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Glycoprotein D of Herpes Simplex Virus (HSV) Binds Directly to HVEM, a Member of the Tumor Necrosis Factor Receptor Superfamily and a Mediator of HSV Entry

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Glycoprotein D (gD) is a structural component of the herpes simplex virus (HSV) envelope which is essential for virus entry into host cells. Chinese hamster ovary (CHO-K1) cells are one of the few cell types which are nonpermissive for the entry of many HSV strains. However, when these cells are transformed with the gene for the herpesvirus entry mediator (HVEM), the resulting cells, CHO-HVEM12, are permissive for many HSV strains, such as HSV-1(KOS). By virtue of its four cysteine-rich pseudorepeats, HVEM is a member of the tumor necrosis factor receptor superfamily of proteins. Recombinant forms of gD and HVEM, gD-1(306t) and HVEM(200t), respectively, were used to demonstrate a specific physical interaction between these two proteins. This interaction was dependent on native gD conformation but independent of its N-linked oligosaccharides, as expected from previous structure-function studies. Recombinant forms of gD derived from HSV-1(KOS)rid1 and HSV-1(ANG) did not bind to HVEM(200t), explaining the inability of these viruses to infect CHO-HVEM12 cells. A variant gD protein, gD-1(Δ290-299t), showed enhanced binding to HVEM(200t) relative to the binding of gD-1(306t). Competition studies showed that gD-1(Δ290-299t) and gD-1(306t) bound to the same region of HVEM(200t), suggesting that the differences in binding to HVEM are due to differences in affinity. These differences were also reflected in the ability of gD-1(Δ290-299t) but not gD-1(306t) to block HSV type 1 infection of CHO-HVEM12 cells. By gel filtration chromatography, the complex between gD-1(Δ290-299t) and HVEM(200t) had a molecular mass of 113 kDa and a molar ratio of 1:2. We conclude that HVEM interacts directly with gD, suggesting that HVEM is a receptor for virion gD and that the interaction between these proteins is a step in HSV entry into HVEM-expressing cells.

The envelope of herpes simplex virus (HSV) is complex, as it contains at least 10 virus-encoded glycoproteins (53). However, only a subset of these mediate virus entry in cell culture. The initial interaction of HSV with cell surface heparan sulfate proteoglycans is mediated by glycoprotein C (gC) and/or gB (19, 20, 62). This is presumably followed by interaction of one or more of the viral glycoproteins with cellular receptors (6, 25, 26, 29). Then gD, gB, and the complex of gH and gL act individually or in combination to trigger pH-independent fusion of the viral envelope with the host cell plasma membrane (53).

Several lines of evidence have implicated gD as an HSV receptor-binding protein. For example, UV-inactivated wild-type HSV virions, but not UV-inactivated virions lacking gD, are able to block infection by HSV (26, 29). Second, wild-type strains of HSV do not infect cells expressing gD (5, 27). This gD-mediated interference occurs at the level of penetration and is dependent on the structure of gD in the infecting virus (6, 11, 12, 27, 44). Lastly, cells incubated in the presence of soluble, truncated gD (gDt) are resistant to infection (17, 25, 39, 55). A unifying explanation for these observations is that inhibition is primarily due to binding of nonvirion gD to cellular receptor(s) which prevents them from binding to gD in the virus.

gD is a typical type I integral membrane glycoprotein. In its ectodomain of 319 amino acids, gD has three sites for the addition of N-linked oligosaccharides (N-CHO) and six cysteine residues arranged into three disulfide bonds (31). gD function is dependent on its native conformation but is independent of the three N-CHO (9, 15, 51, 52, 54). Mutagenesis coupled with complementation analysis identified four separate regions of gD that are important for virus entry (7, 37). Wild-type and mutant forms of gDt were cloned into a baculovirus expression system, and the abilities of these proteins to block HSV infection were studied. The ability of gDt to inhibit HSV infection depends on its native conformation (39) but is independent of the presence of the three N-CHO (60). Some mutations ablated the ability of gDt to block infection, whereas others, such as gD-1(Δ290-299t), markedly enhanced blocking activity. We speculated that changes in gDt structure altered its ability to interact with cellular protein(s), i.e., receptor(s) (38, 39). This hypothesis could best be verified by identifying the cellular receptor(s) which interacts with gD to mediate HSV entry.

Recently, expression cloning was used to isolate and identify a HeLa cell gene product which upon expression in normally nonpermissive Chinese hamster ovary (CHO) cells allows for

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entry of many HSV strains (35). This gene product, the herpesvirus entry mediator (HVEM), is a 230-amino-acid type I integral membrane protein. Because it contains a motif of four cysteine-rich pseudorepeat sequences, it is considered a new member of the tumor necrosis factor receptor (TNFR) superfamily (1, 50). A soluble form of HVEM (consisting of the ectodomain of HVEM fused in frame to the C_H2 and C_H3 domains of rabbit immunoglobulin G [IgG] heavy chain), HVEM:Fc, blocks HSV type 1 (HSV-1) infection of CHO cells stably transformed to express HVEM on the cell surface (CHO-HVEM12 cells). Moreover, polyclonal antibodies to HVEM:Fc block HSV-1 infection of CHO-HVEM12 cells. Three HSV-1 strains with changes in the gD sequence infected CHO-HVEM12 cells with markedly reduced efficiencies, suggesting that HVEM interacts directly with gD.

In this study, our goal was to test the hypothesis that gD can interact directly with HVEM. The approach was to use purified truncated forms of both HVEM and viral glycoproteins in direct binding assays. Among the five herpesvirus glycoproteins involved in entry, only gD was able to bind to HVEM. The binding was dependent on the native conformation of gD but independent of its N-CHO. These and other studies presented here show that HVEM meets the criteria for a receptor which interacts with gD and leads to HSV entry.

MATERIALS AND METHODS

Cells and virus. African green monkey (Vero) cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Vero-gL cells, stably transfected with a plasmid expressing HSV-1 gL (35, 40), were grown in DMEM–10% FBS with G418 (400 μg/ml). CHO-HVEM12 cells (35) were grown in Ham's F-12 medium supplemented with 10% FBS and 200 μg of G418 per ml. Sf9 (*Spodoptera frugiperda*) cells (GIBCO BRL) were grown in Sf900II medium (GIBCO BRL). KOS-gL86 is a mutant KOS virus in which the *Escherichia coli lacZ* gene under the control of the cytomegalovirus immediate-early promoter replaces part of the gL open reading frame (ORF) (35, 40). This virus was propagated on Vero-gL cells.

Construction of a baculovirus recombinant expressing a truncated and secreted form of HVEM. The strategy used was the same as that employed in the construction of a baculovirus recombinant expressing gD-1(306t) (49). Plasmid pBEC10 contains the entire coding sequence for the HVEM ORF (35). A 486-bp fragment of HVEM, corresponding to amino acids 39 to 200 (where amino acid 39 is the first amino acid after the predicted signal peptide sequence), was amplified by PCR. The amino-terminal primer, 5′-GCG**AGATCT**GCCATCAT GCAAGGAGGACGAGTA-3′, hybridized to the noncoding strand of HVEM and added a *Bgl*II site (bold letters) just upstream from the codon for amino acid 39. The carboxy-terminal primer, 5′-GCG**TGATCA**GTGGTGGTGGTGGTGG TGGGAGCTGCTGGTTCCAGCT-3′, added five histidine codons after the histidine at amino acid 200 of HVEM, a stop codon, and a *Bcl*I site (bold letters). The histidine codons were added to provide a binding site for nickel-nitriloacetic acid-agarose resin (Qiagen) for purification (see below). The PCR-amplified product was digested with *Bgl*II and *Bcl*I and ligated with DNA from plasmid pVT-Bac (56) which had been digested with *Bam*HI. The mellitin signal sequence, coded for by pVT-Bac, replaced the HVEM signal. An extra aspartic acid codon was added to the N terminus of HVEM as a result of cloning (Fig. 1A). The ligated plasmid was used to transform XL2-Blue (Stratagene) competent *E. coli* cells. The resulting plasmid, pCW275, was recombined into baculovirus (*Autographa californica* nuclear polyhedrosis virus) by cotransfection with Baculogold DNA (Pharmingen) (49). Plaques were picked and amplified. Culture supernatants were screened for HVEM expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). Blots were probed with rabbit anti-HVEM peptide serum R133 (see below) (Fig. 1A). Baculovirus recombinants expressing HVEM were subjected to two additional rounds of plaque purification (49). The recombinant baculovirus was named bac-HVEM(200t). The recombinant protein was designated HVEM(200t) (Fig. 1A).

Purification of HVEM(200t). Sf9 cells grown in 3-liter suspension cultures (61) were infected with bac-HVEM(200t) at a multiplicity of infection of 4. At 48 h postinfection, the supernatant was clarified by centrifugation (1,500 × *g* for 30 min at 4°C) and then by filtration (0.22-μm-pore-size filter). The supernatant was concentrated and exchanged into 600 ml of phosphate-buffered saline (PBS) by tangential flow filtration (10-kDa molecular mass cutoff membrane; Millipore). The protein solution was mixed with 2.5 ml of nickel-nitriloacetic acid resin (Qiagen; preequilibrated with PBS) and incubated overnight at 4°C on a rotary shaker. The resin was pelleted (100 × *g* for 10 min at 4°C), resuspended in PBS, transferred to a column, and washed first with PBS and then with stepwise increasing concentrations of imidazole (0.01 to 0.25 M) in 0.02 M phosphate buffer (pH 7.5) containing 0.5 M NaCl. The 0.25 M eluate, containing most of the HVEM(200t), was dialyzed against PBS and concentrated (10-kDa molecular mass centrifugal membrane; Millipore). The yield of purified HVEM(200t) was 2 to 3 mg/liter of supernatant.

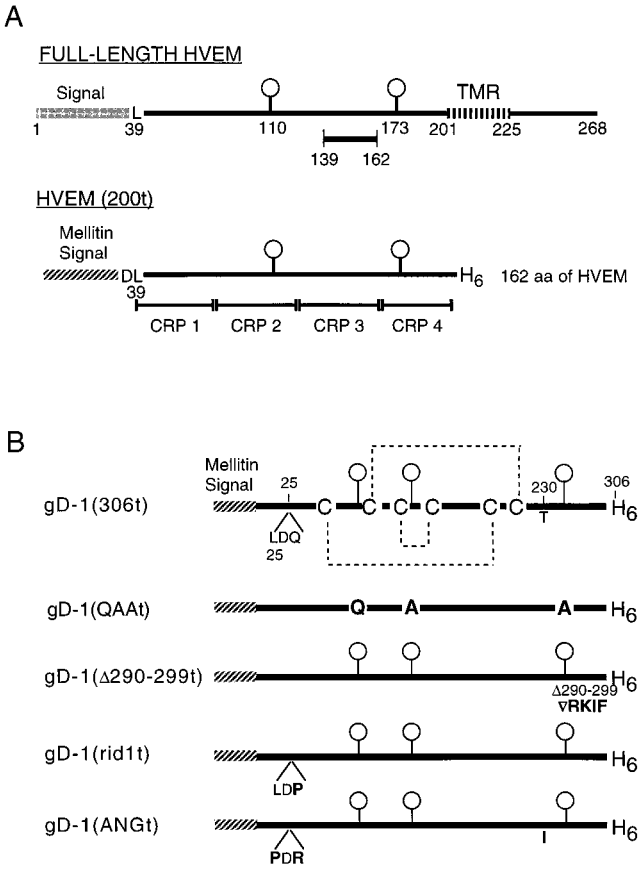

FIG. 1. Schematic representations of gD and HVEM proteins. (A) Diagrams of the full-length HVEM from HeLa cells (35) and the recombinant baculovirus protein HVEM(200t). Leucine 39 is the first amino acid residue of the mature protein (after removal of the predicted signal sequence). In HVEM(200t), the natural signal was replaced by the honeybee mellitin signal encoded by the insertion vector pVT-Bac (56). Cloning of the HVEM ectodomain ORF into pVT-Bac also added an aspartic acid residue to the N-terminus. HVEM(200t) was truncated at histidine 200 prior to the predicted transmembrane region (TMR) and five additional histidine residues were added to the C terminus. Consensus sites for the addition of N-CHO are indicated by balloons. The 24-amino-acid (aa) peptide (aa residues 139 to 162) used to prepare R133 antiserum is shown below the full-length form of HVEM. The boundaries of the four cysteine-rich pseudorepeat (CRP) elements present in the HVEM ectodomain are shown below HVEM(200t). (B) Forms of HSV-1 gDt produced by recombinant baculovirus-infected cells (38, 39, 49). Each form is truncated prior to the TMR and contains a six-histidine tag at the carboxy terminus. gD-1(306t) (Patton strain) has the same sequence as gD from strain KOS through amino acid 306. Not shown is gD-2(306t) from HSV-2 strain 333 (39). N-CHO sites are indicated by balloons. Cysteine residues, along with the disulfide bonding pattern determined for full length gD-1 (31), are also shown. gD-1(QAAt) has three amino acid changes relative to gD-1(306t), N94Q, S123A, and T264A, resulting in a molecule lacking N-CHO (49). gD-1(Δ290-299t) contains a combined deletion and linker-insertion mutation where amino acid residues 290 through 299 of gD-1(306t) (IPPNWHIPSI) are replaced by the amino acid residues RKIF (7, 39). gD-1(rid1t) has a single amino acid change (Q27P), and gD-1(ANGt) has three amino acid changes (L25P, Q27R, and T230I) relative to gD-1(306t) (38).

Production and purification of soluble forms of HSV glycoproteins. The production and purification of gD-1(306t), gD-2(306t), gD-1(QAAt), gD-1(Δ290-299t), gD-1(rid1t), gD-1(ANGt), and gC-1(457t) (Fig. 1B) from recombinant baculovirus-infected cells have been described elsewhere (38, 39, 49, 55). HSV-1 gH truncated at amino acid 792 and complexed to full-length HSV-1 gL was produced by mouse L cells stably transformed with plasmids pCMV3gH(792) and pCMV3gL-1 (14). This cell line, HL-7, was kindly provided by Gary Dubin.

The properties of these cells and gHt-gL purification protocols are described in detail elsewhere (41). gB-1s was produced in 293 (human kidney) cells stably transformed with plasmid pRP-RSV-gBs (32). These cells, which were kindly provided by R. Manservigi, were maintained in DMEM supplemented with 10% FBS and 3 μg of methotrexate per ml. To obtain secreted protein, cells were grown for 24 h in serum-free medium lacking methotrexate and the supernatant was applied to a column of anti-gB-1 monoclonal antibody (MAb) A22-Sepharose. The column was washed with 0.01 M Tris (pH 7.5) containing 0.15 M NaCl and then eluted with 3 M KSCN. The eluate was dialyzed against PBS and concentrated (PM10 membrane; Amicon). Soluble purified CD4 was kindly provided by Robert Doms, and sTva, a recombinant baculovirus-produced form of Tva, the receptor for Rous sarcoma virus (18), was kindly provided by Paul Bates.

Antibodies. Monospecific antiserum (R133) to HVEM was generated by immunizing a rabbit with peptide RRYATSSPGQRVQKGGTESQDTLC (Fig. 1A) coupled to keyhole limpet hemocyanin as previously described (8). R133 specifically recognizes the synthetic peptide used for immunization. The production of antiserum to HVEM:Fc was previously described (35). R7 antiserum was raised against gD-2 isolated from virus-infected cells (23). R46 antiserum was raised against gC isolated from HSV-1-infected cells (16). R69 antiserum was prepared against reduced and alkylated gB isolated from HSV-1-infected cells (16). MAbs 37S (48) and 8H4 (14) were used to detect gH and gL, respectively. MAb A22.1, used for gB purification, was kindly supplied by Becton Dickinson.

SDS-PAGE analysis. Purified glycoproteins were separated by SDS-PAGE under reducing conditions in precast Tris-glycine gels (Novex). After SDS-PAGE, separated proteins were either silver stained (Pharmacia) or transferred to nitrocellulose and reacted with the appropriate antiserum. Blots were blocked with PBS containing 5% milk and 0.2% Tween 20 and incubated with secondary antibody (goat anti-mouse or goat-anti rabbit) coupled to horseradish peroxidase in PBS containing 5% milk and 0.2% Tween 20. Blots were washed with 0.2% Tween 20 in PBS, and bands were visualized by exposure to X-ray film after the addition of chemiluminescent substrate (ECL; Amersham). Purified glycoproteins were digested with glycosidases as previously described (45, 49).

Blocking of HSV-1 entry into CHO-HVEM12 and Vero cells by soluble gD or soluble HVEM. Vero and CHO-HVEM12 cells were plated on 96-well dishes and incubated overnight. For experiments with soluble gD, cells were chilled to 4°C for 10 min and the medium was replaced with DMEM–5% FBS containing various concentrations of gD-1(306t) or gD-1(Δ290-299t). The plates were rocked for 90 min at 4°C, at which time KOS-gL86 (5×10^5 PFU/well) was added. The plates were rocked for an additional 90 min at 4°C, and then DMEM–5% FBS containing the appropriate soluble gD was added to maintain the initial concentration of soluble gD during the subsequent 6 h of incubation at 37°C. The experiments with soluble HVEM were done as previously described (35). Briefly, KOS-gL86 was mixed with various concentrations of HVEM(200t), HVEM:Fc (35), or rabbit IgG as a control and then incubated for 30 min at 37°C before addition to CHO-HVEM12 cells on 96-well plates. After 2 h of incubation at 37°C, cells were treated with 0.1 M citrate buffer (pH 3.0) to inactivate extracellular virus. Cells were washed and incubated in PBS containing glucose and 1% calf serum for 4 h at 37°C. In both types of assays, cells were washed with PBS at 6 h postinfection, substrate (*o*-nitrophenyl-β-D-glucopyranoside in PBS containing 0.5% Nonidet P-40) was added to each well, and β-galactosidase activity was measured at various time points with a Spectromax 250 enzyme-linked immunosorbent assay (ELISA) reader. The results were plotted as percentages of controls in which no soluble gD or HVEM was present.

ELISA. Soluble receptor proteins [HVEM(200t), CD4, and sTva] in PBS were bound to wells of microtiter plates for 3 h at room temperature (RT). Plates were washed with 0.1% Tween 20 in PBS (PBS-Tween) and incubated in 5% nonfat milk–0.2% Tween 20 in PBS (blocking solution) for 30 min at RT. Plates were washed with PBS-Tween and incubated with gD (or other HSV-1 envelope glycoproteins) at various concentrations in blocking solution for 16 h at 4°C. Plates were washed with PBS-Tween and incubated for 30 min at RT with the appropriate antiserum diluted in blocking solution. Plates were washed three times with PBS-Tween and incubated with horseradish peroxidase-conjugated secondary antibody diluted in blocking solution. Plates were washed once with PBS-Tween and then with 20 mM citrate buffer (pH 4.5). A substrate of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) (Moss, Inc.) in citrate buffer (pH 4.5) was added, and the A_{405} was read with a microtiter plate reader (Bio-Tek).

Competition binding assay with ^{125}I-labeled gD-1(Δ290-299t). HVEM(200t) in PBS at a concentration of 200 nM was used to coat modular 96-well microtiter plates for 3 h at RT. Plates were washed and blocked as described above for ELISA. Serial threefold dilutions of unlabeled competitor proteins in blocking solution were added to duplicate wells of the plate, and ^{125}I-labeled gD-1(Δ290-299t), iodinated as previously described (36), was added to a final concentration of 14 nM. Plates were incubated for 16 h at 4°C and washed with PBS-Tween. Wells were separated and counted in a gamma counter (Wallac). At each concentration of competitor, the percentage of ^{125}I-labeled gD-1(Δ290-299t) bound was calculated as follows: [counts per minute of ^{125}I-labeled gD-1(Δ290-299t) bound in the presence of competitor/counts per minute of ^{125}I-labeled gD-1(Δ290-299t) without competitor] × 100.

Mass spectrometry, gel filtration, and N-terminal sequencing. Matrix-assisted laser desorption-ionization mass spectrometry (21) was performed with samples of HVEM(200t) dissolved in 50% acetonitrile containing 1% trifluoroacetic acid and diluted with 2-(4-hydroxyphenylazo)benzoic acid (Aldrich) as previously described (45). For gel filtration, proteins were diluted with PBS and were applied to a calibrated Superdex 200 column (HR 10/30; Pharmacia). Fractions were collected and analyzed for HVEM(200t) and gD-1(Δ290-299t) by SDS-PAGE and subsequent immunoblotting with R133 antiserum to visualize HVEM and R7 antiserum to visualize gD. Samples of HVEM(200t) and gD-1(Δ290-299t), as well as the complex of the two proteins isolated by size exclusion chromatography, were sequenced by adsorptive protocols with a Perkin-Elmer/Applied Biosystems 473A microsequencer (45).

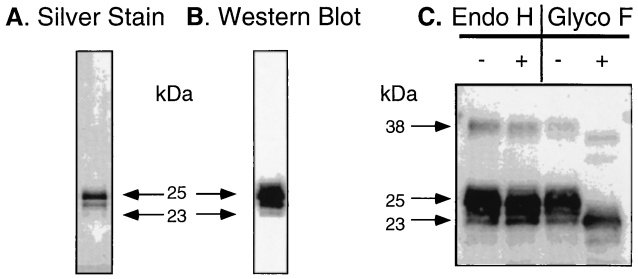

FIG. 2. SDS-PAGE analysis of purified HVEM(200t). Purified HVEM(200t) was electrophoresed through a 12% Tris–glycine–polyacrylamide gel under reducing conditions and visualized by silver staining (A) or Western blotting with R133 antiserum (B). The sizes of stained protein bands were calculated from the migration of molecular mass standards. (C) Purified HVEM(200t) was incubated under identical conditions without (−) or with (+) endo H or glyco F. Mock- and enzyme-digested samples were electrophoresed under reducing conditions through a 12% Tris–glycine–polyacrylamide gel, Western blotted, and probed with R133 antiserum.

RESULTS

Characterization of baculovirus-produced HVEM(200t). Previously we constructed a plasmid which upon transfection into mammalian cells produced the ectodomain of HVEM fused to the Fc region of rabbit IgG (HVEM:Fc) (35). For the production of large quantities of HVEM, we expressed the ectodomain with a C-terminal, six-histidine tag, HVEM(200t), in the baculovirus system (Fig. 1A). HSV glycoproteins expressed in insect cells from baculovirus recombinants have properties similar to those of proteins produced in mammalian cells (38, 39, 45, 49, 55).

HVEM(200t) was purified by nickel-agarose chromatography and analyzed by SDS-PAGE (Fig. 2A and B). The protein was present primarily in three closely migrating silver-stained bands (Fig. 2A), ranging from 23 to 25 kDa. A faint band which migrated to 38 kDa was sometimes seen (Fig. 2C). On Western blots, the same bands also reacted with both R133 and anti-HVEM:Fc (35) (data not shown).

The deduced HVEM amino acid sequence includes two potential sites for the addition of N-CHO, and HVEM:Fc was shown to be N glycosylated (35). Purified HVEM(200t) was digested with endoglycosidase H (endo H) and glycopeptidase F (glyco F) to verify that it also contained such posttranslational N-CHO modifications (Fig. 2C). Treatment with endo H had no effect, whereas treatment with glyco F increased the mobility of HVEM(200t). The simplest interpretation is that the closely migrating bands in the control lane represent HVEM(200t) with two, one, or no mature (Golgi-modified) forms of N-CHO. The mobility of the 38-kDa band was also altered by glyco F, suggesting that it was a dimeric form of HVEM(200t) which survived the denaturation conditions.

Because HVEM(200t) exhibited heterogeneity by SDS-PAGE, we examined it by mass spectrometry (Fig. 3A). HVEM(200t) was detected as a major peak of 20,020 Da and two minor peaks, with each one about 700 Da smaller. The minor peaks may represent HVEM(200t) with one or no N-

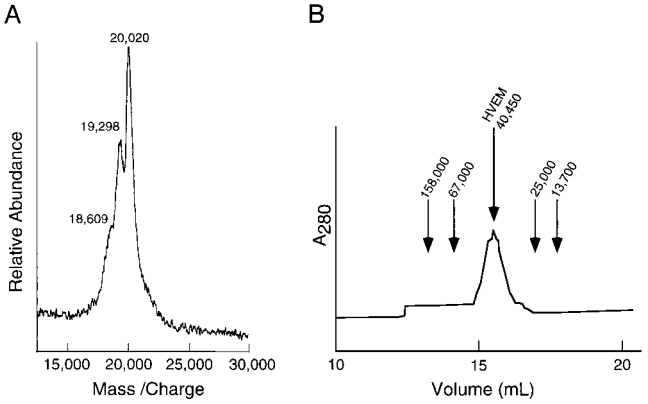

FIG. 3. Analysis of the molecular size of purified HVEM(200t). (A) Purified HVEM(200t) was dissolved in 50% acetonitrile containing 1% trifluoroacetic acid, diluted with 2-(4-hydroxyphenylazo)benzoic acid, and analyzed by mass spectrometry. The calculated masses of the three peaks of singly charged species are indicated. (B) HVEM(200t) (35 μM in PBS) was loaded onto a Superdex 200 gel filtration column and eluted with PBS. The eluate was monitored for protein by A_{280}. The major A_{280} peak had a calculated molecular mass of 40,450 Da, based on the peak positions of molecular mass standards (indicated by arrows).

CHO (45). No covalently linked dimeric species were detected. N-terminal sequencing of purified HVEM(200t) confirmed the expected amino acid sequence through 10 residues (data not shown). No other sequences were present, suggesting that the size heterogeneity of HVEM(200t) is due primarily to heterogeneity of N-CHO.

By gel filtration (Fig. 3B), HVEM(200t) eluted with a calculated mass of 40 kDa, suggesting that it is a dimer in solution. SDS-PAGE and immunoblot analyses of the peak fractions from the gel filtration column indicated that the 40-kDa form contained all three glycosylation isoforms (data not shown).

It was previously shown that HVEM:Fc blocks HSV-1(KOS) infection of CHO-HVEM12 cells (35). To determine whether HVEM(200t) had similar biological activity, HVEM(200t), HVEM:Fc, and rabbit IgG were tested for the ability to block HSV-1(KOS) entry into CHO-HVEM12 cells (Fig. 4). The results showed that the two recombinant forms of HVEM were equally effective in blocking HSV infection. In both cases, the concentration necessary to inhibit infection by 50% was 1 μM. In this experiment, the rabbit IgG control had some inhibitory effect when it was present at high concentrations, possibly due to interaction with gE and gI (13). The results suggested that both HVEM(200t) and HVEM:Fc interacted with at least one virion component essential for virus entry.

Evidence that gD binds directly to HVEM. Having demonstrated that HVEM(200t) had biological activity, we next used ELISA to examine the in vitro interaction between gD-1(306t) and HVEM(200t) (Fig. 5). Microtiter plates were coated with various concentrations of HVEM(200t), and then the plates were incubated with various concentrations of gD-1(306t) (49), ranging from 1 nM to 1 μM. Bound gD was detected with anti-gD serum R7. Increasing amounts of gD-1(306t) bound as the concentration of HVEM(200t) increased, up to a concentration of 200 nM (Fig. 5A). No binding was detected in control wells containing no HVEM(200t). The experiment was repeated at 200 nM HVEM(200t) and with a greater range of gD-1(306t) concentrations (up to 20 μM) to be certain that the reaction was saturable (Fig. 5B). Saturation occurred at 5 μM gD-1(306t). In subsequent experiments, plates were coated with 200 nM HVEM(200t).

Is the reaction specific for HVEM and gD? To be certain we were not detecting nonspecific binding to a glycoprotein receptor, we carried out two experiments (Fig. 6). First, we asked whether secreted forms of other HSV glycoproteins involved in entry (i.e., gB, gC, and gH-gL) could bind to HVEM(200t). Each of these proteins was purified by immunoaffinity chromatography, and each reacted as expected with several different MAbs (14, 33, 39, 42, 55) (data not shown). Various concentrations of gD-1(306t), gD-2(306t) (39), gC-1(457t) (55), gB-1s (33), and gHt-gL (42) were incubated on ELISA plates coated with HVEM(200t), and binding was detected with antibody to each glycoprotein. gD-1(306t) and gD-2(306t) bound similarly to HVEM(200t) (Fig. 6A); however, gC-1(457t), gB-1s, and gHt-gL failed to bind, indicating the specificity of HVEM binding for gDt. The fact that gD-1(306t) and gD-2 (306t) bound similarly to HVEM(200t) is consistent with the finding that the entry of both HSV-1 and HSV-2 strains into CHO cells was enhanced by the expression of HVEM (35). Both forms of gD can also block HSV infection of Vero cells (39), although the mediator(s) of entry into these cells has not yet been identified.

As a second test of specificity, we examined the binding of gD-1(306t) to soluble forms of extraneous viral receptors. gD-1 (306t) bound to ELISA plates coated with HVEM(200t) but not to plates coated with CD4 (the HIV-1 receptor) or sTva (a soluble form of the Rous sarcoma virus receptor) (18) (Fig. 6B). In separate experiments, neither gD-1(306t) nor gD-1 (Δ290-299t) bound to purified soluble mannose-6-phosphate receptor (4) (data not shown). Thus, the observed binding was specific both for gD and for HVEM.

Binding of HVEM to gD depends on gD conformation but not on N-CHO on gD. The antigenic structure of gD and its function in infection are highly dependent on its native conformation and the maintenance of its three disulfide bonds (15, 31). Denaturation of gDt destroys its ability to interact with

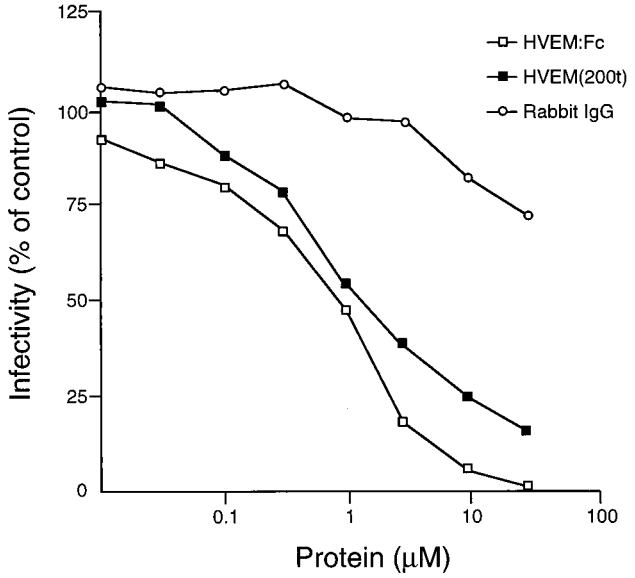

FIG. 4. Effects of soluble HVEM on HSV-1 infection CHO-HVEM12 cells. Various concentrations of HVEM(200t), HVEM:Fc, and rabbit IgG were incubated with KOS-gL86 for 30 min at 37°C prior to inoculation of cells on 96-well plates. After 2 h of incubation at 37°C, cells were treated with low-pH buffer to inactivate extracellular virus, washed, and then incubated for an additional 4 h. Cells were lysed for quantitation of β-galactosidase activity, which was proportional to the number of infected cells and was expressed as a percentage of the activity detected in the absence of soluble HVEM.

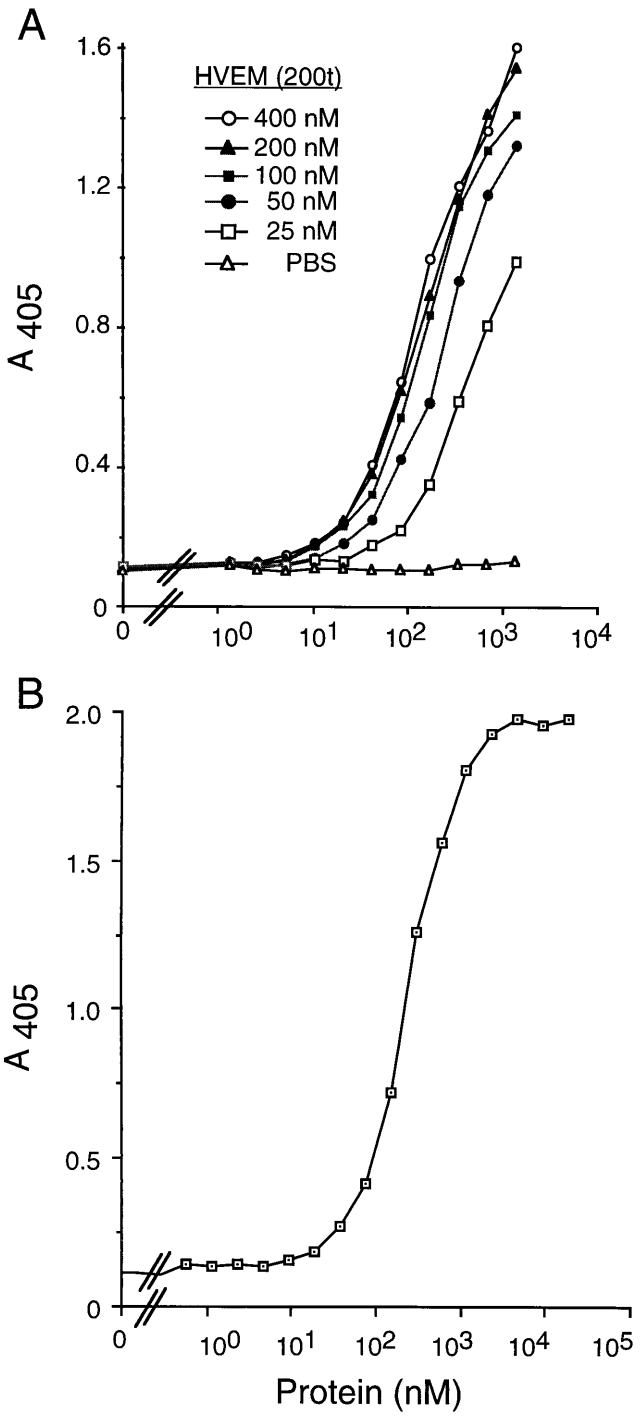

FIG. 5. Binding of gD-1(306t) to HVEM(200t). (A) Various concentrations of HVEM(200t) were bound to wells of an ELISA plate in PBS. Various concentrations of gD-1(306t) were added to the bound HVEM(200t) for 16 h at 4°C. Bound gDt was detected with anti-gD serum R7, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of duplicate wells. The experiment was repeated twice with similar results. (B) HVEM(200t) at a concentration of 200 nM in PBS was bound to an ELISA plate and then incubated with various concentrations of gD-1(306t) for 16 h at 4°C. Bound gDt was detected with R7 antiserum, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of duplicate wells.

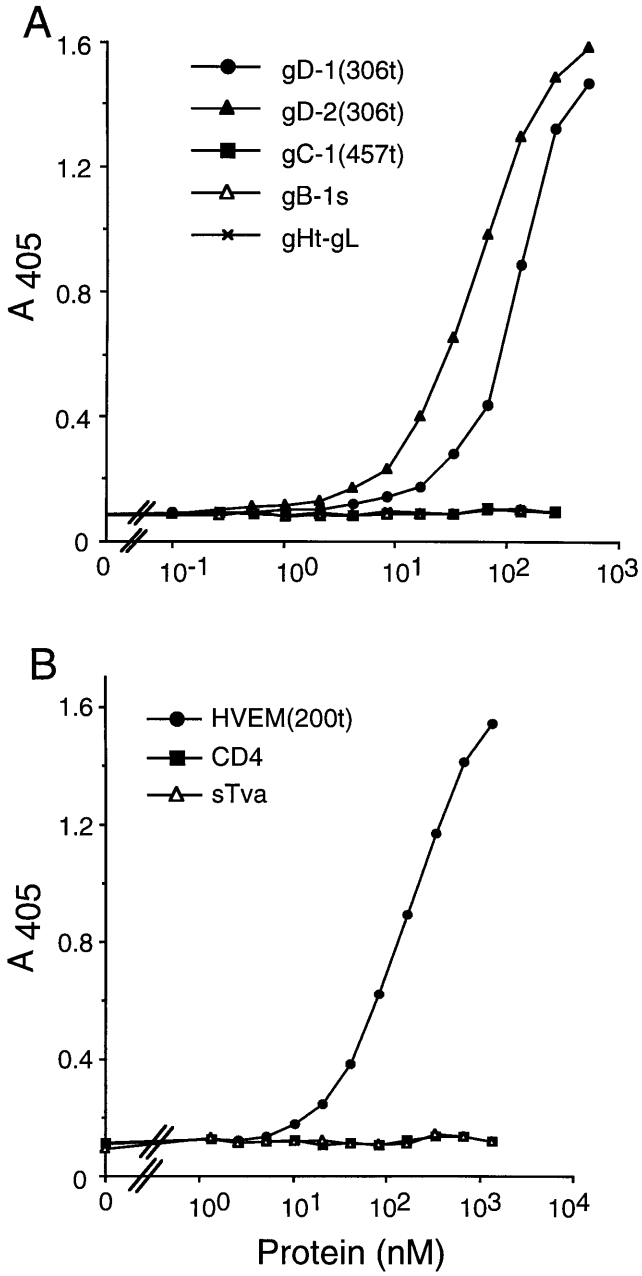

FIG. 6. Specificity of the gD-HVEM interaction. (A) HVEM(200t) at 200 nM in PBS was adsorbed to ELISA plates and incubated with various concentrations of gD-1(306t), gD-2(306t), gC-1(457t), gB-1s, and gHt-gL for 16 h at 4°C. Bound proteins were detected with antiserum R7 (for gD), R47 (for gC), R69 (for gB), or a mixture of MAbs 37S and 8H4(for gH and gL, respectively), followed by peroxidase-conjugated secondary antibody and substrate. The data are averages of duplicate wells. The experiment was repeated twice with similar results. (B) Wells of an ELISA plate were coated with 200 nM HVEM(200t), CD4, or sTva in PBS and then incubated with various concentrations of gD-1 (306t) for 16 h at 4°C. Bound gDt was detected with antiserum R7, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of triplicate wells. The experiment was repeated twice with similar results.

conformation-dependent MAbs and results in the loss of its biological activity (39). In contrast, gD function is retained when the three sites for N-CHO addition are mutated (51, 52). A variant form of gDt, gD-1(QAAt), which lacks signals for N-CHO (Fig. 1B) (49) is able to block infection of Vero cells as effectively as wild-type gDt does (46, 60). These properties

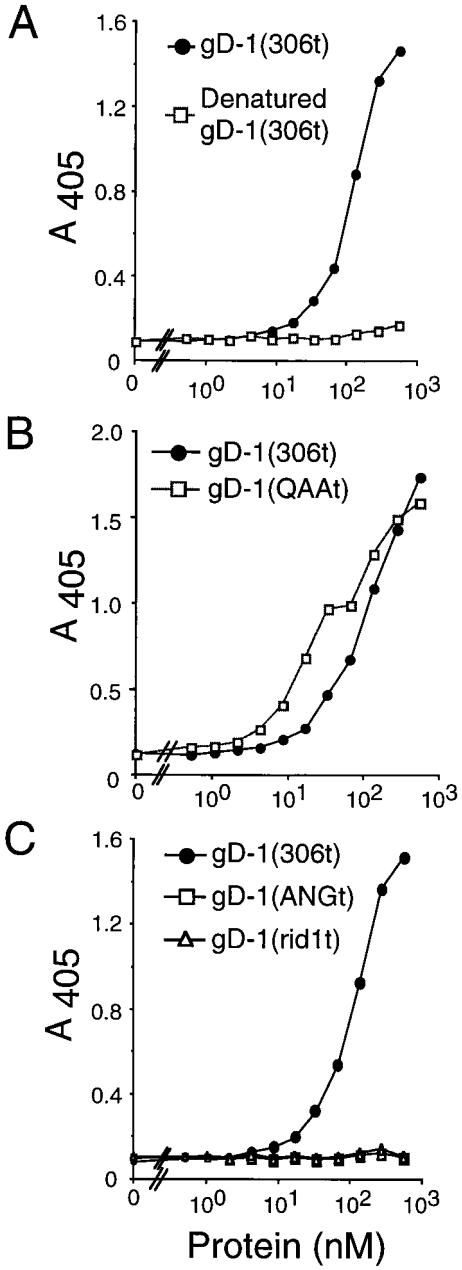

FIG. 7. Binding of denatured and mutant forms of gD to HVEM(200t). ELISA plates were coated with 200 nM HVEM(200t) in PBS, blocked, and incubated with various concentrations of gDt. Bound gD was detected with antiserum R7, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of duplicate wells, and each experiment was repeated twice with similar results. (A) The binding of gD-1(306t) was compared with that of reduced and alkylated gD-1(306t). gDt was reduced and alkylated by previously described methods (45), desalted, and exchanged into PBS on a Pierce polyacrylamide 6000 desalting column. (B) The binding of gD-1(306t) was compared with that of gD-1(QAAt). (C) The binding of gD-1(306t) was compared with those of gD-1(ANGt) and gD-1(rid1t).

predicted that if binding of gD to HVEM is relevant to gD function in vivo, then in vitro binding of gD to HVEM should be dependent on disulfide bonds but independent of N-CHO on gD. gD-1(306t) was reduced and alkylated to ensure that there would be no refolding during the incubation period with HVEM, and this treatment ablated its binding to HVEM(200t) (Fig. 7A). In contrast, gD-1(QAAt) bound as well as gD-1 (306t) did to HVEM(200t) (Fig. 7B). Thus, HVEM has requisite properties of a gD-binding viral receptor.

HVEM does not bind to gD proteins containing the rid1 and ANG mutations. The expression of HVEM by CHO-HVEM12 cells enhances the entry of several wild-type strains of HSV-1 and HSV-2 (35). However, three strains of HSV-1 known to carry mutations in the gD gene did not exhibit enhanced entry into CHO-HVEM12 cells, although they are fully infectious for other cell types such as Vero cells. These viruses were HSV-1(KOS)rid1 (KOS-rid1), KOS-rid2, and HSV-1(ANG). KOS-rid1 and KOS-rid2 were selected for resistance to gD-mediated interference, and both exhibited a change in amino acid 27 of gD (Q27P and Q27R, respectively) (11). HSV-1 (ANG) gD has the same substitution at amino acid 27 as KOS-rid2 gD does as well as changes at amino acids 25 (L25P) and 230 (T230I) (24). There are also several changes within the cytoplasmic tail of HSV-1(ANG) relative to HSV-1(KOS). The failure of these viruses to infect CHO-HVEM12 cells could be due to the inability of gD from these strains to bind to HVEM. Therefore, we tested the abilities of truncated versions of these proteins, gD-1(rid1t) and gD-1(ANGt) (Fig. 1B), to bind to HVEM(200t). The biological and structural properties of these proteins have been described previously (38).

Neither gD-1(rid1t) nor gD-1(ANGt) bound to HVEM (200t) in an ELISA (Fig. 7C), thus accounting for the inability of viruses containing these gDs to infect CHO-HVEM12 cells. It is worth noting that both of these proteins are able to block HSV infection of Vero cells (38). The results support the concept that the entry of KOS-rid1 and HSV-1(ANG) into permissive cells involves cellular proteins other than (or in addition to) HVEM (35, 57).

gD-1(Δ290-299t) shows enhanced binding to HVEM(200t). Baculovirus-produced gDt blocks HSV-1 and HSV-2 infections of Vero and other mammalian cells (38, 39, 55). Among several gDt variants, gD-1(Δ290-299t) (Fig. 1B) exhibited unexpectedly enhanced inhibitory activity. This observation predicted that gD-1(Δ290-299t) might bind to HVEM(200t) better than gD-1(306t) did, and we tested this by ELISA (Fig. 8A). Comparable binding to HVEM(200t) occurred at concentrations of gD-1(Δ290-299t) that were considerably lower than those of gD-1(306t).

The differences in the binding of the two forms of gDt to HVEM(200t) could be either simply quantitative [i.e., gD-1 (Δ290-299t) has a higher affinity for HVEM] or qualitative as well (i.e., the two proteins could interact with different regions of HVEM). To distinguish between these possibilities, we carried out binding competition studies with ^{125}I-labeled gD-1 (Δ290-299t) (Fig. 8B). A fixed amount of iodinated gDt [the concentration necessary to give half-maximal binding to HVEM(200t)] was mixed with increasing concentrations of unlabeled gD-1(Δ290-299t) or gD-1(306t) and then added to HVEM(200t). Both proteins competed with the iodinated probe for binding to HVEM(200t), indicating that both forms of gD bound to the same site. Approximately 50-fold-more gD-1(306t) was needed to achieve the same level of blocking as that of a given concentration of gD-1(Δ290-299t), suggesting that the enhanced binding of gD-1(Δ290-299t) is due to a higher affinity of interaction for HVEM(200t).

The results of the binding experiment (Fig. 8A) suggest a larger difference in the affinities of the two proteins for HVEM than do the results of the competition experiment (Fig. 8B). Because the signal in Fig. 8A depends on antibodies for the detection of bound gDt, it is possible that some of this apparent enhanced binding is due to increased binding of antibody to the variant protein. However, in separate experiments, we did not see substantial differences in the binding of R7 to gD-1

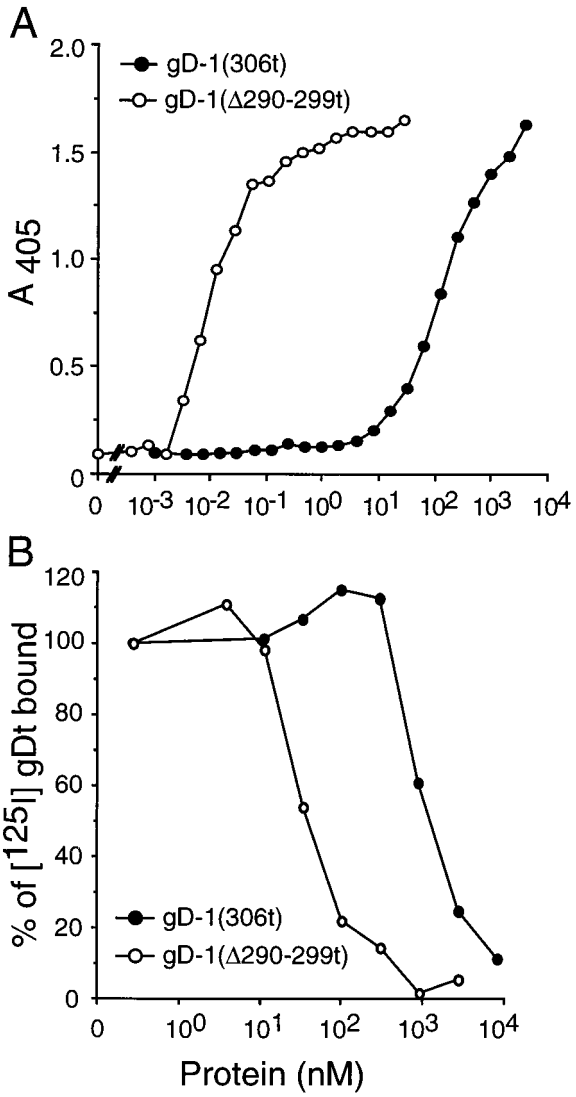

FIG. 8. Binding of gD-1(Δ290-299t) to HVEM(200t). (A) An ELISA plate was coated with 200 nM HVEM(200t) in PBS, blocked, and incubated with various concentrations of gD-1(306t) and gD-1(Δ290-299t). Bound gDt was detected with antiserum R7, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of duplicate wells, and the experiment was repeated several times with similar results. (B) An ELISA plate was coated with 200 nM HVEM(200t) in PBS, blocked, and incubated with 14 nM ^{125}I-labeled gD-1(Δ290-299t) in the presence of various concentrations of unlabeled gD-1(306t) and gD-1(Δ290-299t). Individual wells were separated and counted in a gamma counter. The data are the averages of duplicate wells at each concentration of competitor. The percentage of ^{125}I-labeled gD-1(Δ290-299t) bound was calculated as follows: [counts per minute of ^{125}I-labeled gD-1(Δ290-299t) bound in the presence of competitor/counts per minute of ^{125}I-labeled gD-1(Δ290-299t) without competitor] × 100.

(Δ290-299t) versus that to gD-1(306t) (data not shown). Alternatively, alterations in gD structure resulting from iodination could have affected the ability of gD-1(Δ290-299t) to bind to HVEM(200t), thereby influencing the extent of competition (Fig. 8B). Experiments to address the issue of affinity without the use of antibodies and iodinated forms of the glycoproteins are in progress.

Blocking of HSV-1 infection of CHO-HVEM12 cells with forms of gDt. After a demonstration that gD binds to HVEM under in vitro conditions, it was of interest to determine whether gD blocked HSV infection of CHO-HVEM12 cells. Therefore, Vero and CHO-HVEM12 cell monolayers were incubated with increasing concentrations of gD-1(306t) and gD-1(Δ290-299t) at 4°C for 90 min. Then cells were infected with the β-galactosidase reporter virus KOS-gL86 (35, 40). As previously reported (38, 39) and shown in Fig. 9, both forms of gD blocked HSV entry into Vero cells, with gD-1(Δ290-299t) exhibiting enhanced blocking relative to that of gD-1(306t). In the case of CHO-HVEM12 cells, gD-1(Δ290-299t) but not gD-1(306t) blocked infection. The differences seen in blocking by the two forms of gD are consistent with the differences seen in in vitro binding. Another point of interest is that at least 10-fold-more gD-1(Δ290-299t) was needed to inhibit infection of CHO-HVEM12 cells compared with that for Vero cells, consistent with the high probability that Vero and CHO-HVEM12 cells express different levels and kinds of mediators of HSV entry.

Characterization of the gD-HVEM complex by size exclusion chromatography. To study the interaction between gD and HVEM by another method and to assess the stoichiometry of the interaction, we mixed gD-1(Δ290-299t) and HVEM (200t) in solution and examined complex formation by size exclusion chromatography on a Superdex 200 column. Column fractions were analyzed by SDS-PAGE and immunoblotting, probing duplicate blots for gD and HVEM (Fig. 10). The molecular masses of gD-1(Δ290-299t) alone and HVEM(200t) alone were 58 and 40 kDa, respectively (Fig. 10, blots 1). These sizes suggest that both proteins were present in solution in dimeric form. Complex formation was then assessed by mixing various amounts of gD-1(Δ290-299t) with a constant amount of HVEM(200t) to give molar ratios of gD to HVEM of 1:1, 1:2, and 1:3. Each mixture was incubated overnight at 4°C and then applied to a Superdex 200 column. When the proteins were mixed at a 1:1 ratio (Fig. 10, blots 2), approximately 50% of the gD-1(Δ290-299t) and all of the HVEM(200t) eluted in fractions corresponding to a higher molecular mass (113 kDa). These results indicated that the two proteins were in a complex which remained associated during size exclusion chromatography. The presence of a large proportion of free gD-1(Δ290-299t) but not free HVEM(200t) suggested that gD was in excess when the proteins were mixed at a 1:1 ratio, i.e., there was more HVEM than gD in the complex. Alternatively, a

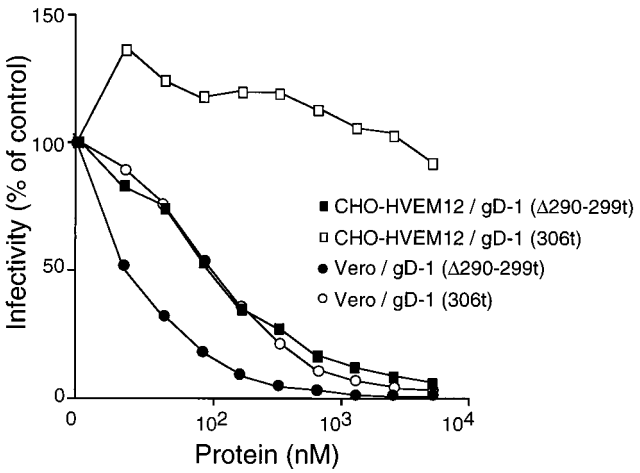

FIG. 9. Effects of soluble gDt on infection of Vero and CHO-HVEM12 cells. Cells on 96-well plates were pretreated with various concentrations of gD-1(306t) and gD-1(Δ290-299t) at 4°C for 90 min. KOS-gL86 was then added for an adsorption period of 90 min at 4°C. Then cells were shifted to 37°C for 6 h and lysed for the quantitation of β-galactosidase activity. The data are percentages of β-galactosidase activity detected in the absence of gDt.

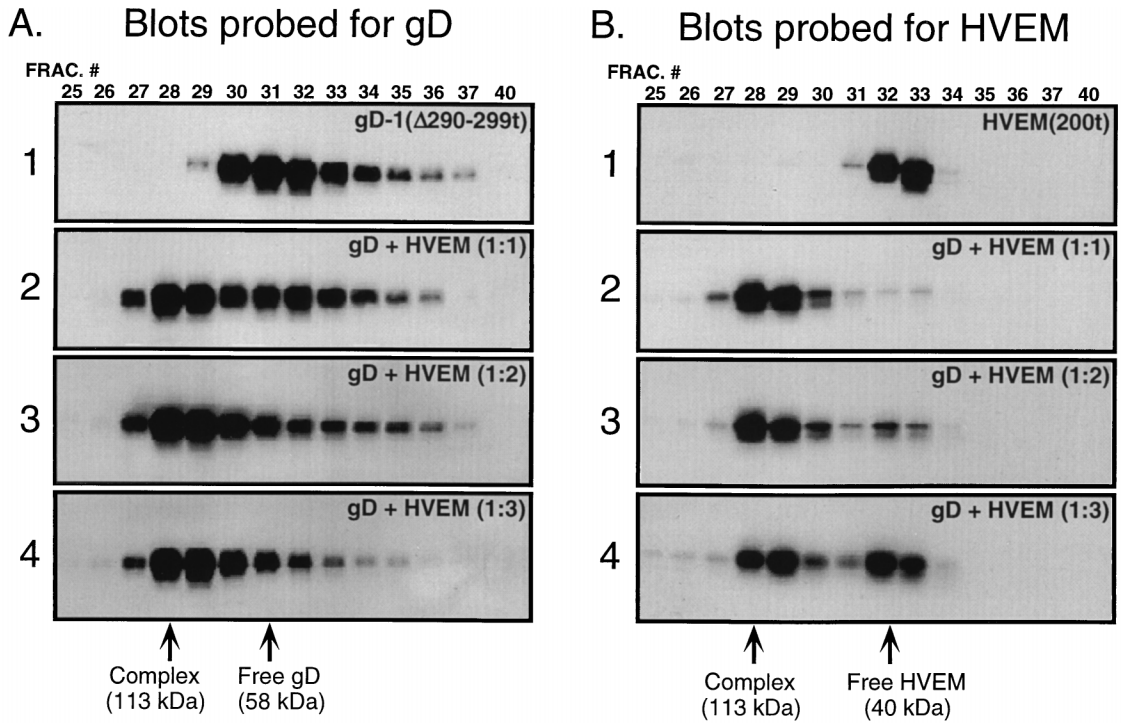

FIG. 10. Gel filtration of the gD-1(Δ290-299t)–HVEM(200t) complex. Samples of gD-1(Δ290-299t) at a concentration of 35 μM in PBS, HVEM(200t) (35 μM in PBS), and the two proteins mixed at molar ratios of gD to HVEM of 1:1 (35 μM each), 1:2 (17.5 μM gD and 35 μM HVEM), and 1:3 (11.6 μM gD and 35 μM HVEM) were incubated for 16 h at 4°C. Each sample was then chromatographed on a Superdex 200 column. Fractions (FRAC.) of 0.5 ml were collected for each column run and analyzed by SDS-PAGE, followed by Western blotting. (A) Immunoblots were reacted with antiserum R7 to detect gD. Blots: 1, gD-1(Δ290-299t) alone; 2, gD-1 (Δ290-299t) and HVEM(200t), mixed at a 1:1 molar ratio; 3, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:2 molar ratio; 4, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:3 molar ratio. Arrows indicate the positions of highest A_{280} for the complex and for gD-1(Δ290-299t) run alone. (B) Immunoblots were reacted with antiserum R133 to detect HVEM. Blots: 1, HVEM alone; 2, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:1 molar ratio; 3, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:2 molar ratio; 4, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:3 molar ratio. Arrows indicate the positions of highest A_{280} for the complex and for HVEM(200t) run alone.

fraction of the gD-1(Δ290-299t) was unreactive. However, when the starting concentration of gD was decreased and the amount of HVEM was kept constant prior to mixing, a greater proportion of the total gD was eluted from the column in association with HVEM. At a mixing ratio of 1 mol of gD to 2 mol of HVEM, very little free HVEM or gD-1(Δ290-299t) was seen (Fig. 10, blots 3). At a 1:3 mixing ratio of gD to HVEM, the complex still formed but there was a greater proportion of free HVEM (Fig. 10, blots 4). A simple interpretation of these results is that at a gD/HVEM ratio of 1:2, maximal amounts of both proteins were present as a complex and that this ratio therefore represents the stoichiometry. When the experiment was done with gD-1(306t), no complex was detected by gel filtration (data not shown). This result was not unexpected based on the ELISA data, i.e., the affinity of gD-1(306t) for HVEM(200t) may be too low to maintain a stable complex for the time needed to detect it by gel filtration.

To further evaluate the stoichiometry of the gD-HVEM complex, we carried out SDS-PAGE, followed by silver staining, of fraction 28 (Fig. 10, blots 3) and compared the intensities of the gD and HVEM bands in the complex to the intensities of known concentrations of each protein run separately (Fig. 11). Quantitation of the proteins was carried out by densitometry, and the molar ratio of gD-1(Δ290-299t) to HVEM(200t) was calculated to be 1:2.2.

In addition, we carried out N-terminal sequencing of the complex. As controls, we sequenced unfractionated mixtures of the two proteins at different molar ratios (data not shown). This experiment confirmed the molar ratio of gD-1(Δ290-299t) to HVEM(200t) (1:2) suggested by gel filtration and SDS-PAGE analysis of the complex (Fig. 10 and 11).

DISCUSSION

Over the past decade, a number of laboratories have endeavored to identify a cellular receptor for HSV by in some cases focusing on potential interactions with gD (3, 4, 22, 28, 34, 36, 47). Most recently, a member of the TNFR family of proteins, HVEM, was identified as a mediator of HSV entry into normally nonpermissive CHO-K1 cells (35). The inability of three strains of HSV with mutations in the gD gene to infect CHO-HVEM12 cells (35) strongly suggested that gD binds directly to HVEM.

If in fact gD does bind to HVEM to mediate virus entry, we should be able to demonstrate that (i) gD interacts specifically with HVEM; (ii) the native conformation, i.e., maintenance of three intact disulfide bonds of gD, may be critical for this interaction (31); (iii) the three N-CHO on gD are not necessary for the HVEM interaction (51, 52); and (iv) gD isolated from the rid and ANG strains of HSV-1 may fail to interact with HVEM (35, 57). The results presented here verify these predictions by showing that purified soluble forms of native gD ectodomain, with or without N-CHO, can bind specifically to a purified form of HVEM ectodomain, whereas denatured gDt or rid1 and ANG forms of gDt failed to bind. These results demonstrate that gD is a receptor-binding protein for HSV entry into cells via HVEM. Although other glycoproteins, including gB and gH-gL, are also required for entry, their roles

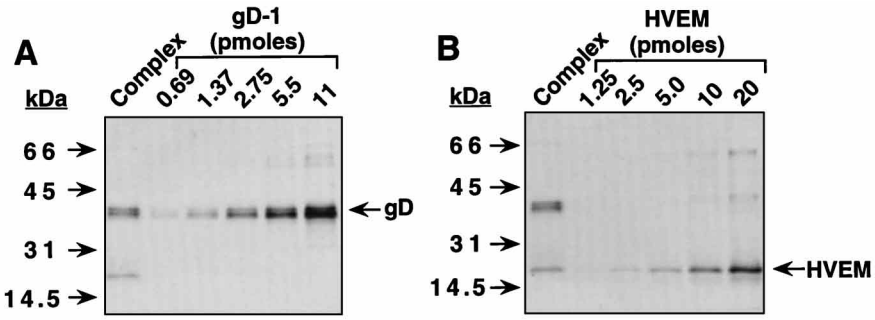

FIG. 11. Analysis of the composition of the complex formed between gD-1(Δ290-299t) and HVEM(200t). gD-1(Δ290-299t) at a concentration of 17.5 μM in PBS was mixed with HVEM(200t) (35 μM in PBS) and incubated for 16 h at 4°C. The complex was chromatographed on a Superdex 200 column. A portion of the fraction which contained the highest concentration of complex (fraction 28 [Fig. 10, blots 3]) was analyzed by SDS-PAGE, followed by silver staining. (A) Silver-stained gel of the complex (first lane) and of various concentrations of gD-1(Δ290-299t); (B) silver-stained gel of the complex (first lane) and various concentrations of HVEM(200t). Densitometry was used to construct standard curves of A_{600} versus protein concentration for both proteins, and the amounts of gD and HVEM in the complex were determined from these curves to be 2.7 pmol of gD-1(Δ290-299t) and 6.0 pmol of HVEM(200t).

do not seem to involve stable interactions with HVEM, at least none that can be detected with isolated soluble forms of these proteins.

Binding of gDt to HVEM(200t). To test our predictions, we chose the baculovirus system for producing large amounts of secreted HVEM(200t). Physical-chemical studies confirmed the purity of the nickel-agarose-purified glycoprotein and showed that it is a dimer in solution. Like HVEM:Fc, this protein blocked HSV entry into CHO-HVEM12 cells. We also found that gD-1(306t) bound directly to HVEM(200t) immobilized on an ELISA plate by utilizing antisera to gD to detect the interaction. When the assay was reversed by coating the plate with gD-1(306t) and adding HVEM(200t), we did not detect bound HVEM(200t) with the antipeptide serum R133. Although the reason for this is not clear, it is possible that residues 139 to 162 of HVEM (corresponding to those used for preparing the antiserum) interact with gD, thereby sterically blocking the binding of the antipeptide antibodies. Studies to localize the domains of HVEM which interact with gD are in progress.

Our results show that gD-1(306t) bound to HVEM(200t) specifically and in a saturable manner. No other HSV glycoprotein bound to HVEM, and gD did not bind to other soluble receptors. We further found that native gD conformation was critical for its interaction with HVEM but that N-CHO on gD were irrelevant. These two properties meet the criteria for gD function in virus infection. As predicted, forms of gDt from the rid1 and ANG strains of HSV-1 did not bind to HVEM(200t). These data suggest that at least residue 27, and perhaps residues 25 and 230 of gD, is directly involved in binding to HVEM. Alternatively, alteration of these residues may indirectly affect the structure of the interacting residues on gD. We previously showed that both gD-1(rid1t) and gD-1(ANGt) exhibited structural differences compared with gDt from the KOS strain (38). Interestingly, gD-2(306t) bound to HVEM (200t) as well as gD-1(306t) did, even though they differ in amino acid sequence at 35 residues. Both proteins also block infection of Vero cells by HSV-1 and HSV-2 equally well (39). Significantly, they have the same amino acids at residues 25, 27, and 230 as do the gDs from many strains of HSV-1 and HSV-2.

A gD-1 variant protein carrying a combined deletion and linker-insertion mutation, gD-1(Δ290-299t), was previously shown to have an enhanced ability to block HSV infection of Vero cells (38, 39). Here we showed that comparable binding to HVEM(200t) occurred at concentrations of gD-1(Δ290-299t) considerably lower than those of gD-1(306t). Our studies further indicated that gD-1(Δ290-299t) and gD-1(306t) competed for the same site(s) on HVEM but that the interaction between gD-1(Δ290-299t) and HVEM(200t) was stronger. Experiments to examine the affinity of the interaction are in progress. From the results of ELISA experiments, we estimate that the K_d for gD-1(Δ290-299t) binding to HVEM is in the nanomolar range or less and that the K_d for gD-1(306t) binding to HVEM is in the micromolar range or less. However, these estimates assume that the complex consists of equimolar amounts of gD and HVEM.

In addition to its ability to bind HVEM(200t) in vitro, we found that gD-1(Δ290-299t) blocked HSV-1 infection of CHO-HVEM12 cells. However, more gD-1(Δ290-299t) was needed to block infection of CHO-HVEM12 cells than was needed to block Vero cells and gD-1(306t) did not block infection of CHO-HVEM12 cells. One possibility is that CHO-HVEM12 cells overexpress HVEM and that therefore larger amounts of gD-1(306t) (which are not practical to use) are required for inhibition; alternatively, the Vero mediator(s) of HSV entry may bind to both forms of gDt with higher affinity than does HVEM. Thus, gD-1(306t) may bind to HVEM on cells with too low an affinity to compete effectively with virion-associated gD. We are in the process of isolating additional HVEM-expressing CHO cell clones to determine whether there is a correlation between the level of HVEM expression and the ability of gD to block infection. It is worth noting that gD-1 (Δ290-299t) was unable to rescue the infectivity of a gD null virus in complementation assays (7). One could argue that enhanced binding of gD to receptor interferes with later steps of viral entry and that a weak interaction, such as that displayed by wild-type gD-1(306t), is beneficial. These possibilities will be addressed in future studies.

Stoichiometry of the gD-HVEM interaction. According to the results of gel filtration experiments, the stoichiometry of the gD-HVEM complex is 1:2. This stoichiometry was further supported by SDS-PAGE analysis as well as by N-terminal sequencing of the fractionated complex. Determination of the mass of the complex by gel filtration (113 kDa) did not permit us to speculate on its exact composition. Assuming that two dimers of HVEM(200t) associate with one dimer of gD-1 (Δ290-299t), the mass of such a complex should be approximately 140 kDa. Alternatively, one dimer of HVEM(200t) complexed with a monomer of gD-1(Δ290-299t) should have a mass of approximately 75 kDa. Perhaps relevant to our observations, it has been noted that the ectodomain of TNFR migrates anomalously by SDS-PAGE as well as by gel filtration

(30, 43). We are carrying out additional experiments to more accurately determine the size of the complex.

Receptor oligomerization is a mechanism by which many extracellular hormones transmit their signals to the inside of a cell (58, 59). Thus, it is not surprising that a number of receptor-ligand complexes have been shown to contain multiple receptor molecules. Gel filtration studies similar to those used here showed that the ectodomain of the TNFR bound in a 3:3 molar ratio to TNF alpha (three monomers of TNFR and one trimer of ligand) (43). This ratio is in agreement with that determined from crystallography data (2). A stoichiometry of 1:2 was reported for the complex formed by human growth hormone and the ectodomain of its receptor-binding protein (10). In that case, a sequential binding mechanism that leads to receptor dimerization and cell signalling was postulated. Since the gel filtration studies between gD and HVEM were carried out with mixtures that had been incubated overnight, we would not expect to see 1:1 intermediates even if sequential interaction occurred. Measurements of real-time interaction will be needed to examine this possibility. Whether the 1:2 stoichiometry of the gD-HVEM interaction has significance for subsequent events in HSV infection remains to be examined.

HVEM may be one of several mediators of HSV entry. It was previously noted that the rid and ANG strains of HSV did not exhibit enhanced entry into CHO-HVEM12 cells, although they did infect Vero and HeLa in a normal fashion (35). Moreover, gD-1(rid1t) and gD-1(ANGt) proteins blocked infection of Vero cells by the KOS strain (38) and gD-1(306t) can block infection of Vero cells by many HSV strains, including KOS, but not infection by ANG and rid1 viruses. Here, we found that neither gD-1(rid1t) nor gD-1(ANGt) protein bound to HVEM (200t) in vitro. Experiments are under way to identify molecules which mediate infection by the rid and ANG strains of HSV. Should they work in a fashion analogous to that of HVEM, we would expect them to bind to gD-1(rid1t) and gD-1 (ANGt).

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REFERENCES

1. **Baker, S. J., and E. P. Reddy.** 1996. Transducers of life and death: TNF receptor superfamily and associated proteins. Oncogene **12:**1–9.
2. **Banner, D. W., A. D'Arcy, W. Janes, R. Gentz, H.-J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer.** 1993. Crystal structure of the soluble human 55 kd receptor-human TNFβ complex: implications for the TNF receptor activation. Cell **73:**431–445.
3. **Brunetti, C. R., R. L. Burke, B. Hoflack, T. Ludwig, K. S. Dingwell, and D. C. Johnson.** 1995. Role of mannose-6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission. J. Virol **69:**3517–3528.
4. **Brunetti, C. R., R. L. Burke, S. Kornfeld, W. Gregory, F. R. Masiarz, K. S. Dingwell, and D. C. Johnson.** 1994. Herpes simplex virus glycoprotein D acquires mannose 6-phosphate residues and binds to mannose 6-phosphate receptors. J. Biol. Chem. **269:**17067–17074.
5. **Campadelli-Fiume, G., M. Arsenakis, F. Farabegoli, and B. Roizman.** 1988. Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. J. Virol. **62:**159–167.
6. **Campadelli-Fiume, G., S. Qi, E. Avitabile, L. Foà-Tomasi, R. Brandimarti, and B. Roizman.** 1990. Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. J. Virol. **64:**6070–6079.
7. **Chiang, H.-Y., G. H. Cohen, and R. J. Eisenberg.** 1994. Identification of functional regions of herpes simplex virus glycoprotein gD by using linker-insertion mutagenesis. J. Virol. **68:**2529–2543.
8. **Cohen, G. H., B. Dietzschold, M. Ponce de Leon, D. Long, E. Golub, A. Varrichio, L. Pereira, and R. J. Eisenberg.** 1984. Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates production of neutralizing antibody. J. Virol. **49:**102–108.
9. **Cohen, G. H., M. I. Muggeridge, D. Long, D. A. Sodora, and R. J. Eisenberg.** 1992. Structural and functional studies of herpes simplex virus glycoprotein D, p. 217–228. *In* J. E. Ciardi (ed.), Genetically engineered vaccines. Plenum Press, New York, N.Y.
10. **Cunningham, B. C., M. Ultsch, A. M. de Vos, M. G. Mulkerrin, K. R. Clauser, and J. A. Wells.** 1991. Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. Science **254:**821–825.
11. **Dean, H. J., S. S. Terhune, M. Shieh, N. Susmarski, and P. G. Spear.** 1994. Single amino acid substitutions in gD of herpes simplex virus 1 confer resistance to gD-mediated interference and cause cell-type-dependent alterations in infectivity. Virology **199:**67–80.
12. **Dean, H. J., M. S. Warner, S. S. Terhune, R. M. Johnson, and P. G. Spear.** 1995. Viral determinants of the variable sensitivity of herpes simplex virus strains to gD-mediated interference. J. Viol. **69:**5171–5176.
13. **Dubin, G., N. O. Fishman, R. J. Eisenberg, G. H. Cohen, and H. M. Friedman.** 1992. The role of the herpes simplex viruses in immune evasion. p. 111–120. *In* B. T. Rouse (ed.), Herpes simplex viruses. Springer-Verlag, New York, N.Y.
14. **Dubin, G., and H. Jiang.** 1995. Expression of herpes simplex virus type 1 glycoprotein L (gL) in transfected mammalian cells: evidence that gL is not independently anchored to cell membranes. J. Virol. **69:**4564–4568.
15. **Eisenberg, R. J., D. Long, D. L. Sodora, H.-Y. Chiang, W. C. Wilcox, W. R. Abrams, M. I. Muggeridge, and G. H. Cohen.** 1994. Structure and function of glycoprotein D of herpes simplex virus, p. 43–65. *In* Y. Becker and G. Darai (ed.), Frontiers in virology, vol. 3. Springer-Verlag, Heidelberg, Germany.
16. **Eisenberg, R. J., M. Ponce de Leon, H. M. Friedman, L. F. Fries, M. M. Frank, J. C. Hastings, and G. H. Cohen.** 1987. Complement component C3b binds directly to purified glycoprotein C of herpes simplex virus types 1 and 2. Microb. Pathog. **3:**423–435.
17. **Fuller, A. O., and W.-C. Lee.** 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. J. Virol. **66:**5002–5012.
18. **Gilbert, J. M., P. Bates, H. E. Varmus, and J. M. White.** 1994. The receptor for the subgroup A avian leukosis sarcoma viruses binds to subgroup A but not to subgroup C envelope glycoprotein. J. Virol. **68:**5623–5628.
19. **Herold, B. C., R. J. Visalli, N. Sumarski, C. Brandt, and P. G. Spear.** 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulfate and glycoprotein B. J. Gen. Virol. **75:**1211–1222.
20. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the absorption of virus to cells and in infectivity. J. Virol. **65:**1090–1098.
21. **Hillenkamp, F., M. Karas, R. C. Beavis, and B. T. Chait.** 1991. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. Anal. Chem. **63:**1192A–1203A.
22. **Huang, T., and G. Campadelli-Fiume.** 1996. Anti-idiotypic antibodies mimicking glycoprotein D of herpes simplex virus identify a cellular protein required for virus spread from cell to cell and virus-induced polykaryocytosis. Proc. Natl. Acad. Sci. USA **93:**1836–1840.
23. **Isola, V. J., R. J. Eisenberg, G. R. Siebert, C. J. Heilman, W. C. Wilcox, and G. H. Cohen.** 1989. Fine mapping of antigenic site II of herpes simplex virus glycoprotein D. J. Virol. **63:**2325–2334.
24. **Izumi, K. M., and J. G. Stevens.** 1990. Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene. J. Exp. Med. **172:**487–496.
25. **Johnson, D. C., R. L. Burke, and T. Gregory.** 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. Virol. **64:**2569–2576.
26. **Johnson, D. C., and M. W. Ligas.** 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. **62:**4605–4612.
27. **Johnson, R. M., and P. G. Spear.** 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J. Virol. **63:**819–827.
28. **Kaner, R. J., A. Baird, A. Mansukhani, C. Basilico, B. D. Summers, R. Z. Florkiewicz, and D. P. Hajjar.** 1990. Fibroblast growth factor receptor is a portal of cellular entry for herpes simplex virus type 1. Science **248:**1410–1413.
29. **Lee, W.-C., and A. O. Fuller.** 1993. Herpes simplex virus type 1 and pseudorabies virus bind to a common saturable receptor on Vero cells that is not

heparan sulfate. J. Virol. **67:**5088–5097.
30. **Loetscher, H., R. Gentz, M. Zulauf, A. Lustig, H. Tabuchi, E.-J. Schlager, M. Brockhurst, H. Gallati, M. Manneberg, and W. Lesslauer.** 1991. Recombinant 55-kDa tumor necrosis factor (TNF) receptor. J. Biol. Chem. **266:** 18324–18329.
31. **Long, D., W. C. Wilcox, W. R. Abrams, G. H. Cohen, and R. J. Eisenberg.** 1992. Disulfide bond structure of glycoprotein D of herpes simplex virus types 1 and 2. J. Virol. **66:**6668–6685.
32. **Manservigi, R., M. P. Grossi, R. Gualandri, P. G. Balboni, A. Marchini, A. Rotola, P. Rimessi, D. Di Luca, E. Cassai, and G. Barbanti-Brodano.** 1990. Protection from herpes simplex virus type 1 lethal and latent infections by secreted recombinant glycoprotein B constitutively expressed in human cells with a BK virus episomal vector. J. Virol. **64:**431–436.
33. **Manservigi, R., R. Gualandri, M. Negrini, L. Albonici, G. Milanesi, E. Cassai, and G. Barbanti-Brodano.** 1988. Constitutive expression in human cells of herpes simplex virus type 1 glycoprotein B gene cloned in an episomal eukaryotic vector. Virology. **167:**284–288.
34. **Mirda, D. P., D. Navarro, P. L. Lee, P. Paz, L. Pereira, and L. T. Williams.** 1992. The fibroblast growth factor receptor is not required for herpes simplex virus type 1 infection. J. Virol. **66:**448–457.
35. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87:**427–436.
36. **Muggeridge, M. I., G. H. Cohen, and R. J. Eisenberg.** 1992. Herpes simplex virus infection can occur without involvement of the fibroblast growth factor receptor. J. Virol. **66:**824–830.
37. **Muggeridge, M. I., W. C. Wilcox, G. H. Cohen, and R. J. Eisenberg.** 1990. Identification of a site on herpes simplex virus type 1 gD that is essential for infectivity. J. Virol. **64:**3617–3626.
38. **Nicola, A. V., C. Peng, H. Lou, G. H. Cohen, and R. J. Eisenberg.** 1997. Antigenic structure of soluble herpes simplex virus (HSV) glycoprotein D correlates with inhibition of HSV infection. J. Virol. **71:**2940–2946.
39. **Nicola, A. V., S. H. Willis, N. N. Naidoo, R. J. Eisenberg, and G. H. Cohen.** 1996. Structure-function analysis of soluble forms of herpes simplex virus glycoprotein D. J. Virol. **70:**3815–3822.
40. **Novotny, M. J., and P. G. Spear.** Unpublished data.
41. **Peng, T., G. Dubin, R. J. Eisenberg, and G. H. Cohen.** Unpublished data.
42. **Peng, T., M. Ponce de Leon, H. Jiang, G. Dubin, G. H. Cohen, and R. J. Eisenberg.** 1996. Studies of a soluble form of the HSV gH-gL complex expressed by a mammalian cell line, abstr. 130. *In* 21st International Herpesvirus Workshop, De Kalb, Ill.
43. **Pennica, D., V. T. Lam, R. F. Weber, W. J. Kohr, L. J. Basa, M. W. Spellman, A. Ashkenazi, S. J. Shire, and D. V. Goeddel.** 1993. Biochemical characterization of the extracellular domain of the 75-kilodalton tumor necrosis factor receptor. Biochemistry **32:**3131–3138.
44. **Roller, R. J., and B. Roizman.** 1994. A herpes simplex virus US11-expressing cell line is resistant to herpes simplex virus infection at a step in viral entry mediated by glycoprotein D. J. Virol. **68:**2830–2839.
45. **Rux, A. H., W. T. Moore, J. D. Lambris, W. R. Abrams, C. Peng, H. M. Friedman, G. H. Cohen, and R. J. Eisenberg.** 1996. Disulfide bond structure determination and biochemical analysis of glycoprotein C from herpes simplex virus. J. Virol. **70:**5455–5465.
46. **Rux, J. J., S. H. Willis, A. V. Nicola, R. M. Burnett, G. H. Cohen, and R. J. Eisenberg.** 1996. Crystallization of truncated HSV glycoprotein D produced in a baculovirus expression system, abstr. 119. *In* 21st International Herpesvirus Workshop, De Kalb, Ill.
47. **Shieh, M.-T., and P. G. Spear.** 1991. Fibroblast growth factor receptor: does it have a role in the binding of herpes simplex virus? Science **253:**208–209.
48. **Showalter, S. D., M. Zweig, and B. Hampar.** 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. Infect. Immun. **34:**684–692.
49. **Sisk, W. P., J. D. Bradley, R. J. Leipold, A. M. Stoltzfus, M. Ponce de Leon, M. Hilf, C. Peng, G. H. Cohen, and R. J. Eisenberg.** 1994. High-level expression and purification of secreted forms of herpes simplex virus type 1 glycoprotein gD synthesized by baculovirus-infected insect cells. J. Virol. **68:**766–775.
50. **Smith, C. A., T. Farrah, and R. G. Goodwin.** 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell **76:**959–962.
51. **Sodora, D. L., G. H. Cohen, M. I. Muggeridge, and R. J. Eisenberg.** 1991. Absence of asparagine-linked oligosaccharides from glycoprotein D of herpes simplex virus type 1 results in a structurally altered but biologically active protein. J. Virol. **65:**4424–4431.
52. **Sodora, D. L., R. J. Eisenberg, and G. H. Cohen.** 1991. Characterization of a recombinant herpes simplex virus which expresses a glycoprotein D lacking asparagine-linked oligosaccharides. J. Virol. **65:**4432–4441.
53. **Spear, P. G.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Inc., Boca Raton, Fla.
54. **Tal-Singer, R., R. J. Eisenberg, T. Valyi-Nagy, N. W. Fraser, and G. H. Cohen.** 1994. N-linked oligosaccharides on herpes simplex virus glycoprotein gD are not essential for establishment of viral latency or reactivation in the mouse eye model. Virology. **202:**1050–1053.
55. **Tal-Singer, R., C. Peng, M. Ponce de Leon, W. R. Abrams, B. W. Banfield, F. Tufaro, G. H. Cohen, and R. J. Eisenberg.** 1995. The interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. J. Virol. **69:**4471–4483.
56. **Tessier, D. C., D. Y. Thomas, H. E. Khouri, F. Laliberte, and T. Vernet.** 1991. Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide. Gene **98:**177–183.
57. **Warner, M. S., R. I. Montgomery, and P. G. Spear.** 1996. A herpes simplex virus type 1 mutant resistant to gD mediated interference is altered in utilization of cell surface receptors for entry, p. 94, abstr. W7-3. *In* American Society for Virology Annual Meeting, London, Ontario, Canada.
58. **Wells, J. A.** 1996. Binding in the growth hormone complex. Proc. Natl. Acad. Sci. USA **93:**1–6.
59. **Wells, J. A.** 1994. Structural and functional basis for hormone binding and receptor oligomerization. Curr. Opin. Cell. Biol. **6:**163–173.
60. **Willis, S. H., A. V. Nicola, J. J. Rux, R. M. Burnett, R. J. Eisenberg, and G. H. Cohen.** Unpublished data.
61. **Willis, S. H., C. Peng, M. Ponce de Leon, A. V. Nicola, A. H. Rux, G. H. Cohen, and R. J. Eisenberg.** Expression and purification of secreted forms of herpes simplex virus glycoproteins from baculovirus-infected insect cells. *In* M. S. Brown and A. R. MacLean (ed.), Methods in molecular medicine, in press. Humana Press, Totowa, N.J.
62. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63:**52–58.