a concentration of 50 μM almost completely inhibited the binding of gC to C3b. Therefore, there may be overlap between C3b and HS binding sites on gC. Our inhibition data are consistent with a previous study that showed that heparin inhibited the binding of C3b-coated erythrocytes to HSV-1-infected Vero cells (Huemer *et al.,* 1992). Trybala *et al.* (1994) identified residues between 143 and 150 and residue 247 as part of the HS-binding domain on gC1. We previously identified four noncontiguous domains (Regions I–IV) in the central portion of gC1 that bind to C3b (Hung *et al.,* 1992). In this model, Region I is adjacent to residues 143–150 and Region IV contains residue 247. However, we also determined that the HS binding domains on gC1 include residues 33–123 (Tal-Singer *et al.,* 1995), which are not part of the C3b binding domains. Taken together, these data suggest that the HS and C3b binding sites on gC1 may overlap but are not identical. Alternatively, HS may alter the conformation of gC such that the C3b binding site is no longer accessible.

We can also compare the binding constants for the gC–HS complex with that of the gC–C3b complex for each serotype of gC (Table 1). gC1(457t) has a roughly threefold higher affinity for C3b than for HS, while gC2(426t) has a sevenfold higher affinity for C3b than for HS. In each case, changes in both the rate of complex formation (k_{on}) and the complex stability (k_{off}) contribute to the higher affinities. The functional implications of these differences remain to be explored.

To our knowledge, this is the first report of affinity and kinetic values for gC binding to heparin, HS, and C3b. Previously, we used optical biosensor technology to determine the affinity of gD for cellular receptors HveA (Rux *et al.,* 1998; Willis *et al.,* 1998) and HveC (Krummenacher *et al.,* 1999; Whitbeck *et al.,* 1999). Therefore, it is now possible to quantitatively compare the interaction of gC with HS to that of gD with receptors. The ectodomain of gD, gD1(306t), bound to both HveA and HveC with a K_D of 30×10^{-7} M. The data in this paper show that the affinity of the gC1–HS complex is 30-fold higher (1×10^{-7} M) than the affinity of gD for any of its receptors. This trend in affinities was surprising to us, in light of the prevailing idea in the HSV field, which is that gC and/or gB bind to HS proteoglycans with a low affinity that allows virus to attach loosely to cells (grazing over the surface) and position virion gD in close enough proximity to interact with higher affinity to a specific cellular receptor (Flint *et al.,* 2000; Roller and Rauch, 1998). Our data suggest that the opposite may in fact be the case, i.e., that the gC–HS interaction has a higher affinity than the gD–receptor interaction. Thus, we propose that a more appropriate model for the initial steps in HSV entry into cells should include the following: (1) HSV gC attaches tightly to the cell via abundant heparan sulfate proteoglycans; and (2) if a less abundant cellular receptor for virion gD is nearby, the entry process can continue. If no gD receptor is available near the site where initial attachment occurs, then this particular virion would remain attached to the surface and be unable to carry out the later steps of entry. The caveat here is the assumption that the interactions between soluble molecules mimic those between virion and cell-surface molecules. Even if the absolute affinities are different, we propose that the binding of virion gC to heparan sulfate is tighter than the binding of virion gD to cellular receptors.

MATERIALS AND METHODS

Construction, production, and purification of gC baculovirus recombinants

The construction, production, and purification of gC1(457t), gC1(Δ33–123t), and gC2(426t) were described previously (Tal-Singer *et al.,* 1995). All recombinants were produced in a baculovirus expression system and truncated (gCt) prior to the transmembrane region. gC1(457t) contains the ectodomain of gC1, being truncated at residue 457. gC1(Δ33–123t) is identical to gC1(457t) except that it lacks amino acids 33–123. gC2(426t) is truncated at residue 426. We previously showed that these baculovirus-expressed proteins are similar in conformation to full-length gC isolated from HSV-infected cells and that all bind to C3b (Tal-Singer *et al.,* 1995). Purified protein was used in all experiments.

Biotinylation of heparin, heparan sulfate, and C3

Heparin from bovine kidney (Sigma) or heparan sulfate from porcine intestinal mucosa (Seikagaku) were dissolved in PBS and biotinylated at primary amino groups for 1 h at room temperature with a molar excess of sulfo-*N*-hydroxysuccinimide-biotin (Pierce) (Williams and Straus, 1997). Excess biotin was removed using a 100-fold buffer exchange via ultrafiltration (3000 MW cutoff).

The production of site-specifically biotinylated C3b from human serum was previously described (Sarrias *et al.,* 2001). Briefly, the thioester bond of native C3 from human serum was hydrolyzed by methylamine and the product was biotinylated using EZ-Link PEO-maleimide-activated biotin (Pierce, Rockford, IL). Upon removal of free biotin, C3 was cleaved with trypsin to produce biotinylated C3b, which was purified by ion-exchange chromatography as previously described (Sarrias *et al.,* 2001). Biotinylated-C3b, -heparin, and -heparan sulfate were aliquoted and stored at −80°C.

Optical biosensor analysis

All surface plasmon resonance (SPR) experiments were carried out on a BIACORE X (Biacore, AB) at 25°C as previously described (Krummenacher *et al.,* 1999; Rux *et al.,* 1998; Willis *et al.,* 1998). The running buffer was HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 0.05% P-20 (surfactant). The SA sensor chip (Biacore) was used for all experiments and consisted of streptavidin precoupled to all cells. The streptavidin surface was preconditioned with four 1-min pulses of 50 mM NaOH in 1 M NaCl before addition of the biotinylated species. In each case, the biotinylated ligand was coupled to flow cell 2 (Fc2). Flow cell 1 was left as a streptavidin control to compensate for nonspecific binding and for changes in refractive index upon addition of sample. All data were corrected by subtracting the data on Fc1 from that on Fc2. In all cases, the nonspecific binding was less than 5% of the total binding. Binding rates were unaffected by flow rate, demonstrating that these gC–ligand interactions were not limited by diffusion and thus the rates determined are truly reflective of the gC–ligand interaction (data not shown) (Morton and Myszka, 1998).

(1) Binding of gC to biotinylated heparin or heparan sulfate

Biotinylated heparin or biotinylated heparan sulfate was diluted in running buffer to approximately 25 μg/mL and flowed over preconditioned flow cell 2 at 5 μL/min until approximately 70 resonance units (RU) were immobilized. To characterize the binding of gC variants to heparin or heparan sulfate, the flow path was set to include both flow cells, the flow rate was set to 30 μL/min, and the data collection rate was set to high (5 Hz). Each gC sample was injected over a 3-min period to follow association. Then the sample was replaced with buffer and the dissociation phase followed for 3 min. For equilibrium experiments with gC2–heparin the flow rate was set to 2 μL/min to allow for longer contact times. The chip surface was regenerated with 0.2 M Na_2CO_3, pH 9.0, to remove any residually bound gC.

(2) Binding of gC to biotinylated C3b

Biotinylated C3b was diluted in running buffer to a final concentration of 10 μg/mL and flowed over flow cell 2 at 5 μL/min until approximately 700 RU were immobilized. Binding of serial dilutions of gC to C3b and regeneration of the C3b surface were carried out as above.

Data analysis

SPR data were analyzed using BIAevaluation software, version 3.0, which employs global fitting. Model curve fitting was done using a 1:1 Langmuir interaction with drifting baseline. For the interaction of gC2(426t) with heparin or heparan sulfate, Scatchard analyses were performed to determine the affinities and a maximum k_{off} was estimated by using the equation $\ln(R_0/R_n) = k_{off}(t_n - t_0)$, where R_0 is the response at time zero (t_0) of dissociation and R_n is the response at time n (t_n) (Karlsson *et al.,* 1991).

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants NS 36731 and NS30606 to R.J.E. and G.H.C. and Grant HL 28220 to H.M.F.

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