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Soluble Forms of Herpes Simplex Virus Glycoprotein D Bind to a Limited Number of Cell Surface Receptors and Inhibit Virus Entry into Cells

DAVID C. JOHNSON,[1]* RAE LYN BURKE,[2] AND TIMOTHY GREGORY[3]

Molecular Virology and Immunology Program, Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5[1]; Chiron Corporation, Emeryville, California 94608[2]; and Department of Process Development, Genentech, Inc., South San Francisco, California 94080[3]

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Herpes simplex virus type 1 (HSV-1) and HSV-2 plaque production was inhibited by treating cells with soluble forms of HSV-1 glycoprotein D (gD-1t) and HSV-2 glycoprotein D (gD-2t). Both glycoproteins inhibited entry of HSV-1 and HSV-2 without affecting virus adsorption. In contrast, a soluble form of HSV-2 glycoprotein B had no effect on virus entry into cells. Specific binding of gD-1t and gD-2t to cells was saturable, and approximately 4×10^5 to 5×10^5 molecules bound per cell. Binding of gD-1t was markedly reduced by treating cells with certain proteases but was unaffected when cell surface heparan sulfate glycosaminoglycans were enzymatically removed or when the binding was carried out in the presence of heparin. Together, these results suggest that gD binds to a limited set of cell surface receptors which may be proteins and that these interactions are essential for subsequent virus entry into cells. However, binding of gD to its receptors is not required for the initial adsorption of virus to the cell surface, which involves more numerous sites (probably including heparan sulfate) than those which mediate gD binding.

The entry of herpes simplex viruses (HSVs) into cells appears to be a complicated process involving at least four viral glycoproteins and more than one type of interaction with the cell surface. The initial adsorption of HSV type 1 (HSV-1) to cells apparently involves very numerous sites and is difficult to saturate (14). Experiments involving monoclonal antibodies or virion-derived liposomes suggested a role for gB, gC, and gD in virus adsorption to cells (8, 15). More recently, evidence has been presented that HSV adsorbs to heparan sulfate (36), and this initial interaction of HSV with cells may be mediated by gB and gC (D. WuDunn, R. Santos, and P. G. Spear, personal communication). In addition, glycoprotein gIII of pseudorabies virus, a homolog of HSV gC, binds to heparan sulfate (23). However, none of these viral glycoproteins alone is required for the initial interactions of HSV with the cell surface because viruses lacking gB, gC, or gD can adsorb to cells (4, 11, 12, 20). Nevertheless, gB and gD are essential for secondary interactions at the cell surface which lead to virus entry into cells (4, 20).

Johnson and Ligas (14) recently defined a set of cell surface receptors which are required for entry of HSV-1 and HSV-2 into cells. These receptors are much more limited in number than the sites to which HSV can adsorb and require the presence of gD in the envelope of virions. UV-inactivated virus particles containing gD were able to block the entry process of HSV, whereas UV-inactivated particles lacking gD were unable to block HSV entry. Both types of virus particles, containing or lacking gD, adsorbed equally well to cells, and at least 50-fold more radiolabeled virus particles adsorbed to the surfaces of cells than were needed to block virus entry (14). Similarly, cell lines constitutively expressing HSV-1 gD were found to be resistant to infection with HSV-1 and HSV-2 (5, 16). Here, it is likely that gD present in the plasma membrane of transfected cells interacts with receptors blocking virus entry. These observations suggested that gD in the virion of HSV interacts with a limited set of cell surface molecules, which we will designate gD receptors, during or shortly before virus penetration into cells, a process which apparently involves fusion of the virion envelope with the plasma membrane (9, 15, 25). In contrast, cell lines expressing gB or gC are not resistant to infection (1, 27), and there is no evidence, to date, for saturable receptors for these viral proteins.

Genetically engineered forms of HSV-1 gD and HSV-2 gD and gB have been constructed as subunit vaccines (2, 3, 18, 28, 30, 32; R. L. Burke et al., unpublished results). These are soluble forms of the viral glycoproteins, denoted here gD-1t, gD-2t, and gB-2t, which are truncated by removal of the transmembrane domains of the polypeptides so that they are secreted rather than being maintained in cell membranes and are purified from culture supernatants of CHO cell transformants. We tested the effects of gD-1t and gD-2t on replication of HSV-1 and HSV-2 and found that the purified glycoproteins inhibited HSV-1 and HSV-2 plaque production and virus penetration. In addition, gD-1t and gD-2t bound to a limited number of cell surface receptors.

MATERIALS AND METHODS

Cells and viruses. Vero cells and human R970-5 cells (26) were grown in α minimal essential medium (α-MEM) (GIBCO Laboratories, Burlington, Ontario) supplemented with 7% fetal bovine serum (FBS). HSV-1 strain F and HSV-2 strain G were obtained from P. G. Spear, Northwestern University, Chicago, Ill.

Enzymes and reagents. Trypsin (type XIII, tolylsulfonyl phenylalanyl chloromethyl ketone treated), chymotrypsin (type VII, TLCK treated), and protease type XIV (pronase) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Heparinase, heparitinase, and chondroitin ABC lyase were obtained from ICN ImmunoBiologicals (Mississauga, Ontario). The units are expressed in conventional units, where

* Corresponding author.

1 conventional unit equals 6 mIU. Heparin was obtained from Calbiochem. Bovine serum albumin (BSA) (fraction V) was obtained from Boehringer Mannheim.

Soluble forms of HSV-1 gD (gD-1t), HSV-2 gD (gD-2t), and HSV-2 gB (gB-2t) and radioiodination. gD-1t was harvested from culture supernatants of the gD10.2 cell line (18) and was purified by immunoaffinity chromatography (3). Purified gD-1t is 275 amino acids in length. gD-2t is 326 amino acids in length and was similarly purified by chromatography from amplified CHO cells (R. L. Burke et al., unpublished results). A soluble form of HSV-2 gB, denoted gB-2t, which is 696 amino acids in length, was constructed in a fashion similar to that reported by Stuve et al. (32) and purified from culture supernatants of transfected CHO cells by immunoaffinity chromatography. gD-1t and gD-2t were radiolabeled by incubating the protein with $Na^{125}I$ (1 to 3 mCi) and 2 Iodobeads (Pierce Chemicals, Rockford, Ill.) in a total volume of 0.5 ml of phosphate-buffered saline (PBS) for 15 to 30 min. The protein was separated from free $Na^{125}I$ by chromatography on Sephadex G-25.

Inhibition of HSV-1 and HSV-2 plaque production by gD-1t and gD-2t. Vero or R970-5 cell monolayers in 24-well dishes (approximately 1.5×10^5 cells per well) were treated for 1.5 h at 4°C with gD-1t, gD-2t, or BSA (Sigma Chemical Co., St. Louis, Mo.) at 200, 100, or 25 μg/ml diluted in 150 μl of α-MEM containing 1% FBS. HSV-1 or HSV-2 diluted in α-MEM containing 1% FBS (150 to 200 PFU/20 μl) were added to the wells for a further 1.5 h at 4°C. The virus and glycoproteins were removed, and either α-MEM containing 1% FBS and 0.1% human globulin (Canadian Red Cross, Hamilton, Ontario) or α-MEM containing 1% FBS, 0.1% human globulin, and gD-1t, gD-2t, or BSA at the appropriate concentrations was added to the wells. After 2 days, the medium was removed and the cells were stained with crystal violet.

Inhibition of HSV-1 and HSV-2 entry into cells by gD-1t and gD-2t. Human R970-5 cells growing in 24-well dishes (≈1.0 × 10^5 cells per well) were treated with gD-1t, gD-2t, gB-2t, or BSA diluted in 150 μl of α-MEM containing 1% FBS for 1.5 h at 4°C. HSV-1 or HSV-2 diluted in α-MEM containing 1% FBS (20 μl containing 5 PFU/cell) was added, and the cells were incubated for a further 2 h at 4°C. The virus and glycoproteins were removed, and the cells were washed twice with warm medium lacking methionine and incubated at 37°C with medium lacking methionine and containing gD-1t, gD-2t, gB-2t, or BSA at the indicated concentrations. After 2 h, $[^{35}S]$methionine (50 μCi/ml; Amersham, Oakville, Ontario) was added for 3 to 4 h. Detergent extracts of the cells were prepared as described before (13), and thymidine kinase was immunoprecipitated.

Immunoprecipitation and gel electrophoresis. Cell extracts were sonicated, clarified by centrifugation at 80,000 × *g* for 1 h, and mixed with rabbit polyclonal serum specific for thymidine kinase (20), which was kindly provided by W. Summers, Yale University, New Haven, Conn. The antibody-antigen complexes were precipitated with protein A-Sepharose as described before (13, 14). Samples of precipitated proteins were electrophoresed in 10% *N,N'*-diallytartardiamide cross-linked polyacrylamide gels infused with 2,5-diphenyloxazole and exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.) as described previously (13).

Adsorption of radiolabeled HSV-1 and HSV-2 to cells. $[^{35}S]$methionine-labeled HSV-1 and HSV-2 virions were purified by using dextran T-10 gradients as described previously (20) except that Vero cells infected with HSV-1 or HSV-2 were labeled from 4 to 12 h postinfection in medium 199 lacking methionine and supplemented with 1% FBS and 50 to 100 μCi of $[^{35}S]$methionine; the cells were then incubated until 20 to 24 h postinfection with α-MEM containing 1% FBS. R970-5 cells growing in 24-well dishes were pretreated with gD-1t, gD-2t, BSA, or heparin diluted in α-MEM containing 5 mg of BSA per ml and 2% FBS for 1.5 h at 4°C and then incubated with labeled virions (approximately 100 PFU/cell and a total of 2×10^5 to 5×10^5 cpm/well) in the presence of gD-1t, gD-2t, BSA, or heparin for 2 h at 4°C. The cells were washed three times with α-MEM containing 10% FBS and lysed in PBS containing 1% Nonidet P-40 and 0.5% sodium deoxycholate, and radioactivity in the cell lysates was counted with ACS aqueous scintillation counting fluid (Amersham) and a scintillation counter.

RESULTS

Soluble gD-1t and gD-2t inhibit HSV-1 and HSV-2 plaque production. Vero cell monolayers were incubated with soluble gD-1t or gD-2t, using two courses of treatment. In one case, the cells were treated for 1.5 h with soluble gD-1t, gD-2t, or BSA and then HSV-1 or HSV-2 was added for a further 1.5 h at 4°C. The glycoproteins and unbound virus were removed, medium without gD-1t or gD-2t was added, and plaques were allowed to develop for 2 days (Fig. 1, wells A). Alternatively, the cells were incubated with soluble gD or BSA, virus was added, and then the medium containing glycoproteins and unbound virus was removed and fresh medium containing gD-1t, gD-2t, or BSA was added to the wells for the following 2 days (Fig. 1, wells B). A reduction of 40 to 75% in the number of plaques was observed when cells were treated with 100 or 200 μg of gD-1t or gD-2t per ml when the glycoproteins were present before and during the virus adsorption stage but not during the 2-day virus growth phase. Under these conditions, lower concentrations of either gD-1t or gD-2t (25 μg/ml) had less of an effect. If instead the soluble gD-1t or gD-2t was present continuously, plaque production was completely inhibited at concentrations of 200 and 100 μg/ml and inhibited by over 75% when 25 μg/ml was used. Therefore, for complete inhibition of HSV infection, soluble gD-1t or gD-2t must be continuously present in the culture medium. Similar results were obtained with human R970-5 cells, although the plaques were more difficult to visualize and, overall, were fewer in number.

To test the possibility that gD-1t or gD-2t could bind to HSV virions, causing inactivation of the viruses by unknown mechanisms, infectious HSV-1 was incubated with 100 μg of gD-1t per ml for 2 h at 4°C, and then the soluble gD-1t was removed by pelleting the virus in an ultracentrifuge. A preparation of HSV-1 treated with gD-1t produced as many plaques as virus similarly treated with BSA (100 μg/ml) (results not shown).

Soluble gD-1t and gD-2t inhibit HSV entry into cells. To examine the possibility that gD-1 and gD-2 block interactions of virus with gD receptors, the entry of HSV into cells was examined. Cells were treated with gD-1 or gD-2 at 4°C, and then HSV-1 or HSV-2 was added for a further period at 4°C. The glycoproteins and unbound virus were removed, and the cells were incubated at 37°C for 2 h in the presence of gD-1t or gD-2t and then briefly labeled with $[^{35}S]$methionine. To quantitate virus infection, a viral early polypeptide, thymidine kinase, was immunoprecipitated from cell extracts. Soluble gD-1t markedly inhibited the entry of HSV-1 at concentrations of 25 to 200 μg/ml (Fig. 2). Similarly, gD-2t

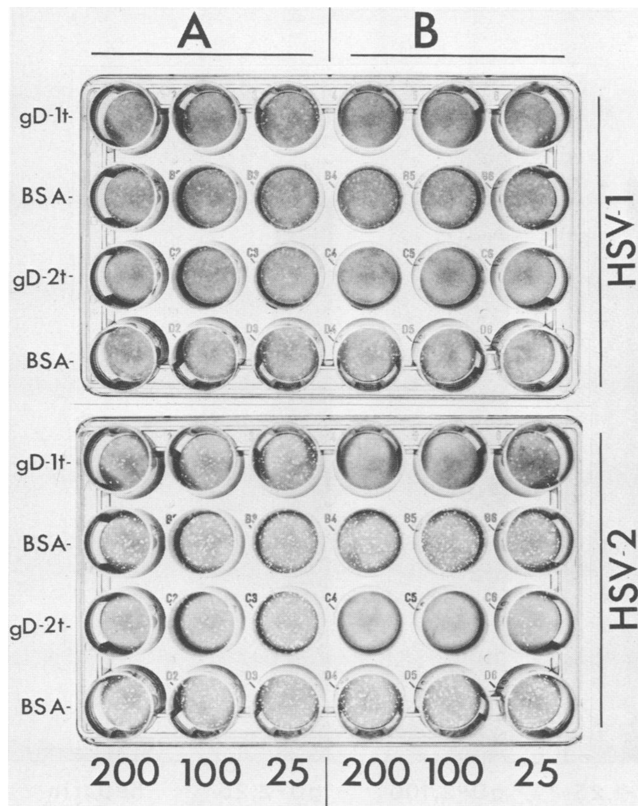

FIG. 1. Soluble gD-1t and gD-2t inhibit HSV-1 and HSV-2 plaque production. Vero cells growing in 24-well dishes were treated with either gD-1t, gD-2t, or BSA (horizontal columns) at 200, 100, or 25 μg/ml (vertical columns) in 150 μl of α-MEM containing 1% FBS for 1.5 h at 37°C. HSV-1 (upper plate) or HSV-2 (lower plate) was added as 150 PFU diluted in 20 μl for 1.5 h at 37°C, and then the glycoproteins and virus were removed. Wells on the left side of the plate (A) were then incubated for 2 days with α-MEM containing 1% FBS and 0.1% human globulin. Wells on the right side of the plate (B) were treated similarly except that the virus and glycoproteins were removed and the cells were incubated for 2 days with α-MEM containing 1% FBS, 0.1% human globulin, and either gD-1t, gD-2t, or BSA at the indicated concentrations. The monolayers were stained with crystal violet and photographed.

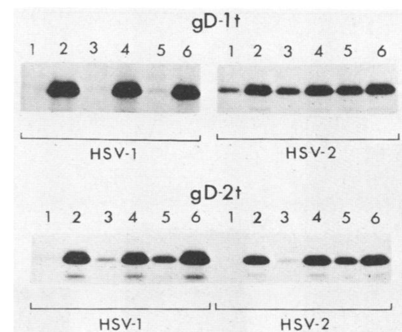

FIG. 2. HSV-1 and HSV-2 entry into cells is inhibited by gD-1t and gD-2t. Human R970-5 cells were treated with gD-1t, gD-2t, or BSA diluted in α-MEM containing 1% FBS for 1.5 h at 4°C, and then HSV-1 or HSV-2 was added and the cells were incubated for a further 2 h at 4°C. The unbound virus and glycoproteins were removed, and the cells were washed twice with warm medium lacking methionine and incubated at 37°C with medium lacking methionine and containing gD-1t, gD-2t, or BSA at the indicated concentrations. After 2 h, [^{35}S]methionine was added for 4 h. Detergent extracts of the cells were prepared, and the viral early protein thymidine kinase was immunoprecipitated as a measure of virus infection. Lanes 1, gD-1t or gD-2t, 200 μg/ml; lanes 2, BSA, 200 μg/ml; lanes 3, gD-1t or gD-2t, 100 μg/ml; lanes 4, BSA, 100 μg/ml; lanes 5, gD-1t or gD-2t, 25 μg/ml; lanes 6, BSA, 25 μg/ml.

markedly inhibited HSV-1 and HSV-2 entry at concentrations of 100 or 200 μg/ml, and some inhibition was evident at 25 μg/ml. However, the inhibitory effects of gD-1t on HSV-2 entry into cells were less pronounced. We are unable to explain the differential inhibition of HSV-1 and HSV-2 entry by this preparation of gD-1t, although this finding may be related to the observation that our stocks of HSV-2 contain much higher particle-PFU ratios than our stocks of HSV-1. Since equal numbers of infectious HSV-1 and HSV-2 particles were used in these experiments, gD-1t may be less effective in blocking the expression of thymidine kinase induced by larger numbers of HSV-2 particles. This explanation assumes that noninfectious HSV-2 particles can be transcriptionally active. Alternatively, HSV-1 and HSV-2 receptors may share determinants but differ in affinities for gD-1t and gD-2t. We note that gD-1t is significantly shorter than gD-2t and may lack sequences involved in gD-2 receptor recognition.

Soluble form of gB does not inhibit entry of HSV. The effects of soluble gD on virus entry into cells may result from specific interactions with virus receptors or, alternatively, nonspecific effects of viral glycoproteins on cells or virus replication. In part to exclude the latter interpretation and to further examine the role of gB during virus entry, we treated cells with a soluble form of HSV-2 gB, denoted gB-2t. Cells treated with gB-2t at a concentration of 200 μg/ml were infected with HSV-1, whereas cells treated with 50 μg of gD-2t per ml were not infected (Fig. 3). Thus, inhibition of HSV entry appears to be a property of gD and not of gB.

HSV-1 and HSV-2 adsorption to cells is unaffected by gD-1t and gD-2t. The initial interactions of HSV with the cell surface do not depend on gD and apparently involve very numerous or nonsaturable adsorption sites (14, 20). The adsorption of radiolabeled HSV-1 and HSV-2 virions to cells treated continuously with gD-1t or gD-2t was measured. Both soluble glycoproteins had little effect on the adsorption of HSV-1 or HSV-2 to cells (Fig. 4). In contrast, heparin inhibited the adsorption of both labeled HSV-1 and HSV-2

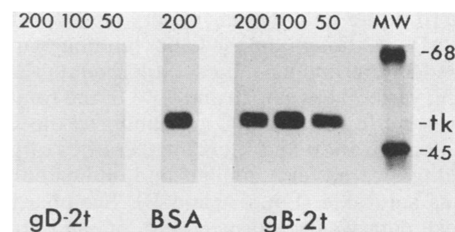

FIG. 3. Entry of HSV-1 is unaffected by treating cells with soluble gB. R970-5 cells were treated with 200, 100, or 50 μg of gD-2t, BSA, or gB-2t per ml diluted in medium for 1.5 h at 4°C, and then HSV-1 was added for a further 1.5 h at 4°C. The unbound virus and glycoproteins were removed, and the cells were washed twice with warm medium lacking methionine and incubated at 37°C with medium lacking methionine and containing gD-2t, BSA, or gB-2t at the indicated concentrations. After 2 h, [^{35}S]methionine was added for 4 h. Detergent extracts of the cells were prepared, and thymidine kinase (tk) was immunoprecipitated. Molecular size markers (lane MW) of 68 and 45 kilodaltons are indicated.

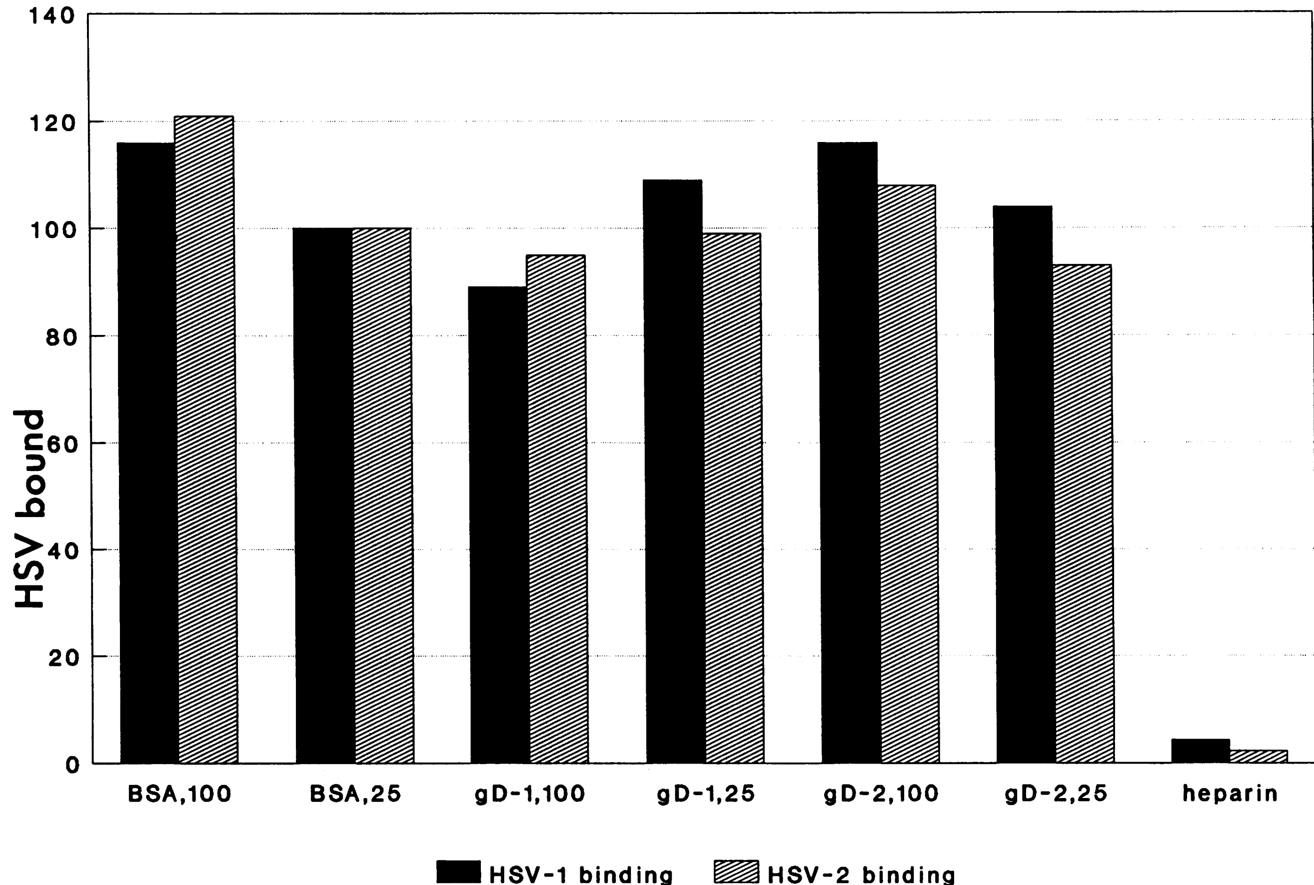

FIG. 4. HSV-1 and HSV-2 adsorption to cells is unaffected by gD-1t and gD-2t. R970-5 cells growing in 24-well dishes were treated with BSA, gD-1t, or gD-2t at 25 or 100 μg/ml or with heparin (5 μg/ml diluted in 150 μl of α-MEM containing BSA [5 mg/ml] and 2% FBS) at 4°C for 1.5 h, and then 20 μl of medium containing [^{35}S]methionine-labeled gradient-purified HSV-1 or HSV-2 was added for 2 h at 4°C. The cells were washed three times with medium containing BSA (5 mg/ml) and 2% FBS, and cell extracts were counted. Adsorption of radioactive virus to cells in the presence of BSA (25 μg/ml) was arbitrarily set at 100.

by over 90%. Thus, the adsorption of HSV to cells is unaffected by soluble forms of gD.

Specific binding of gD-1t and gD-2t to cells. Human R970-5 cells were incubated with radiolabeled gD-1t or gD-2t in the presence or absence of excess unlabeled gD to control for nonspecific binding. Nonspecific binding observed in the presence of a 40- to 500-fold excess of unlabeled gD was subtracted from the total binding observed in the absence of unlabeled gD. Although nonspecific binding varied from experiment to experiment, in most experiments the nonspecific binding varied between 20 and 70% of the total binding. Binding was performed at 4°C to minimize the effects of receptor internalization and recycling. Profiles of gD-1t and gD-2t binding were very similar and indicated that the binding was saturable (Fig. 5A and B). Scatchard analysis (29) of these data was performed (Fig. 5C and D), and the results indicated that 5.2×10^5 molecules of gD-1t and 4.0×10^5 molecules of gD-2t were bound per cell. The dissociation constants calculated from the slopes of these lines were 2.6×10^{-7} M for gD-1t and 2.3×10^{-7} M for gD-2t.

Specific binding of gD-1t and gD-2t is reduced by protease treatment but not by heparinase treatment or heparin. In order to begin to characterize the receptors which bind gD, cells were treated with various proteases to determine whether the receptors were sensitive to proteolysis. Cells were scraped from the dishes untreated, removed by using EDTA or EGTA (ethylene glycol tetraacetic acid), or treated for a brief period with trypsin, chymotrypsin, or pronase, which caused rapid cell rounding and detachment from the plastic. The cells in suspension were then extensively washed with large excesses of BSA to remove exogenous proteases or chelators and incubated with 1 μg of labeled gD-1 in the presence or absence of a 50-fold excess of unlabeled gD-1. Specific binding of gD-1t was affected only marginally by treating the cells with EGTA or EDTA; however, trypsin, chymotrypsin, and pronase reduced binding more extensively (Fig. 6). The most pronounced effects were observed with pronase, which reduced binding by 60% when 0.1 mg/ml was used and 90% when 1.0 mg/ml was used. Cells treated with proteases for these brief periods remained able to exclude trypan blue stain. Therefore, gD receptors are sensitive to proteolysis, and although the results suggest that receptors for gD are proteins, it is also possible that major alterations to the surfaces of cells caused by proteolysis affect nonprotein receptors.

Cells treated with heparinase or heparitinase, which remove heparan sulfate glycosaminoglycans, or heparin do not bind HSV-1 or HSV-2 (36). We tested the effects of enzymatic removal of cell surface heparan sulfate and heparin on the binding of soluble gD. Treatment of R970-5 cells with heparinase, heparitinase, or chondroitin ABC lyase, which removes surface chondroitin and dermatan sulfate gly-

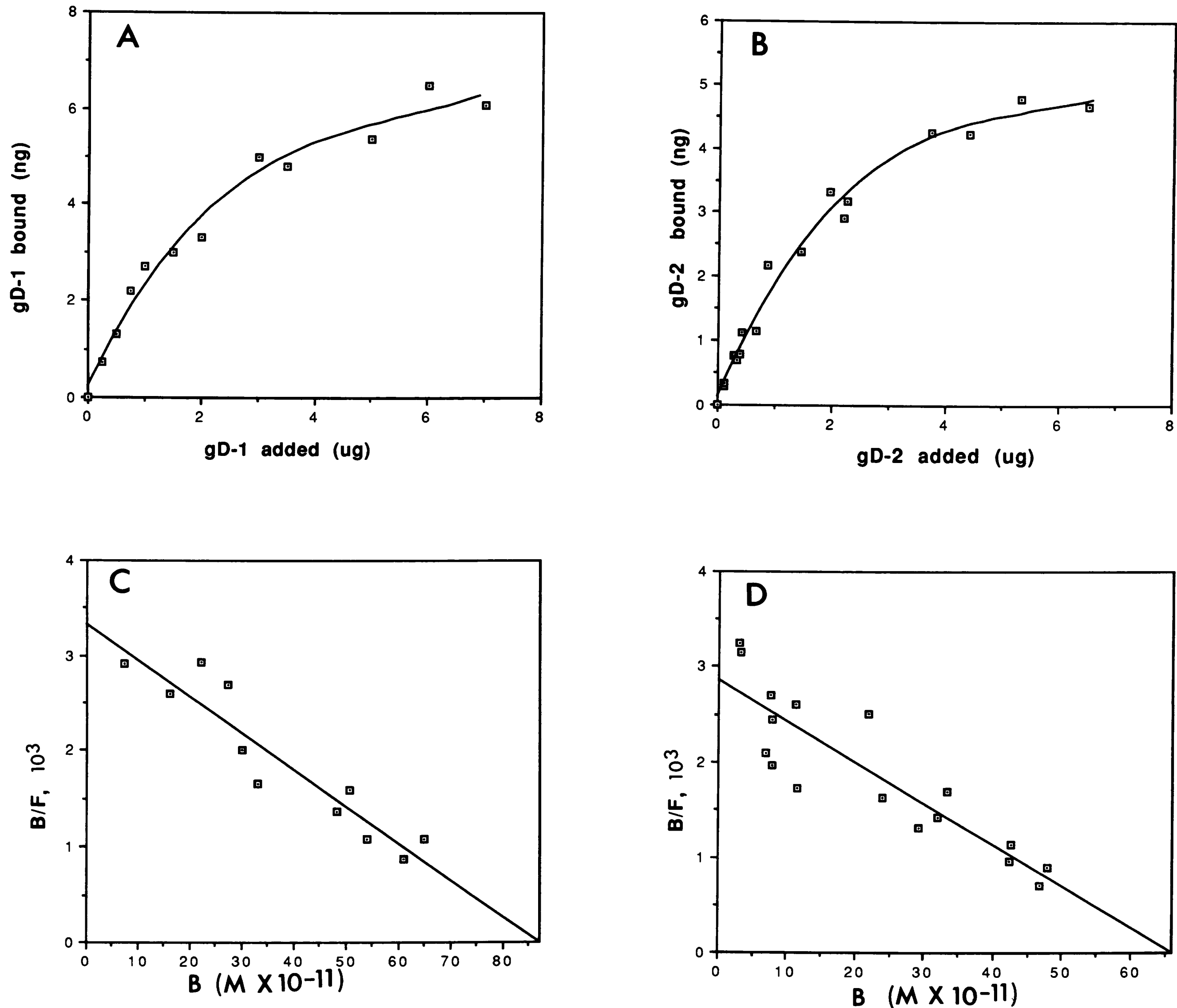

FIG. 5. Binding of radiolabeled gD-1t and gD-2t to cells. Iodinated gD-1t (A) or gD-2t (B) was diluted in PBS containing 2% FBS and BSA (5 mg/ml) (PBS/FBS/BSA) and incubated for 4 h at 4°C with confluent monolayers of R970-5 cells in 24-well dishes in the presence or absence of a 40- to 500-fold excess of unlabeled gD-1t or gD-2t. The cells were rapidly washed three times with PBS/FBS/BSA, and the cells were extracted with PBS containing 1% Nonidet P-40 and 0.5% sodium deoxycholate. The amount of bound gD-1t or gD-2t was determined by counting radioactivity in cell extracts. The specific binding of gD-1t (A) or gD-2t (B) was calculated by subtracting the nonspecific binding observed in duplicate wells incubated with labeled gD-1t or gD-2t in the presence of excess unlabeled gD from the total binding observed in duplicate wells incubated with gD-1t or gD-2t in the absence of unlabeled protein. Scatchard analysis (29) was performed on the binding of gD-1t (C) and gD-2t (D). B, Bound; B/F, bound-free ratio.

cosaminoglycans, had little effect on the binding of gD-1t to cells (Fig. 7). The small reduction (20%) produced by heparinase in this experiment was not observed in other experiments. Similar results were obtained with gD-2t, and treatment of R970-5 cells with heparinase or heparitinase before addition of approximately 100 PFU of HSV-1 per 2-cm^2 well inhibited plaque production by over 85%, although when cells were treated with heparinase or heparitinase and incubated with larger quantities of virus (multiplicity of infection, 0.5) the cells became infected. Heparin, which completely abolished HSV binding and plaque production when present at concentrations of 5 μg/ml, had little effect on the specific binding of gD to cells (Fig. 7).

DISCUSSION

Of the many animal viruses which have been described, only a very few have been shown to utilize defined cell surface molecules as receptors. Notably, human immunodeficiency virus was found to interact with the CD4 molecule found on helper T cells (6, 21, 22). Epstein-Barr virus uses the CR2 receptor found on B cells as a receptor (7, 24), and more recently, ICAM-1 was found to be the major receptor for human rhinoviruses (10, 31). These viruses replicate in a restricted set of cell types expressing virus receptors. In contrast, viruses such as HSV bind to and infect a wide spectrum of cell types derived from diverse species. This

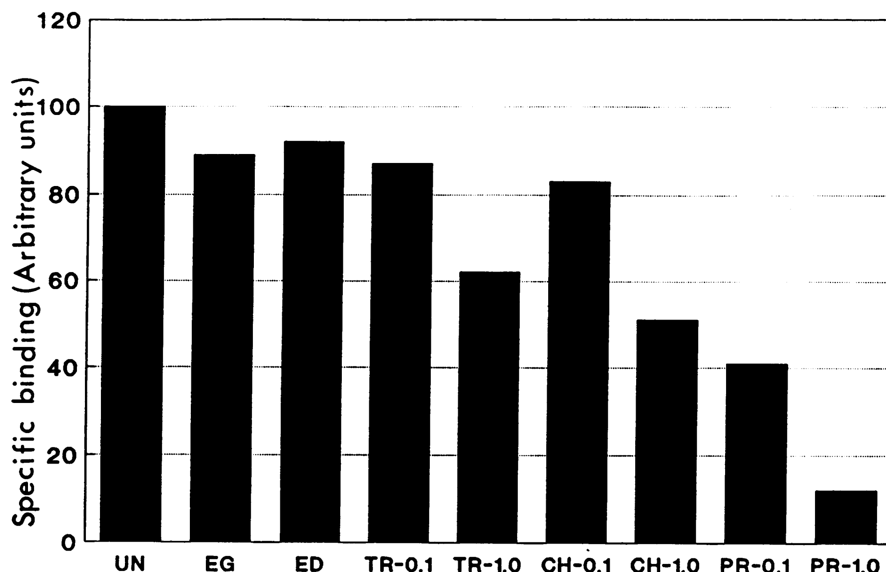

FIG. 6. Specific binding of gD-1t is reduced by treating cells with proteases. R970-5 cells growing in 24-well dishes were washed twice with PBS and either scraped from the wells (untreated [UN]) or treated with PBS containing 5 mM EDTA (ED) 5 mM EGTA (EG), trypsin (TR) at 0.1 or 1.0 mg/ml, chymotrypsin (CH) at 0.1 or 1.0 mg/ml, or pronase (PR) at 0.1 or 1.0 mg/ml for 30 min at 20°C, except that with pronase the cells were incubated at 37°C. The cells were rapidly pelleted, washed four times with PBS containing 2% FBS and BSA (five mg/ml), and incubated with 1 μg of iodinated gD-1t in the presence or absence of 50 μg of unlabeled gD-1t. The specific binding of gD-1t (see Fig. 5) is shown. The binding of gD-1t to untreated cells was arbitrarily set at 100.

observation suggests either that numerous HSV receptors exist or that HSV interacts with molecules which are widely distributed.

WuDunn and Spear (36) have suggested that HSV initially interacts with cell surface heparan sulfate based on the observations that heparin inhibits HSV adsorption to cells and HSV was unable to adsorb to and infect cells when heparan sulfate has been enzymatically removed. Previously, it was reported that other polyanionic compounds, i.e., dextran sulfate and protamine sulfate, and polycationic compounds, i.e., polylysine and Polybrene, inhibit HSV adsorption to cells and virus replication (17, 33, 35), suggesting that HSV adsorption is ionic in nature. Johnson and Ligas (14) showed that there are very numerous or nonsaturable cell surface sites to which HSV can adsorb, and it seems likely that these nonsaturable or numerous adsorption sites may include heparan sulfate moieties, which are abundant cell surface components. Here, we use the term adsorption to describe the initial interactions of HSV with the cell surface.

However, there is also good evidence from the studies of Johnson and Ligas (14) as well as others (5, 16) that gD

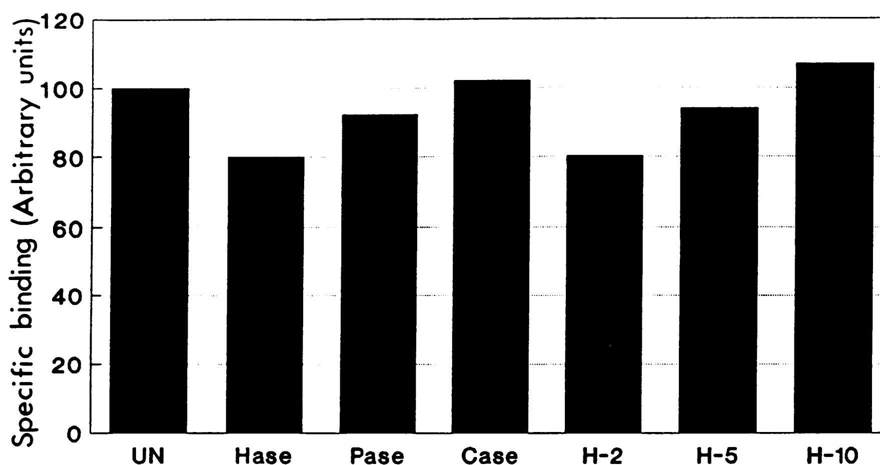

FIG. 7. Binding of gD-1t to cells treated with heparinase or in the presence of heparin. R970-5 cells growing in 24-well dishes were washed twice with PBS containing 0.1% glucose, BSA (0.1 mg/ml), and 500 U of aprotinin per ml and then incubated with this buffer (UN) or heparinase (1 U/ml, Hase), heparitinase (1 U/ml, Pase), or chondritin ABC lyase (2 U/ml, Case) diluted in this buffer for 40 min at 37°C. The cells were washed with PBS/FBS/BSA and then incubated with PBS/FBS/BSA for 30 min on ice. The specific binding of gD-1t to enzyme-treated cells and to cells in the presence of 2, 5, or 10 μg of heparin per ml (H-2, H-5, and H-10, respectively) was measured as described in the legend to Fig. 5.

molecules in the viral envelope interact with cell surface receptors which are saturable and much less numerous than the sites to which the virus can adsorb. The interactions of gD with its receptors are required for virus entry into cells but not adsorption. Adsorption of HSV occurs whether or not the critical gD receptors are blocked (14, 20), suggesting that most of the adsorption of HSV at the cell surface does not normally lead directly to virus entry into cells.

In this report we have shown that soluble forms of HSV-1 and HSV-2 gD inhibit the entry of HSV-1 and HSV-2 into cells without affecting virus adsorption. Therefore, it would appear that soluble gD interacts with gD receptors and blocks an essential step in the entry pathway. A soluble form of another HSV glycoprotein, gB, also known to be essential for virus entry into cells, had no effect on virus entry. Careful studies on the binding of gB-2t to cells have not been carried out because of problems associated with purifying the relatively large quantities of gB-2t required for these studies. However, gB-2t is recognized by conformationally dependent monoclonal antibodies, binds to heparin-agarose, and is a dimer in solution and in nonreducing PAGE (R. L. Burke et al., unpublished observations), supporting the view that gB-2t is in a native form and capable of binding to cells. There is no evidence that gB binds to saturable cell surface receptors, although this glycoprotein is clearly essential for virus penetration into cells (4). No differences in the abilities of UV-inactivated HSV particles either lacking or containing gB to block infectious HSV were observed (M. W. Ligas and D. C. Johnson, unpublished observations), supporting the idea that gB does not bind to saturable receptors.

In keeping with the observation that HSV entry was blocked by a limited number of gD-containing HSV-1 particles but not by an equal number of particles lacking gD (14), the specific binding of gD-1t and gD-2t was to a relatively restricted number of cell surface receptors. The number of gD receptors, defined by binding of radiolabeled gD-1t and gD-2t, is approximately two orders of magnitude higher than the number of sites described in previous blocking experiments. However, it may be argued that there are hundreds or thousands of gD molecules in the virion envelope, and it seems likely that multiple interactions between gD and gD receptors might occur. The observation that gD receptors number approximately 4×10^5 to 5×10^5 per cell suggests that the receptors are minor components of the plasma membrane and, coupled with the observation that treatment of cells with proteases abolishes gD binding, suggests that gD binds to proteins or structures dependent on cell surface proteins.

Additional evidence that these receptors are not related to the heparan sulfate glycosaminoglycan adsorption sites for the virus came from the observation that enzymatic removal of heparan sulfate or incubation with heparin did not affect the binding of gD. Therefore, although HSV apparently adsorbs to heparan sulfate, it appears unlikely that gD mediates this interaction. Adsorption of virus onto these numerous heparan sulfate adsorption sites may serve to increase the local concentrations of HSV on the cell surface and facilitate more specific interactions with gD receptors required for virus entry into cells.

The affinity of soluble forms of gD for its receptors (k_d, 2.4×10^{-7}) was relatively low compared with those reported for human immunodeficiency virus gp120 (k_d, 4×10^{-9}) (19) and Epstein-Barr virus gp350 (k_d, 1.2×10^{-8}) (34). This finding may partially explain the observation that gD-1t and gD-2t were required continuously in order to completely block HSV plaques. Alternatively, gD receptors may recycle. Concentrations of soluble gD as high as 25 to 100 μg/ml were required to inhibit HSV entry into cells, and although these concentrations translate to less than 2.5 μM, one explanation may involve the low affinity of soluble gD for the gD receptor. It is unclear, at this time, whether these soluble gD molecules, which are truncated near the transmembrane domain and subjected to purification, bind with the same affinity as native gD or whether the soluble forms of gD are functionally equivalent to gD found in the virion envelope. Furthermore, the virion envelope contains many copies of the gD molecule, and this multivalency may stabilize the interactions between the virus and gD receptors. The relatively low affinity of soluble gD for cell surface receptors has made on-going attempts to identify gD receptors difficult.

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