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Characterization of Cell-Binding Properties of Bovine Herpesvirus 1 Glycoproteins B, C, and D: Identification of a Dual Cell-Binding Function of gB†

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Previous studies have suggested that the attachment of bovine herpesvirus 1 (BHV-1) to permissive cells is mediated by its major glycoproteins B (gB), C (gC), and D (gD). In order to gain further insight into the mechanism of the BHV-1 attachment process, we purified authentic gB, gC, and gD from BHV-1-infected cells and membrane anchor-truncated, soluble gB, gC, and gD from stably transfected cell lines by affinity chromatography and examined their cell-binding properties on Madin-Darby bovine kidney cells. All of the glycoproteins tested exhibited saturable binding to Madin-Darby bovine kidney cells. Addition of exogenous heparin or treatment of cells with heparinase to remove cellular heparan sulfate (HS) prevented both gC and gB from binding to cells but had no effect on gD binding. An assessment of competition between gB, gC, and gD for cell binding revealed that gC was able to inhibit gB binding, whereas other combinations showed no effect. Cell-bound gC could be dissociated by heparin or heparinase treatment. The response of bound gB to heparin and heparinase treatments differed for the authentic and soluble forms; while soluble gB was susceptible to the treatment, a significant portion of cell-bound authentic gB was resistant to the treatment. Binding affinity analysis showed that soluble gB and both forms of gC and gD each had single binding kinetics with comparable dissociation constants (*Kd*s), ranging from 1.5×10^{-7} to 5.1×10^{-7} M, whereas authentic gB exhibited dual binding kinetics with $Kd_1 = 5.2 \times 10^{-7}$ M and $Kd_2 = 4.1 \times 10^{-9}$ M. These results demonstrate that BHV-1 gC binds only to cellular HS, gD binds to a non-HS component, and gB initially binds to HS and then binds with high affinity to a non-HS receptor. Furthermore, we found that while authentic gB was able to inhibit viral plaque formation, soluble gB, which retains the HS-binding property but lacks the high-affinity binding property, was defective in this respect. These results suggest that the interaction between gB and its high-affinity receptor may play a critical role in the virus entry process.

Virus infections are initiated through specific interactions between viral attachment proteins and their receptors on the surfaces of permissive cells. For alphaherpesviruses the initial interaction between viral attachment proteins and cellular receptors is followed by virus penetration involving membrane fusion. These two steps constitute the virus entry process. It is now known that for most cell types, the initial attachment of alphaherpesviruses to permissive cells is mediated by an interaction of their gC molecules with the cellular glycosaminoglycan, heparan sulfate (HS) (for a comprehensive review, see reference 36). Early studies using antibodies (5, 9, 14), cell membrane fractionation (19), and virosomes (16) suggested that gB and gD molecules are also involved in the virus attachment process. gB of herpes simplex virus (HSV) has been shown to be able to directly bind to HS (12). More recent studies showed that for gC-negative HSV, the interaction between gB and cellular HS plays a dominant role in attachment (11). gB of pseudorabies virus (PrV) can also bind heparin-coated beads; however, it does so only in conjunction with gC (27, 31). For bovine herpesvirus 1 (BHV-1), gC represents the only viral protein identified to date that has the ability to bind to a heparin-like component (30). HSV gD has been shown to bind to a limited number of specific cellular receptors (14, 15). Recently, Brunetti et al. showed that HSV gD binds to mannose-6-phosphate receptors (4), although the relevance of the gD–mannose-6-phosphate receptor interaction in virus infection has yet to be established. Thaker et al. recently showed that BHV-1 gD binds to a 60-kDa cell surface protein (39). We previously showed that affinity-purified BHV-1 gB, gC, and gD could inhibit virus attachment at 4°C and that gC inhibited not only wild-type (wt) BHV-1 but also a gC-negative mutant to a lesser degree, whereas gB and gD inhibited wt and gC-negative viruses to the same extent. These observations led us to propose that BHV-1 attachment is a complex event in that the wt virus uses gC to carry out the initial interaction with cellular receptors, which is followed by interactions of gB and/or gD with their receptors (24). Karger and Mettenleiter showed that the attachment of PrV and BHV-1 to permissive cells can be divided into two stages, an initial heparin inhibition-sensitive stage and a subsequent heparin-resistant stage (17). By using isogenic viruses with individual viral proteins deleted, they found that although the wt virus and the gD-negative mutant have similar initial binding, the gD-negative mutant was considerably impaired in the heparin-resistant binding, suggesting that the initial attachment of PrV and BHV-1 to cells via a gC-HS interaction is followed by a gD-mediated HS-independent attachment. A similar attachment mechanism has been revealed for HSV (8, 21, 26). The data available to date collectively suggest that attachment of alphaherpesviruses to most permissive cells is a complex event, involving at least gC binding to cellular HS and gD binding to other, undefined cellular receptors.

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In order to further understand the virus attachment mechanism, in the present study we purified both authentic and membrane anchor-truncated forms of BHV-1 gB, gC, and gD and examined each of these glycoproteins for cell-binding properties. By employing exogenous heparin as an inhibitor and heparinase treatment to remove cellular HS, we examined whether BHV-1 gB and gD could also bind to cellular HS and whether binding of any of the glycoproteins to cellular HS could be coupled to secondary HS-independent binding. In addition, we determined the cell-binding kinetics and affinity of each of the three glycoproteins. The results from this study reveal for the first time that BHV-1 gB interacts with cells in a two-step fashion, with initial binding to cellular HS followed by secondary high-affinity binding to a non-HS receptor.

MATERIALS AND METHODS

Virus, cells, and reagents. The BHV-1 Cooper strain was obtained from the National Veterinary Services Laboratories, Ames, Iowa, and propagated in Madin-Darby bovine kidney (MDBK) cells. MDBK cell lines that express secreted gB, gC, or gD under the control of a bovine heat shock hsp70 gene promoter were constructed in this institute (18, 22). All cell lines were maintained in minimum essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS) (GIBCO). Heparin of bovine intestinal mucosa (molecular weight, approximately 3,000 Da) and heparinase of *Flavobacterium heparinum* were purchased from Sigma (St. Louis, Mo.), lactoperoxidase was purchased from Boehringer-Mannheim (Doval, Quebec, Canada), and $Na^{125}I$ was purchased from Amersham (Oakville, Ontario, Canada).

Affinity purification of gB, gC, and gD. Affinity purification of gB, gC, and gD from BHV-1-infected cells was carried out essentially as previously described (42). Briefly, subconfluent MDBK cells were infected with BHV-1 at a multiplicity of infection of 1; at 24 to 48 h postinfection, cells were collected, washed, lysed with 1% Nonidet P-40–1% deoxycholic acid, and sonicated. After centrifugation at 110,000 × *g* for 1 h to remove the cellular debris, the supernatant was collected and applied to immunoadsorbent columns. The columns were sequentially washed with wash buffer (0.1 M Tris-HCl, 0.5 M NaCl, pH 7.5) containing 0.1% Nonidet P-40 and wash buffer without Nonidet P-40. In some cases, the columns were further washed once with 1.0 M NaCl in phosphate buffer (pH 7.2). The bound proteins were eluted from the columns with 50 mM diethylamine (pH 11.0), concentrated by ultrafiltration, and dialyzed extensively against 0.01 M Tris-HCl–0.15 M NaCl–1 mM EDTA (pH 7.5). The purified glycoproteins were stored at −70°C until use. For purification of secreted glycoproteins, cultures of the individual viral protein-expressing cell lines were subjected to daily heat shock treatment (43°C, 4 h) for 4 to 6 days (18); culture media were collected, centrifuged to remove cell debris, and applied to affinity columns as described above.

Protein iodination. Protein iodination was carried out by a lactoperoxidase method as described by Thorell and Johansson (40). About 5 μg of each protein was used for iodination; ^{125}I-labelled protein was separated from free $Na^{125}I$ by chromatography on a Sephadex G-25 column. The labelled proteins were aliquoted and stored at −70°C until use. The specific activities of the labelled proteins ranged between 6 and 20 μCi/μg of protein; the trichloroacetic acid-precipitable radioactivity was more than 90% of the total radioactivity.

Protein binding assays. Confluent MDBK cells in 48-well plates were cooled to 4°C. After removal of the culture medium, the cells in each well were incubated with approximately 2×10^5 cpm of an ^{125}I-labelled protein diluted in MEM-FBS at 4°C for 4 h. After incubation, the cells were washed three times with MEM containing 10% FBS and lysed with 1% Triton X-100 and 1% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (lysis buffer). The total cell lysate was collected, and radioactivity was counted in a gamma counter. In each plate, wells containing an ^{125}I-labelled protein together with 100 μg of unlabelled protein per ml were included and used as a control for nonspecific background binding. Under the conditions tested, the nonspecific binding was always less than 10% of the total binding. Each experiment was performed in triplicate. To assess the effects of heparin and heparinase treatments on glycoprotein binding, four different conditions were employed as follows: (i) heparin competition with ^{125}I-labelled protein binding, in which cells were cooled to 4°C and incubated with 50 μl of heparin at a specified concentration per well for 1 h at 4°C prior to the addition of 50 μl of a labelled protein; (ii) heparinase treatment before addition of labelled proteins, in which cells were treated with 100-μl amounts of different concentrations of heparinase in MEM-FBS containing 0.05% NaN_3 at 37°C for 1 h, washed three times with MEM-FBS, cooled to 4°C, and then incubated with 100 μl of a labelled protein per well; (iii) heparin treatment of cells with bound ^{125}I-protein, in which cells were incubated with a labelled protein for 4 h at 4°C, washed three times with MEM-FBS to remove the free ^{125}I-labelled protein, incubated with 100 μl of heparin at different concentrations in MEM-FBS per well for another hour at 4°C, and then washed and lysed as described above; and (iv) heparinase treatment of cells with bound ^{125}I-protein, which was essentially the same as heparin treatment of cells with bound ^{125}I-protein except that the cells were treated with heparinase at 37°C for 1 h instead of with heparin.

Assay for protein-binding kinetics. The assay for protein-binding kinetics was carried out as described by Lasky et al. (20). Confluent MDBK cells in 48-well plates were cooled at 4°C for 1 h. After removal of medium, the cells were incubated with a constant amount of an ^{125}I-labelled protein (2×10^5 cpm in a 50-μl volume per well) in MEM-FBS in the presence of increasing amounts of an unlabelled protein. After incubation at 4°C for 4 h, the cells were washed three times with MEM-FBS and lysed with 1% Triton X-100–1% SDS. Cell-associated radioactivity was determined in a gamma counter. Amounts of total input, cell-bound, and free proteins were calculated and subjected to Scatchard analysis (32).

Virus adsorption inhibition assay. MDBK cells grown to confluence in 24-well culture plates were cooled at 4°C for 1 h. Viral proteins or heparin was diluted in MEM-FBS to specified concentrations and added to the cells in a volume of 50 μl per well. The plates were incubated for 1 h at 4°C, 50 μl of virus inoculum per well (containing approximately 150 PFU of virus) was added, and the plates were incubated at 4°C for another hour. After adsorption, the plates were washed three times with MEM and overlaid with 0.8% agarose in MEM containing 2% FBS. Plaques were counted 4 days later. The number of viral plaques formed in the absence of an inhibitor was taken as 100% plaque formation and used as a reference for calculating the percentage of viral plaques formed in the presence of an inhibitor. To detect plaque formation on heparinase-treated cells, MDBK cells were incubated with heparinase at 37°C for 1 h before washing and viral inoculation.

RESULTS

Production and characterization of membrane anchor-truncated, soluble gB, gC, and gD. Three stable MDBK cell lines that express membrane anchor-truncated, soluble BHV-1 gB (gBt), gC (gCt), and gD (gDt), respectively, were established in this institute (18, 22). The expression of these viral proteins is controlled by a bovine heat shock hsp70 gene promoter and is highly inducible by subjecting the cells to elevated temperatures (43°C) (18). These truncated glycoproteins are efficiently secreted. Characterization of the truncated glycoproteins, involving immunoprecipitation, reactivity to an extended panel of monoclonal antibodies, and immunogenicity in cattle or in mice, showed that the truncated proteins had properties similar to those of their authentic counterparts; the truncated gB, like its authentic form, is also cleaved and forms disulfide-linked heterodimers (18, 22). Figure 1 shows the predicted transmembrane domains of gB, gC, and gD and the carboxyl termini of the truncated glycoproteins. gBt, gCt, and gDt are terminated at amino acid residues 763, 465, and 356, respectively. During construction of the gCt and gDt genes, several exogenous amino acid residues were introduced at their carboxyl termini; Leu and Asp were added to gCt, and Arg, Val, and Ala were added to gDt.

Effects of heparin or heparinase on the binding of gB, gC, and gD to MDBK cells. To evaluate whether BHV-1 gB, gC, and gD are able to bind to cellular HS, we examined the effects of addition of exogenous heparin and treatment of cells with heparinase to remove cellular HS on the binding of affinity-purified, ^{125}I-labelled glycoproteins to MDBK cells. Prior to this study, a number of preliminary experiments were performed to optimize the conditions for the binding assay. We found that a binding assay in the presence of normal culture medium, i.e., MEM containing 10% FBS, produced the least nonspecific binding and no discernible alteration of cell morphology, and each of the glycoproteins tested reached maximum binding within 4 h of incubation at 4°C (data not shown). These conditions were therefore adopted for the protein binding assays used throughout the present study.

As shown in Fig. 2, authentic (Fig. 2A) and soluble (Fig. 2B) proteins showed similar binding properties in response to heparin and heparinase treatments. Heparin or heparinase treatment inhibited both gC and gB binding to cells in a dose-dependent fashion but had no effect on gD binding. This

FIG. 1. Amino acid sequences of the carboxyl termini of authentic and truncated BHV-1 gB, gC, and gD. Predicted transmembrane domains of authentic gB, gC, and gD are boxed (6, 41, 45). The underlined amino acids of gCt and gDt are exogenous residues added during construction of the mutant molecules. The dashed line in authentic gB represents omitted amino acids in the cytoplasmic tail.

establishes that both BHV-1 gC and gB bind to cellular HS, whereas gD binds to a non-HS cellular component. While binding of both gB and gC was susceptible to heparin inhibition, gB and gC nevertheless differed dramatically in their binding capacities. For example, heparin at a concentration of 0.1 μg/ml inhibited more than 50% of both authentic and soluble gB binding; for the same degree of inhibition of gC binding, a 100-fold higher heparin concentration, i.e., 10 μg/ml, was required. It may be noted that although gB and gC showed different susceptibilities to heparin inhibition, their responses to heparinase treatment are similar. This is likely due to the different operation mechanisms involved in these two assays. The heparin treatment is a competition assay, in which the effectiveness of inhibition by the exogenous heparin depends on the overall binding affinity or avidity of a labelled protein. In contrast, with heparinase treatment, the inhibition of binding of a labelled protein is caused by removal of cell binding sites. Thus, the observation that gB and gC showed similar responses to heparinase treatment may reflect that heparinase treatment was able to remove the binding sites recognized by gB and by gC proportionally.

The observation that both gB and gC were able to bind to MDBK cells in an HS-dependent manner prompted us to further examine the interrelation between the viral proteins in cell binding. Because authentic and soluble proteins exhibited similar binding properties in response to heparin and heparinase treatments, in this particular experiment only authentic proteins were tested. Cells were incubated with a constant amount of an ^{125}I-labelled protein in the presence of different concentrations of a second unlabelled, heterologous protein at 4°C for 4 h; the amount of labelled protein bound to the cells was determined and compared with the amount of labelled protein bound in the absence of the heterologous protein (Fig. 3). gB and gD showed no inhibitory effect on the binding of a second heterologous protein. While gC had no effect on gD binding, it effectively inhibited gB binding. It was noticed that gC at a concentration of 1 μg/ml repeatedly increased the total binding of gB. The reason for this is not clear.

Effects of heparin and heparinase on cell-bound glycoproteins. The experiments described above showed that gC and gB bind to cellular HS, but they could not exclude the possibility that these viral glycoproteins might bind to additional, non-HS cellular receptors following their initial interaction with HS; i.e., the initial binding of these viral proteins to HS might promote their binding to additional, non-HS receptors. Accordingly, we speculated that should gB and gC be able to engage in a secondary non-HS binding, then after being bound to cells, they would become resistant to either heparin or heparinase treatment. Therefore, cells were first incubated with an ^{125}I-labelled glycoprotein at 4°C for 4 h; after removal of free, unbound protein, the cells were treated with exogenous heparin or heparinase (Fig. 4). As expected, bound gD was completely resistant to heparin and heparinase treatments. In contrast, bound gC could be completely removed by either heparin or heparinase treatment. Of interest is that authentic gB and soluble gB showed different responses to heparin and heparinase treatments. While bound soluble gB could be removed completely by either heparin or heparinase treatment, a significant portion of bound authentic gB became resistant to heparin and heparinase treatments. Under the conditions tested, 40% of the bound authentic gB became refractory to treatment. The maximum effects of heparin and heparinase on the bound authentic gB were observed at concentrations of 100 μg/ml and 1 U/ml, respectively; increasing the concentration of either heparin or heparinase did not result in a further reduction of bound authentic gB, indicating the residual bound gB was truly resistant, and not merely reduced in sensitivity, to heparin and heparinase treatments. These results suggest that gC binds solely to cellular HS whereas gB appears to bind first to cellular HS and then to a non-HS cellular receptor.

Binding affinity analysis. To further characterize the binding properties of gB, gC, and gD, we determined the binding affinities of the individual viral glycoproteins on MDBK cells. Cells were incubated with a constant amount of a labelled protein in the presence of increasing levels of an unlabelled homologous protein; the amounts of total input, cell-bound, and free labelled proteins were determined and subjected to Scatchard analyses. The binding affinity plots from one representative experiment are shown in Fig. 5. All of the glycoproteins tested exhibited saturable binding curves; namely, binding of a labelled protein was susceptible to competition by an unlabelled homologous protein. Soluble gB and both forms of gC and gD each showed single binding kinetics; in contrast, authentic gB showed a curve characteristic of dual binding kinetics. The calculated dissociation constant (*Kd*) and number of cell binding sites of each of the glycoproteins are summarized in Table 1. The values presented in Table 1 are the means derived from several independent experiments, whereas the Scatchard plots were drawn from one set of data (similar results were obtained in all experiments). Soluble gB and both

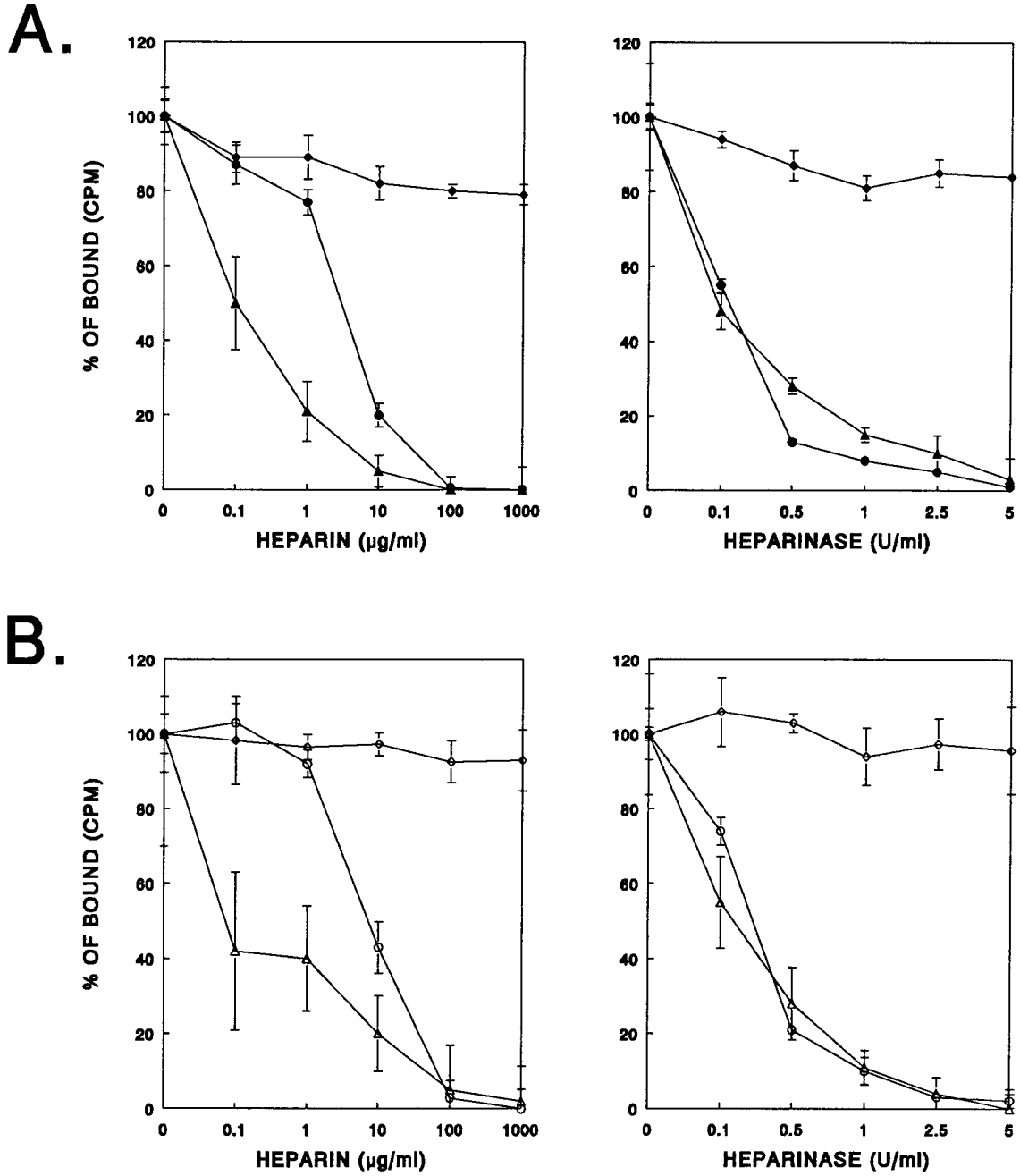

FIG. 2. Effects of heparin and heparinase treatments on binding of ^{125}I-labelled gB, gC, and gD to MDBK cells. For the heparin inhibition assay, confluent MDBK cells grown in 48-well plates were incubated with heparin at the indicated concentrations at 4°C for 1 h; this was followed by addition of approximately 2×10^5 cpm of an ^{125}I-labelled authentic glycoprotein (A) (▲, gB; ●, gC; ◆, gD) or soluble glycoprotein (B) (△, gBt; ○, gCt; ◇, gDt) and incubation at 4°C for 4 h. After incubation, cells were washed with MEM-FBS and lysed with 1% Triton X-100–1% SDS. Cell lysates were collected, and radioactivity was counted in a gamma counter. For heparinase treatment, cells were first treated with heparinase at the indicated concentrations at 37°C for 1 h. After three washes, the cells were incubated with ^{125}I-labelled glycoproteins for 4 h at 4°C; this was followed by the same procedure described above. The counts per minute bound in the presence of 100 μg of an unlabelled protein per ml was defined as background, which was always less than 10% of total counts per minute bound. The percentage of counts per minute bound was calculated as follows: % of cpm bound = [(cpm bound in the presence of inhibitor − background cpm)/(cpm bound in the absence of inhibitor − background cpm)] × 100%. Experiments were performed in triplicate, and the data represent means ± standard deviations for triplicate samples.

forms of gC and gD had similar dissociation constants ranging from 1.5×10^{-7} to 5.1×10^{-7} M. The low-affinity binding of authentic gB had a *Kd* of 5.2×10^{-7} M, which is similar to that of soluble gB, whereas the high-affinity binding had a *Kd* of 4.1×10^{-9} M, which is 2 orders of magnitude higher than that of soluble gB. Among gB, gC, and gD, gC has the largest number of cell binding sites, followed by gD and then gB.

Inhibition of viral plaque formation. In view of the facts that gB was able to bind to cellular HS and its binding to HS was coupled to an additional, non-HS receptor, it was of interest to further evaluate the relevance of these observations at the level of virus infection. We speculated that should the interaction of gB with cellular HS play a role in virus entry, heparin or heparinase treatment to block gB-HS interaction should be

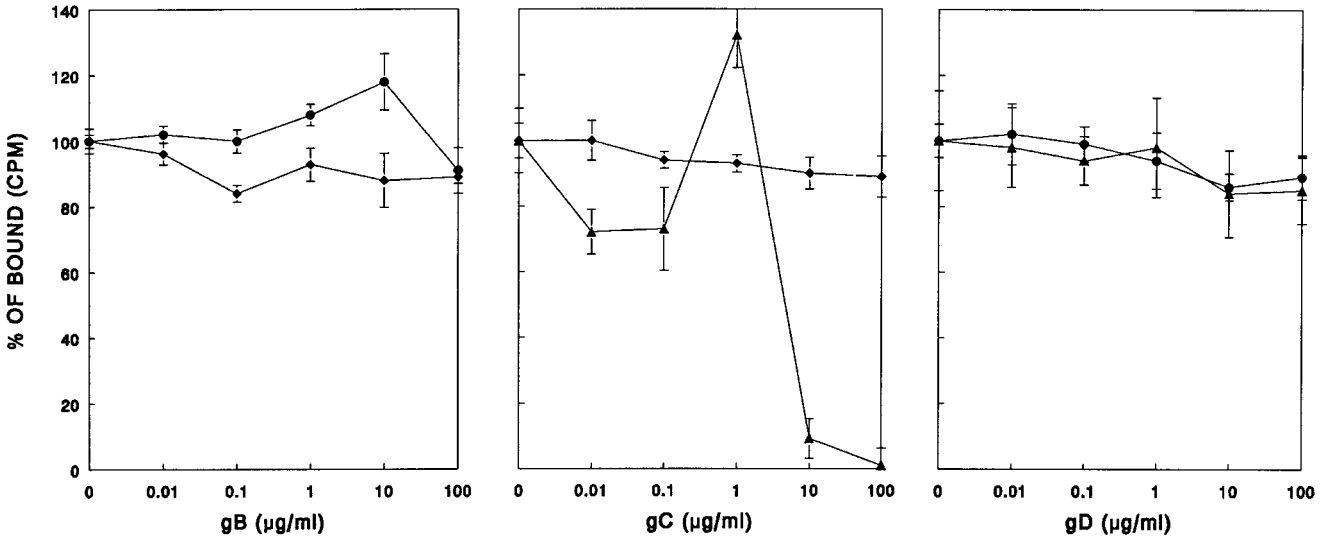

FIG. 3. Competition binding with heterologous proteins. Confluent MDBK cells in 48-well plates were incubated with approximately 2×10^5 cpm of ^{125}I-labelled gB (▲), gC (●), or gD (◆) in the presence of increasing amounts of a second unlabelled, heterologous glycoprotein at 4°C for 4 h. After incubation, cells were washed and lysed. Cell lysates were collected, and radioactivity was counted. The percentage of counts per minute bound was calculated as described for Fig. 2. Experiments were performed in triplicate, and the data represent means ± standard deviations for triplicate samples.

able to inhibit gC-negative virus infection. Therefore, the effects of heparin and heparinase treatments on gC-negative virus plaque formation were determined. As shown in Fig. 6, heparin and heparinase treatments inhibited plaque formation by both wt and gC-negative BHV-1, although heparinase treatment was less effective for the gC-negative mutant than for the wt virus. Of note, the fact that heparinase treatment was not as effective as heparin treatment in inhibiting viral plaque formation was not due to potential damage of the cells after heparinase treatment. We have repeated the same experiment with inclusion of vesicular stomatitis virus and found that either heparin or heparinase treatment had no effect on vesicular stomatitis virus plaque formation.

We previously showed that purified authentic gB, gC, or gD is able to inhibit virus adsorption (24). Since soluble gB was found to be defective in the HS-independent binding function, we next examined whether the soluble gB would retain the ability to inhibit viral plaque formation. As a control, both soluble gC and gD were also included in this experiment. The results are shown in Fig. 7. Consistent with previous results, all three authentic glycoproteins showed various degrees of inhibition of viral plaque formation. Soluble gC showed the same inhibitory effect as authentic gC; soluble gD appears to be slightly less effective than its authentic form. The most dramatic difference observed was that between soluble and authentic gBs. For example, authentic gB at a concentration of 100 μg/ml inhibited about 90% of plaque formation, whereas soluble gB at 200 μg/ml had essentially no effect on plaque formation. These observations from the viral plaque inhibition assay appear to be consistent with what would be predicted on the basis of the bindings studies with purified proteins. Of particular interest was that the loss of the high-affinity binding function of soluble gB was concomitantly associated with its inability to inhibit viral plaque formation. It needs to be pointed out that the virus adsorption inhibition assay was performed at 4°C, a temperature at which virus penetration does not occur. Therefore, the results obtained from this experiment most likely reflect the events during virus attachment rather than those during virus penetration.

DISCUSSION

In this article, we have described cell-binding properties of three major BHV-1 attachment proteins, gB, gC, and gD, in both authentic and soluble forms and have shown that all three viral proteins possess the characteristics for specific saturable binding to MDBK cells; gC and gD bind exclusively to cellular HS and non-HS components, respectively, whereas gB binds to cells in a two-component fashion, with initial binding to HS and secondary binding to a non-HS cellular receptor.

Interaction of gC and gD with MDBK cells. Although prior to this study it had been well established that BHV-1 gC, like its homologs in other alphaherpesviruses, binds to cellular HS, the issue of whether the initial interaction of gC with HS could trigger a further interaction of this molecule with other cellular components had not been directly addressed. It has been well documented that HS has a broad modulatory effect on the functions of a variety of proteins (13) and that binding of basic fibroblast growth factor to its secondary high-affinity receptors takes place only after it binds to HS (46). According to amino acid sequence analysis, BHV-1 gC has an R-G-D motif typical of ligands of cellular adhesion glycoproteins (6). The use of the R-G-D motif as a viral attachment ligand has been recently demonstrated for foot-and-mouth disease virus (25). In addition, it is known that gC homologs can bind serum complement component C3b (7). A more recent study suggested that one of the pathways of HSV type 1 (HSV-1) entry into the cells begins with the gC-dependent attachment of the virus to C3b receptors present on the cell surface (33). We show here that heparin and heparinase treatments not only prevented gC from binding to cells but were also able to remove gC that had bound to cells, indicating that gC, under the conditions tested, binds only to cellular HS. The contention that gC binds only to HS was further corroborated by the single binding kinetics exhibited by gC. While it is still formally possible that gC has non-HS binding sites on other cell types, our results favor the hypothesis that gC does not have non-HS cellular receptors on MDBK cells.

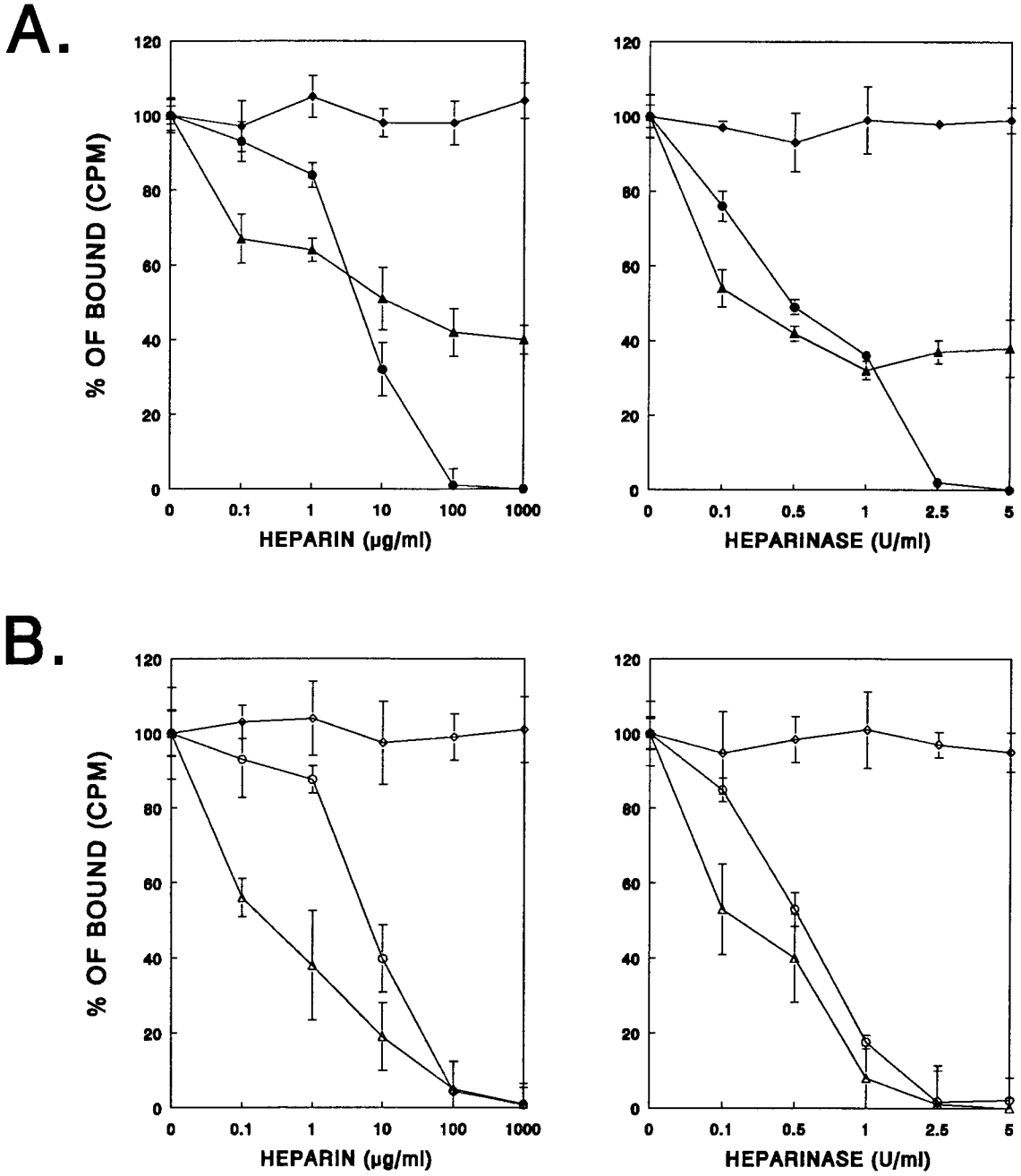

FIG. 4. Effects of heparin and heparinase treatments on bound gB, gC, and gD. Confluent MDBK cells grown in 48-well plates were incubated with approximately 2×10^5 cpm of an ^{125}I-labelled authentic glycoprotein (A) (▲, gB; ●, gC; ◆, gD) or soluble glycoprotein (B) (△, gBt; ○, gCt; ◇, gDt) at 4°C for 4 h. After three washes, cells were incubated either with heparin at 4°C for 1 h or with heparinase at 37°C for 1 h; this was followed by an additional three washes. Cell lysates were collected, and radioactivity was determined. The percentage of counts per minute bound was calculated as described for Fig. 2. Experiments were performed in triplicate, and the data represent means ± standard deviations for triplicate samples.

The observations made with BHV-1 gD in the present study are consistent with what has been previously described for soluble HSV-1 gD (14). Like that of HSV-1 gD, binding of BHV-1 gD to MDBK cells is independent of cellular HS (Fig. 2). According to the binding affinity analysis, BHV-1 gD was estimated to have a dissociation constant of 1.5×10^{-7} to 4.2×10^{-7} M and about 9.5×10^5 to 9.8×10^5 sites per MDBK cell, which also compare favorably with the dissociation constant of 1×10^{-7} to 1.8×10^{-7} M and the number of cell binding sites of 4×10^5 to 5×10^5 per cell reported for HSV gD (14).

Both BHV-1 gC and gD appear to have relatively low binding affinities compared with those of other known virus-cell interactions, which usually range between 10^{-9} and 10^{-10} M (1, 20, 35, 38). It is intriguing that gC has a relatively low binding affinity yet plays a dominant role in virus attachment. It appears, therefore, that factors other than binding affinity must be responsible for the function of gC in virus attachment.

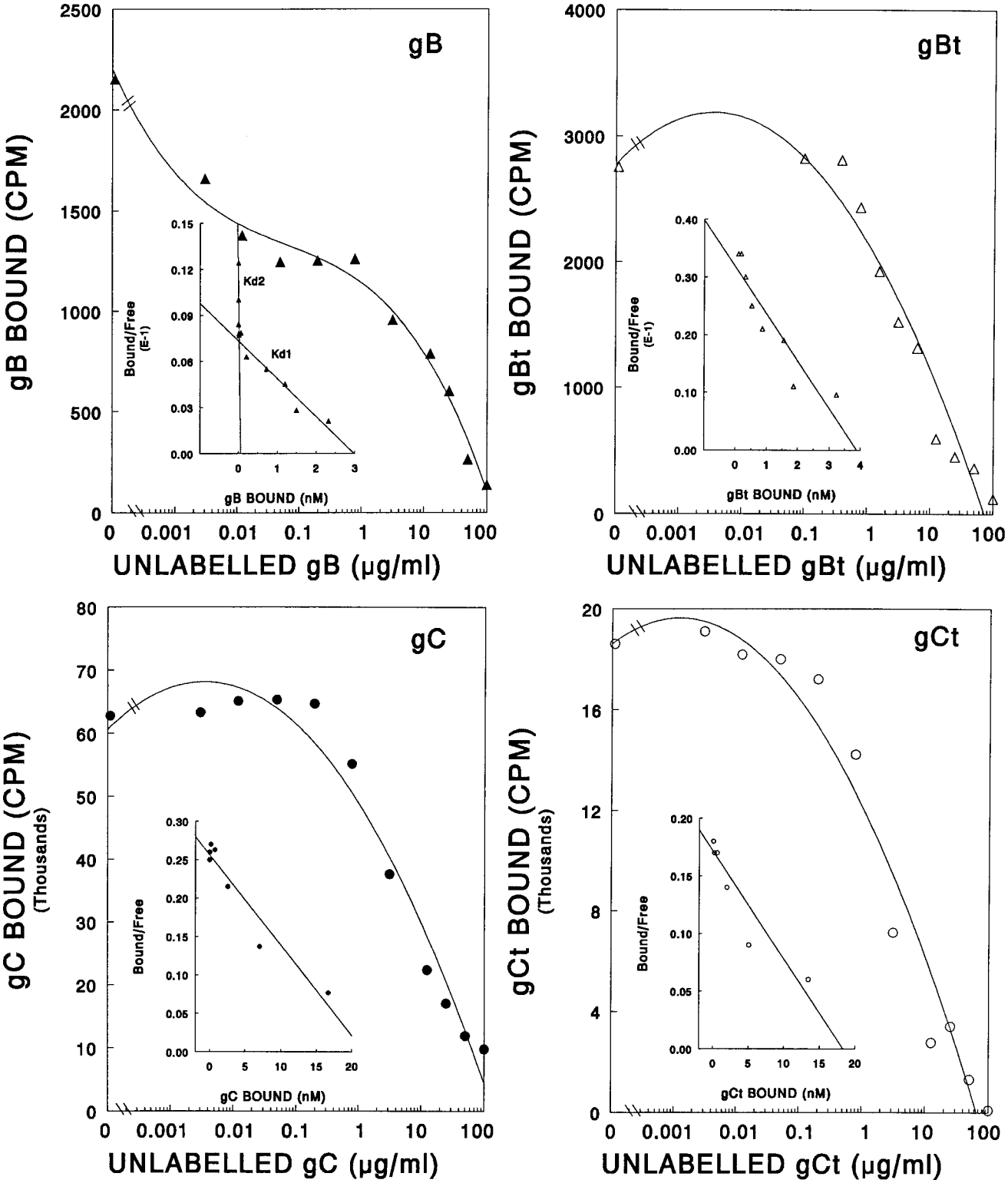

FIG. 5. Competition binding of gB, gC, and gD on MDBK cells. Confluent MDBK cells in 48-well plates were incubated with 2×10^5 cpm of an ^{125}I-labelled protein in the presence of increasing amounts of an unlabelled protein at 4°C for 4 h. After incubation, cells were washed and lysed. Cell lysates were collected, and radioactivity was counted. On the basis of the specific activity of each of the labelled proteins, the amounts of total input, free, and bound proteins were determined and subjected to Scatchard analysis (32). Shown in the insets are lines representing the best fits as determined by a linear regression analysis. E-1, 10^{-1}.

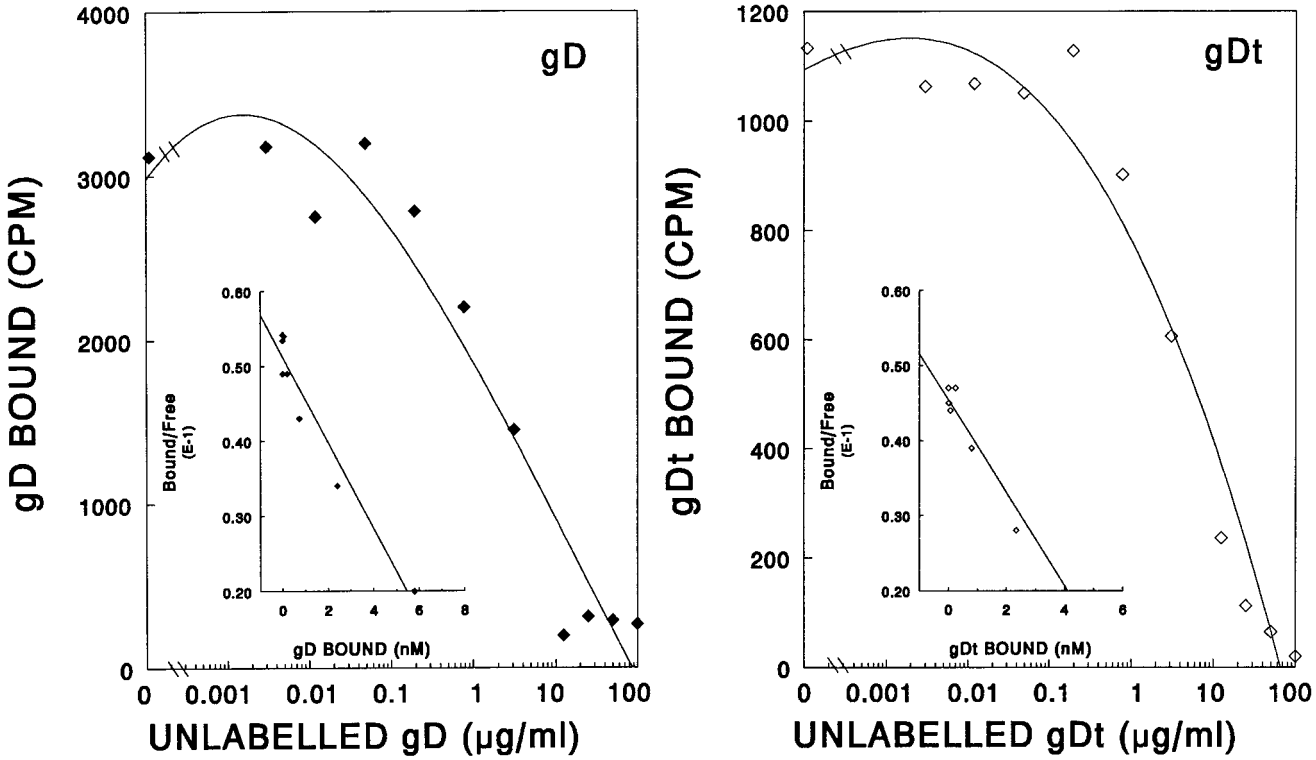

FIG. 5—*Continued.*

Among the three proteins tested, gC has the largest number of binding sites on cells, and it has been shown that gC homologs contain multiple heparin binding sites (23, 29). It is conceivable that the multiple binding epitopes present on a single molecule may provide a mechanism to enhance the overall avidity of binding to its receptors. Furthermore, gC molecules constitute the longest projections present on virions (37). All of these factors may collectively contribute to the preeminent cell-binding function of gC.

Interaction of gB with MDBK cells. Previously, it has been shown that gB of HSV can also bind to cellular HS (11, 12). However, direct binding of gB molecules of other alphaherpesviruses to HS has not been established. On the basis of protein fractionation of radiolabelled virions via heparin affinity chromatography, it was shown that gB of PrV binds to heparin-coated beads only in conjunction with gC (27), whereas gC of BHV-1 represents the only HS-binding protein

TABLE 1. Binding constants and numbers of binding sites of gB, gC, and gD on MDBK cells[a]

Viral protein	*Kd* (M, 10^{-7})	No. of binding sites/cell (10^5)
gB	5.2 ± 1.5, 0.041 ± 0.034	6.1 ± 4.3, 0.084 ± 0.056
gBt	2.2 ± 1.1	5.4 ± 3.4
gC	5.1 ± 1.7	46 ± 27
gCt	3.3 ± 0.4	29 ± 6
gD	1.5 ± 0.5	9.8 ± 2.6
gDt	4.2 ± 0.7	9.5 ± 2.8

[a] The values for authentic glycoproteins are means ± standard deviations for three experiments, and the values for the soluble glycoproteins are means ± standard deviations for two experiments.

(30). In the present study, we showed that both soluble and authentic gBs bind to cellular HS (Fig. 2). The binding of gB to cellular HS seems to be a weak interaction in comparison with the gC-HS interaction because gB was considerably more sensitive to heparin inhibition than gC (Fig. 2). We also found that gC was able to compete against gB binding, but gB was unable to compete against gC binding (Fig. 3). According to the estimated numbers of cell binding sites, gB appears to have about 10 times fewer HS binding sites than gC. This may indicate that gB and gC do not recognize the same spectrum of HS residues but, rather, that gB may recognize only a small fraction of the HS residues that are recognized by gC. However, the possibility that gB and gC have different specificities in response to heparin competition can not be formally excluded, and this remains to be further determined. The relatively weak interaction between gB and HS may provide a potential explanation for the failure to detect BHV-1 gB by heparin affinity chromatography (30). The same explanation may be also applicable to the binding of PrV gB to heparin-coated beads (27). It is possible that initial binding of gC to heparin may increase the local concentration of gB coexisting with gC on the same virion, resulting in a more efficient interaction of gB with heparin.

The most significant finding of the present study is the delineation of a dual binding function of gB; i.e., binding of gB to cellular HS is coupled to an additional binding to a non-HS receptor. This conclusion was established on the basis of the following observations. First, the addition of exogenous heparin or treatment of cells to remove cellular HS prevented both authentic and soluble gBs from binding to cells (Fig. 2); however, once bound to cells, a significant portion of authentic gB became resistant to either heparin competition or heparinase treatment. Second, according to binding affinity analysis, au-

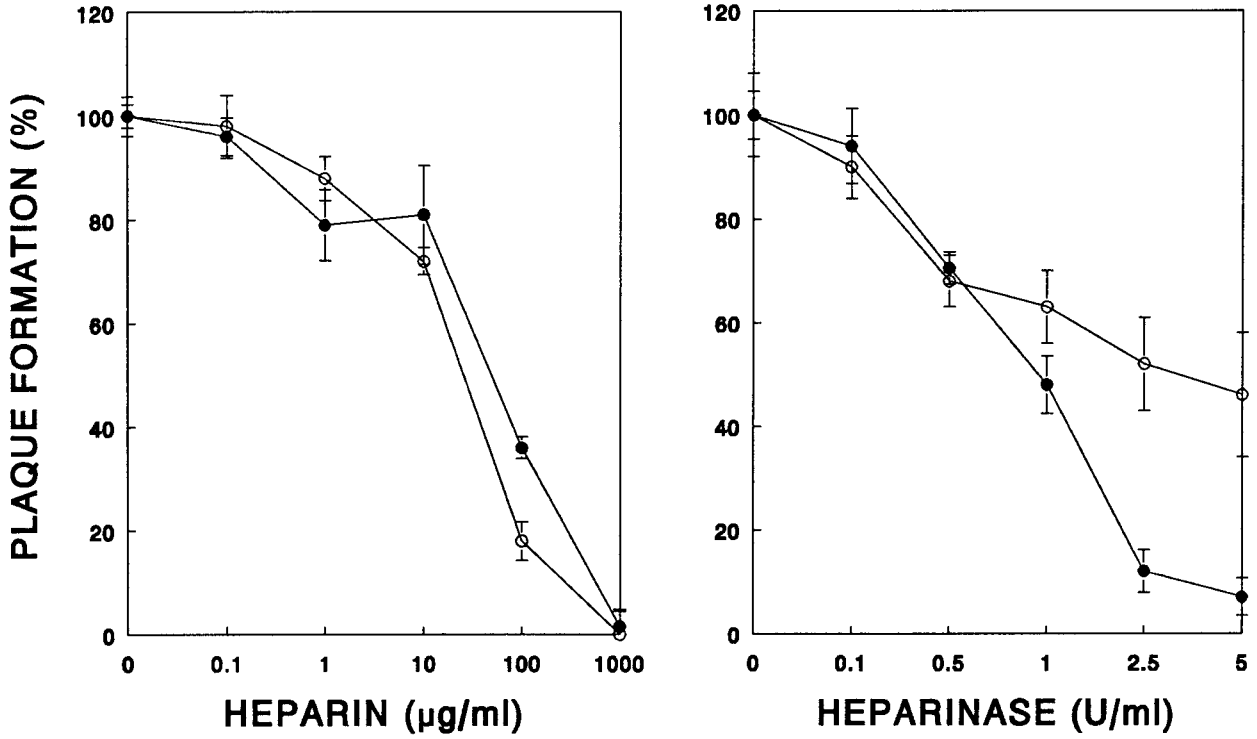

FIG. 6. Effects of heparin and heparinase treatments on viral plaque formation. For the heparin inhibition assay (left), confluent MDBK cells grown in 24-well plates were incubated with heparin at the indicated concentrations at 4°C for 1 h; this was followed by addition of approximately 150 PFU of wt BHV-1 (●) or gC-negative mutant virus (○) per well. The cells were incubated at 4°C for an additional 1 h. For heparinase treatment (right), cells were first treated with heparinase at the indicated concentrations at 37°C for 1 h. The cells were subsequently washed, infected with either wt or mutant virus, and incubated at 4°C for 1 h. After adsorption, the viral inoculum was removed, and cells were washed once with MEM and overlaid with 0.8% agarose in MEM containing 2% FBS. Plaques were counted 4 days later. The number of plaques formed in the absence of an inhibitor was defined as 100% plaque formation. Experiments were performed in triplicate, and the data represent means ± standard deviations for triplicate samples.

thentic gB showed a dual binding kinetics in that one binding component had a dissociation constant similar to that of soluble gB and the other had a significantly higher binding affinity. Finally, we found that authentic gB, but not soluble gB, was able to inhibit viral plaque formation. Since soluble gB and authentic gB both bind to cellular HS, the ability of the authentic gB to inhibit viral plaque formation must be the result of its competition against the virus for the non-HS binding sites but not from its competition for cellular HS binding sites.

According to the dual binding mechanism, binding of gB to its high-affinity cellular receptor requires it to be first bound with HS. Thus, one would expect that soluble gB which binds to cellular HS would also be able to prevent viral gB from binding to HS, by which means it would block viral gB binding to its high-affinity receptor and consequently inhibit viral plaque formation. Nevertheless, we found that soluble gB, under the conditions tested, was incapable of inhibiting viral plaque formation. A possible explanation for this is that gB binding to HS per se is a rather inefficient process, as discussed above, whereas for authentic gB or the gB present on the virions, the weak interaction between gB and cellular HS is stabilized by gB's secondary binding to the high-affinity receptors as well as by other binding forces such as those mediated by gD. As a result, the soluble gB which binds only to HS would not effectively compete against the gB present on virions for cell binding sites.

The secondary binding of gB to MDBK cells has an estimated *Kd* of 4.1×10^{-9} M and 8.4×10^{3} binding sites per cell, which agrees well with the values obtained for other virus-cell interactions. For example, human immunodeficiency virus gp120 binds to CD4-positive cells with a *Kd* of 3×10^{-9} to 4×10^{-9} (20, 35), reovirus binds to cells with a *Kd* of 3×10^{-9} M (1), and Epstein-Barr virus gp350/220 binds to cells with a *Kd* of 1.2×10^{-8} M (38). Because of the presence of the hydrophobic transmembrane domains, authentic gB molecules are expected to exist in solution as micelles. This physical property may have some effect on the accuracy of the estimation of the binding constant, but it is unlikely to affect the overall binding kinetics of gB, i.e., the dual binding property. In fact, binding affinity studies for a number of viruses have been carried out with whole virus particles (1, 3, 43). In the present study, we showed that the authentic and soluble forms of gC and gD, which were tested in parallel with gB, exhibited similar binding constants. Furthermore, the dual binding property of gB as revealed by binding kinetics is fully consistent with the observations made with the heparin and heparinase inhibition studies and plaque inhibition experiments, as described above.

We previously observed that purified gC inhibited not only adsorption of wt BHV-1 but also that of gC mutant virus, suggesting that gC may share binding sites with other viral attachment proteins (24). Here we have shown that gB also binds to cellular HS and, in addition, that the binding to HS constitutes a prerequisite for gB to further bind to a non-HS component. Therefore, it appears that the inhibitory effect of gC on binding of gC-negative virus is due to blockage of gB binding to cellular HS, which in turn blocks gB binding to its high-affinity receptor. The ability of gC to inhibit gB binding may also explain why among the three glycoproteins tested, gC is most effective in inhibiting viral plaque formation.

We found that although heparin was able to completely

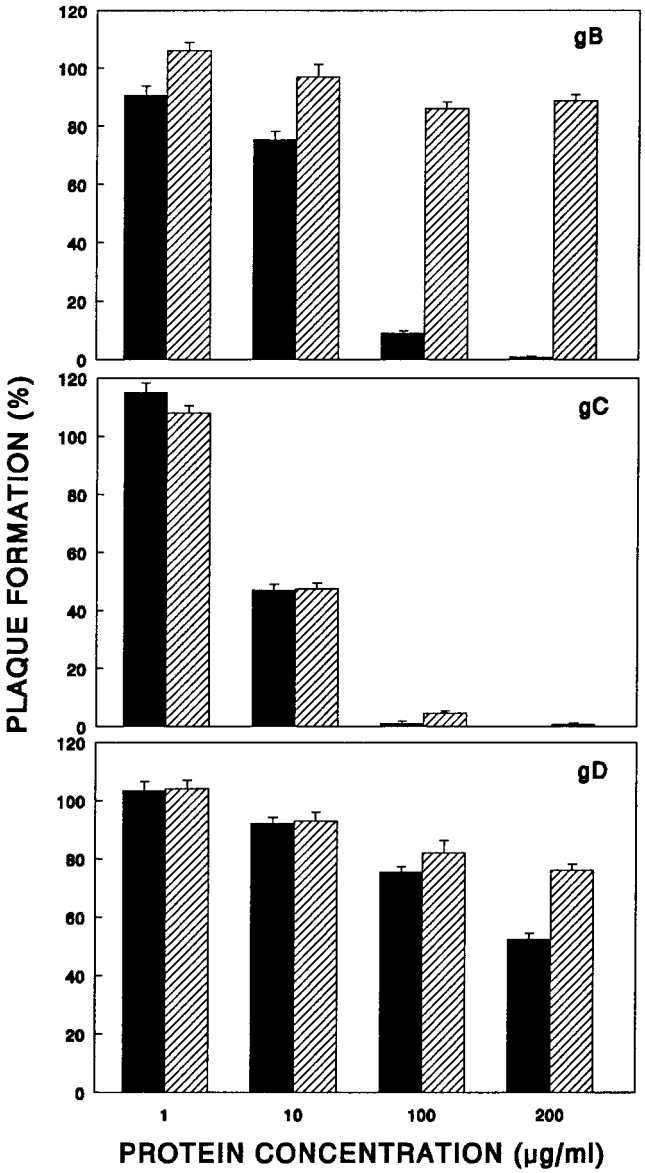

FIG. 7. Effect of purified BHV-1 glycoproteins on viral plaque formation. Confluent MDBK cells grown in 24-well plates were cooled at 4°C and then incubated with 50 μl of a glycoprotein at the indicated concentrations per well at 4°C for 1 h. After incubation, 50 μl of diluted BHV-1 (about 150 PFU) was added to each well of cells in the presence of glycoproteins; this was followed by an additional 1-h incubation at 4°C. After incubation, cells were washed three times with MEM and overlaid with 0.8% agarose. Viral plaques were counted 4 days later. The number of plaques formed in the absence of a glycoprotein was defined as 100% plaque formation. The data represent means plus standard deviations for triplicate samples. Solid bars, authentic protein; hatched bars, truncated protein.

block the infectivity of a gC-negative BHV-1 mutant, heparinase treatment inhibited only about 50% of gC-negative virus plaque formation under the conditions tested (Fig. 6). The lack of complete inhibition by heparinase treatment of the mutant virus plaque formation could be an indication that gB-HS interaction might not be essential for virus infection. However, given the fact that heparin was able to completely inhibit mutant virus infectivity, a more plausible explanation would be that the role of gB-HS interaction is not to provide a dominant binding force for gC-negative virus but rather is to provide a mechanism leading to productive virus entry. According to this premise, a gC-negative mutant would still bind to cells lacking HS, perhaps with a reduced efficiency, but because of the lack of gB-HS interaction it could not produce productive virus entry and therefore plaque formation. By the same premise, a certain fraction of the bound virions might become infectious after interacting with newly synthesized HS. As to exogenous heparin, it may act not simply by inhibiting binding of gC-negative virus; the association of gB with the exogenous heparin molecules may prevent gB from interacting with cellular HS residues, by which it inhibits gB binding to its high-affinity receptor. Nevertheless, further studies are necessary to ascertain whether the gB binding function is an essential function for virus entry. Previously, it has been shown that HSV was able to infect mutant cells which are defective in HS synthesis (10). In addition, the infectivity of gC-negative PrV has been shown to be refractory to inhibition by heparin or heparinase treatment (30).

Recently, Shieh and Spear showed that HSV gB-mediated cell fusion requires the presence of HS (34). In light of the fact that binding of BHV-1 gB to its high-affinity receptor also requires it to be first bound with HS, it is very tempting to speculate that the HS-dependent fusion activity exhibited by HSV-1 gB and the high-affinity binding of BHV-1 gB revealed in this study are related. However, a difference has also been noticed. For HSV gB, the fusion activity can be activated by addition of exogenous heparin, whereas for BHV-1 gB, high-affinity binding was not detected after addition of exogenous heparin to heparinase-treated cells.

The observation that the full-length gB and the truncated gB show different cell-binding activities is intriguing, and the reason for this has yet to be determined. Could the high-affinity binding activity observed with the full-length gB be caused by a nonspecific interaction between an aberrant hydrophobic structure of gB and the cell surface? This seems unlikely for the following reasons. First, the gB high-affinity binding is saturable (Fig. 5), a property which is characteristic of specific interaction between a ligand and its receptor. Second, the full-length gB was able to inhibit viral plaque formation. This suggests that the purified gB was able to compete against the gB present on the virus surface for the same cell binding sites, and thus the binding function of the purified gB resembles that of the authentic gB present on virions. Finally, heparin or heparinase treatment was able to completely prevent the authentic gB from binding to cells (Fig. 1). It is unlikely that a hydrophobic nonspecific binding could be affected by heparin or heparinase treatment. The fact that binding to HS is required for authentic gB to engage in the secondary high-affinity binding suggests that binding to HS may cause a conformational change of authentic gB resulting in exposure of its high-affinity binding sites. Therefore, a more plausible explanation would be that the ability of gB to bind to its high-affinity receptor is a conformation-dependent property and that for soluble gB, the truncation of its carboxyl-terminal amino acid sequence may have caused a defect in its overall conformation state which may preclude the proper exposure of its high-affinity binding site in response to HS binding. In support of this hypothesis, it has been shown that the carboxyl-terminal sequences of HSV gB and PrV gB have a profound effect on the overall gB structural and functional properties, including oligomerization and fusion activity of gB molecules (2, 28, 44). Studies are currently under way to investigate this possibility.

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