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Antigenic Structure of Soluble Herpes Simplex Virus (HSV) Glycoprotein D Correlates with Inhibition of HSV Infection

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Soluble forms of herpes simplex virus (HSV) glycoprotein D (gD) block viral penetration. Likewise, most HSV strains are sensitive to gD-mediated interference by cells expressing gD. The mechanism of both forms of gD-mediated inhibition is thought to be at the receptor level. We analyzed the ability of different forms of soluble, truncated gD (gDt) to inhibit infection by different strains of HSV-1 and HSV-2. Strains that were resistant to gD-mediated interference were also resistant to inhibition by gDt, thereby suggesting a link between these two phenomena. Virion gD was the major viral determinant for resistance to inhibition by gDt. An insertion-deletion mutant, gD-1(Δ290–299t), had an enhanced inhibitory activity against most strains tested. The structure and function of gDt proteins derived from the inhibition-resistant viruses rid1 and ANG were analyzed. gD-1(rid1t) and gD-1(ANGt) had a potent inhibitory effect on plaque formation by wild-type strains of HSV but, surprisingly, little or no effect on their parental strains. As measured by quantitative enzyme-linked immunosorbent assay with a diverse panel of monoclonal antibodies, the antigenic structures of gD-1(rid1t) and gD-1(ANGt) were divergent from that of the wild type yet were similar to each other and to that of gD-1(Δ290–299t). Thus, three different forms of gD have common antigenic changes that correlate with enhanced inhibitory activity against HSV. We conclude that inhibition of HSV infectivity by soluble gD is influenced by the antigenic conformation of the blocking gDt as well as the form of gD in the target virus.

The virion envelope of herpes simplex virus (HSV) contains at least 10 glycoproteins (49). The initial attachment of HSV to cell surface heparan sulfate proteoglycans is mediated by glycoprotein C (gC) and/or gB (20, 21, 56). This is thought to be followed by the interaction of gD with a cellular receptor (4, 26, 27, 31). Then gD along with gB, gH, and gL act alone or in combination to trigger pH-independent fusion of the viral envelope with the host cell plasma membrane (49). gD is essential for entry into mammalian cells (32) and has been implicated in cell fusion (49) and neuroinvasiveness (25).

gD inhibits HSV infection of cultured cells in several ways. For example, wild-type strains of HSV do not infect cells expressing gD (4, 6, 28). This gD-mediated interference phenomenon occurs at the level of penetration and is dependent on the structure of gD in the infecting virus (5, 10, 11). Also, cells pretreated with soluble, truncated gD (gDt) are resistant to infection (18, 26, 42, 50). The mechanism of these forms of gD-mediated inhibition is presumably via interference with HSV binding to its cognate cellular receptor(s).

We previously used mutagenesis and complementation analysis to define regions of gD that are important for virus entry (7). Mutations that did not globally alter the gD structure yet resulted in a molecule that failed to complement a gD-null virus were clustered in four distinct functional regions. These were defined as region I (residues 27 through 43), region II (residues 126 through 161), region III (residues 225 through 246) (16, 40), and region IV (residues 277 through 310). To address why these gD mutants do not function in infection, we recently tested the ability of "nonfunctional" forms of baculovirus-derived gDt representing each functional region to inhibit the infectivity of HSV-1 NS (42). The region I mutant failed to inhibit HSV plaque formation and cell-to-cell spread. The region II and III mutants inhibited these processes although less efficiently than did wild-type gDt. Surprisingly, the region IV mutant, gD-1(Δ290–299t), had a markedly enhanced inhibitory effect on HSV-1 infectivity. Thus, the capacity of mutant forms of gD to inhibit infection did not correlate with the inability of these mutants to complement infection. This suggested that gD has more than a single functional domain.

A better understanding of inhibition of plaque formation by gDt will give insight into the HSV infection process as well as how to prevent it. Here we examined the ability of a larger panel of gDt molecules to inhibit different strains of HSV-1 and HSV-2. We demonstrate that (i) the level of inhibition of HSV infection by gDt is dependent on the allele of gD present in the target virus; (ii) inhibition by gDt is similar but not identical to inhibition by full-length gD expressed in cells (gD-mediated interference); and (iii) variations in the inhibitory activity of wild-type and variant forms of gDt correlate with antigenic differences in gD.

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MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were grown at 37°C in Dulbecco's minimal essential medium supplemented with 5% fetal calf serum. Sf9 (*Spodoptera frugiperda*) cells (GIBCO BRL) used for producing recombinant baculoviruses and recombinant glycoproteins were propagated in Sf900II medium (GIBCO BRL) (42, 48, 55). The following strains of HSV-1 were used: 17, ANG, ANG path (29), HFEM syn (1), KOS, (KOS)rid1, (KOS)rid2 (10) (abbreviated rid1 and rid2, respectively), Patton, and ANG/KOS, a recombinant KOS containing ANG gD in place of KOS gD (11) (kindly provided by M. Warner and P. Spear). In addition, we used HSV-2 333 and G (14). All HSV strains were grown and subjected to titer determination on Vero cells.

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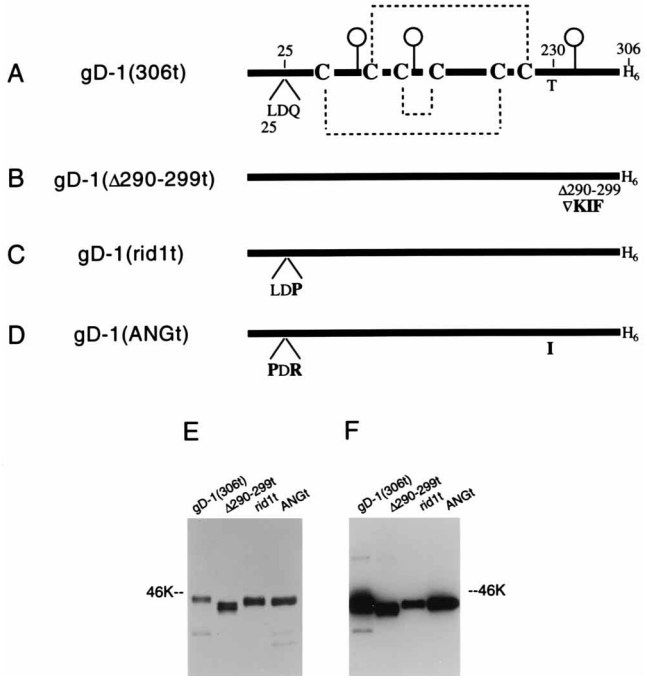

FIG. 1. Schematic representations of truncated forms of HSV gD. Each baculovirus-derived, ectodomain form of gD is truncated at residue 306 and lacks amino acids 307 to 369, which contain the transmembrane and cytoplasmic tail regions present in full-length gD (not shown) from HSV and HSV-infected cell surfaces. The honeybee mellitin signal peptide (not shown) replaces the wild-type signal and is cleaved from the fully processed protein. Each mature protein has amino acids DP (not shown) at the N terminus and a histidine tag at the C terminus. (A) Wild-type gD-1(306t) from HSV-1 Patton. Residues 1 to 306 are identical for gD from HSV-1 strains Patton and KOS (10, 53). The positions of several relevant amino acids are shown. gD has three N-linked sugars (balloons) (8) and three disulfide bonds (dashed lines) (34). (B) gD-1(Δ290–299t) (42) from HSV-1 Patton has amino acids 290 to 299 (IPPNWHIPSI) deleted, R replacing I at residue 290, and amino acids KIF inserted after R. (C and D) Truncated forms of gD from HSV-1 (C) rid1 and (D) ANG. Amino acid changes relative to gD-1(306t) are shown in boldface type (10, 25). (E and F) Purified proteins were electrophoresed under denaturing conditions on 10% polyacrylamide gels and either stained with silver nitrate (E) or Western blotted and probed with rabbit polyclonal antiserum R7 (F). Protein names are indicated above the lanes.

Construction of baculovirus vectors expressing gD-1(rid1t) and gD-1(ANGt). The strategy used to produce gD truncated at amino acid 306 prior to the transmembrane region has been described previously (42, 48). Briefly, DNA fragments were generated by PCR with plasmid pMW13 which contains rid1 gD (10) or a 6.45-kb *Bam*HI fragment of HSV-1 ANG DNA which contains gD as a template and the primers used to construct bac-gD-1(306t) (48). The PCR products were each ligated into the transfer vector pVTBac (51) to produce plasmids pCP269 and pCP271, respectively. These were recombined into the baculovirus (*Autographa californica* nuclear polyhedrosis virus) by cotransfection of Sf9 cells with Baculogold DNA (Pharmingen), resulting in viruses designated bac-gD-1(rid1t) and bac-gD-1(ANGt). The protein products are designated gD-1(rid1t) and gD-1(ANGt), respectively. The cloning of bac-gD-1(306t) and bac-gD-1(Δ290–299t), which express gD-1(306t) and gD-1(Δ290–299t) (from strain Patton), respectively, was described previously (42, 48).

Production and purification of gDt. Detailed protocols exist elsewhere for the purification of gDt (48, 55). In brief, Sf9 cells were grown in suspension cultures and infected with recombinant baculovirus at a multiplicity of infection of 4. At 96 h postinfection, cells were pelleted by centrifugation and the supernatant fluid was passed over a monoclonal antibody (MAb) DL6-Sepharose column. The protein was eluted with freshly prepared 0.1 M ethanolamine (pH 11) (Sigma), neutralized with 2.5 M Tris-HCl, concentrated with a PM10 membrane (Amicon), and dialyzed against phosphate-buffered saline (PBS).

Polyclonal antibodies and MAbs. Rabbit anti-gD serum R7 (24) was used for Western immunoblotting. Rabbit anti-gB (R69 and R91) and anti-gC (R46) sera (13) were used in immunoperoxidase assays. Anti-gD MAb DL6 (group IIb), which recognizes a continuous epitope from residues 272 to 279 (12, 24), was used for immunoaffinity purification and for analysis of antigenic activity. Anti-gD MAbs HD1 (group Ia) (38, 43), DL11 (group Ib) (9, 38), VID (group IIIa) (39, 46), ABD (group IIIb) (46), 45S (group IV) (47), DL2 (group VI) (9), D4 (group IX) (22), and AP7 (group XII) (7, 36), which recognize discontinuous epitopes, and MAb 1D3 (group IV) (17), which recognizes a continuous epitope (residues 11 to 19), were all used for analysis of antigenic structure.

SDS-PAGE analysis. Purified glycoproteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions in 10% polyacrylamide gels. Following SDS-PAGE, the proteins were either stained with silver nitrate (Pharmacia) or transferred to nitrocellulose and reacted with polyclonal antibody R7.

Plaque inhibition assay. The effect of purified forms of gDt on HSV plaque production was assayed essentially as described previously (26, 50). Briefly, Vero cell monolayers were treated with one of the purified glycoproteins for 1.5 h at 4°C. HSV was added (50 PFU per well) for 1.5 h at 4°C. The cells were overlaid and shifted to 37°C for 24 h. The cells were fixed, and plaques were visualized by immunoperoxidase staining.

Antigenic analysis by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96-well; Corning) were coated with proteins at a concentration of 8 μg/ml in PBS. Nonspecific binding was blocked by the addition of blocking buffer (PBS, 1% bovine serum albumin, 1% chicken ovalbumin). Twofold serial dilutions (in blocking buffer) of ascites fluids of anti-gD MAbs were added for 1 h. Plates were washed three times with PBS, and protein A-horseradish peroxidase (Boehringer Mannheim) was added for 1 h. The plates were washed three times with PBS and then rinsed with 20 mM citrate buffer (pH 4.5). Finally, substrate, 2,2′-azino-di(3-ethylbenzthiozoline-6-sulfonic acid) (ABTS; Moss, Inc.) was

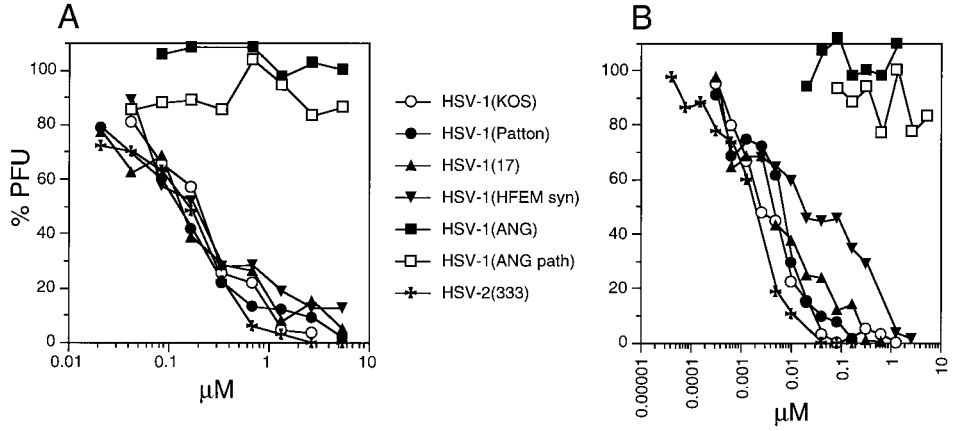

FIG. 2. Effect of gDt on plaque formation by different strains and serotypes of HSV. gD-1(306t) (A) or gD-1(Δ290–299t) (B) diluted in 5% Dulbecco's minimal essential medium was added to Vero cell monolayers in 48-well plates for 90 min at 4°C. The indicated strain of HSV (50 PFU) was added for 90 min at 4°C. Cells were overlaid with medium containing appropriate concentrations of gDt and shifted to 37°C for 24 h. The medium was removed, and the cells were fixed with methanol-acetone (2:1) solution for 20 min at −20°C and air dried. Virus titers were determined by an immunoperoxidase assay with anti-gB and anti-gC rabbit polyclonal antisera. Each point represents the mean of quadruplicate wells. 1 μM gDt = 36 μg/ml.

TABLE 1. Inhibition of HSV plaque formation by variant gDt

Protein	IC_{50}[a] (nM) for:										
	KOS	HFEM syn	17	Patton	ANG	ANG path	ANG/KOS	rid1	rid2	333	G
gD-1(306t)	218	180	139	132	NE[b]	NE	NE	NA[c]	NA	164	263
gD-1(Δ290–299t)	2.6	19.0	4.8	7.5	NE	NE	NE	76.6	65.0	1.5	16.4
gD-1(rid1t)	14.4	19.1	8.3	19.1	NE	NE	NE	71.1	263	24.6	20.6
gD-1(ANGt)	4.1	15.0	4.1	8.2	NE	NE	NE	153	134	8.8	16.3

[a] Concentration necessary for 50% inhibition of 50 PFU of HSV as measured by plaque formation on Vero cells. Several of the values shown represent multiple independent experiments. As an indication of statistical significance, the IC_{50} for inhibition of KOS by gD-1(Δ290–299t) has a standard deviation of 1.7.
[b] NE, no effect (at 5.6 μM inhibitor, there was no measurable effect on plaque formation).
[c] NA, not applicable (at the inhibitor concentrations tested, there was <50% inhibition of plaque formation).

added, and the absorbance at 405 nm was read with a microtiter plate reader (Dynatech).

Determination of the isoelectric point. Purified glycoproteins were resolved on polyacrylamide isoelectric focusing (IEF) gels (Pharmacia) with a pH range from 3 to 9 on a PhastSystem (Pharmacia).

RESULTS

Effect of gDt on different strains and serotypes of HSV. Stick models of several of the proteins used in this study are shown in Fig. 1A through D. All proteins were immunoaffinity purified as evidenced by SDS-PAGE followed by silver staining and Western blot analysis (Fig. 1E and F). Soluble gDt inhibits HSV infection (26, 42, 50). As previously shown, a mutant form of gDt with a linker-insertion mutation in functional region IV, gD-1(Δ290–299t) (Fig. 1B), had an enhanced inhibitory effect on HSV-1 NS compared to the effect of wild type gD-1(306t) (42). We questioned whether HSV-2 and other strains of HSV-1 were inhibited to the same extent. The 50% inhibitory concentration (IC_{50}) is defined as the concentration necessary to inhibit plaque formation by 50%. HSV-1 KOS, 17, HFEM syn, and Patton were similar in their sensitivity to gD-1(306t) (Fig. 2A), which had IC_{50}s ranging from 132 to 218 nM (Table 1). gD-1(306t) inhibited HSV-2 333 and G (Fig. 2A; Table 1), which is consistent with previous reports (26) and supports the notion that HSV-1 and HSV-2 have a similar infectious pathway (26, 40, 42, 45). gD-1(Δ290–299t) was significantly more effective at inhibiting plaque formation (Fig. 2B); the IC_{50}s were 10- to 100-fold lower than those obtained for gD-1(306t) (Table 1). Interestingly, neither form of gDt inhibited HSV-1 ANG and ANG path (Fig. 2).

HSV inhibition by soluble gDt is related to gD-mediated interference. Wild-type HSV-1 KOS, F, MacIntyre, mP, and 17 and HSV-2 G, 186, and 333 do not efficiently penetrate cells expressing gD (4, 6, 11, 28). However, strain ANG is resistant to interference by gD-expressing cells (10). In addition several viruses have been selected for their ability to infect gD-expressing cells (2, 5, 10). The ability of these viruses to overcome gD-mediated interference has been mapped to virion gD. Dean et al. (10) selected two such HSV-1 KOS mutants, rid1 and rid2, which have Pro and Arg substitutions, respectively, at Gln-27 in gD.

Since ANG was resistant to inhibition by both gDt (Fig. 2) and gD-expressing cells, we speculated that inhibition by exogenous gDt was related to inhibition by endogenous full-length gD. To confirm this speculation, we tested whether plaque formation by rid1 or rid2 was inhibited by gDt. gD-1(306t) had little effect on plaque formation by rid1 and rid2 (Fig. 3A; Table 1). However, there was slight inhibition of the rid viruses when concentrations were raised above 0.02 μM gD-1(306t). Since virus strains resistant to gD-mediated interference are also resistant to inhibition by wild-type gDt, we conclude that these two phenomena are related. Although the two rid strains were only minimally inhibited by gD-1(306t), they were inhibited by gD-1(Δ290–299t) in a dose-dependent manner (Fig. 3B; Table 1). However, rid1 and rid2 were more resistant to inhibition by gD-1(Δ290–299t) than were the wild-type strains of HSV (Fig. 3B; Table 1); the IC_{50}s were 3 to 51 times higher. Since ANG and ANG path were resistant to inhibition by both wild-type and mutant forms of gDt (Fig. 2;

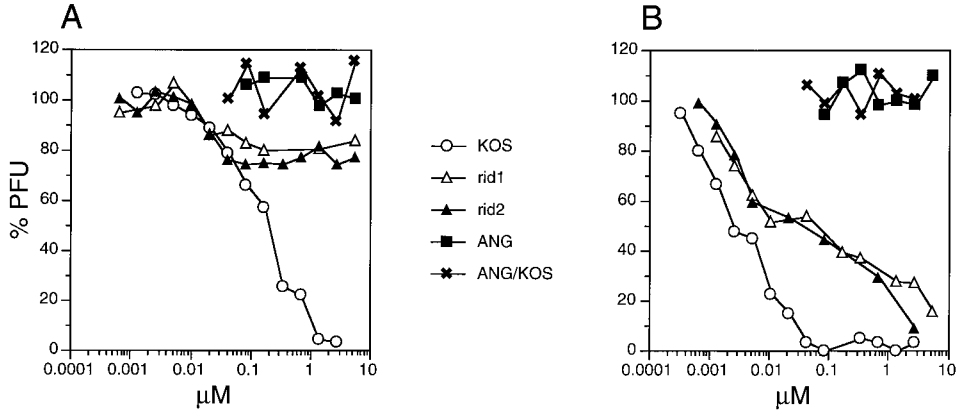

FIG. 3. Effect of gDt on plaque formation by HSV strains that are resistant to gD-mediated interference. gD-1(306t) (A) or gD-1(Δ290–299t) (B) was added to Vero cell monolayers in 48-well plates for 90 min at 4°C. The plates were processed as described in the legend to Fig. 2. Each point represents the mean of quadruplicate wells.

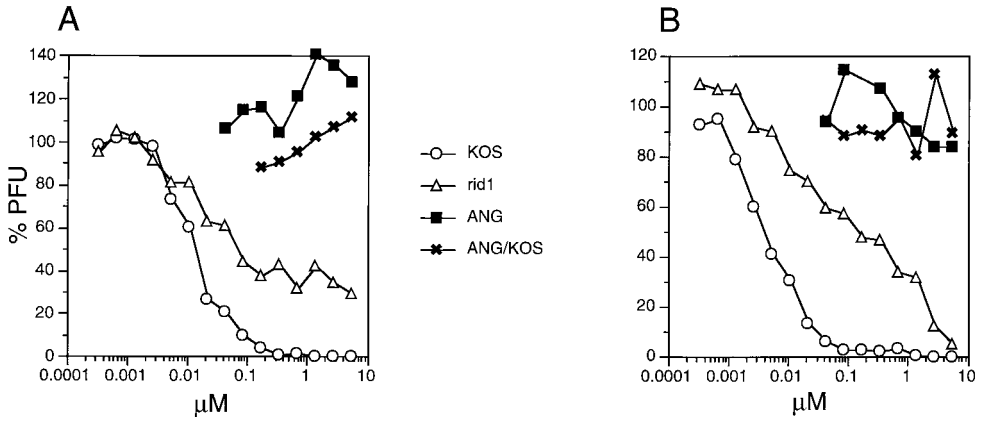

FIG. 4. Effect of gD-1(rid1t) and gD-1(ANGt) on HSV plaque formation. gD-1(rid1t) (A) or gD-1(ANGt) (B) was added to Vero cell monolayers in 48-well plates for 90 min at 4°C. The plates were processed as described in the legend to Fig. 2. Each point represents the mean of quadruplicate wells.

Table 1), this suggests that ANG virus infection of Vero cells is distinct from that of other viruses tested.

HSV-1 ANG was derived from a clinical isolate that was plated under agar and is the only "wild-type" HSV isolate known to have amino acid sequence changes at residues 25 and/or 27 in gD (25). ANG path is a brain-passaged derivative of ANG (29) that has a neuroinvasive phenotype due to an Ala-84–to–Gly mutation in gD (25). When ANG or ANG path are added to cells at a high multiplicity of infection, there is extensive cell fusion in less than 60 min in the absence of viral gene expression (15). This "fusion-from-without" property is due to changes in ANG gB (33). Dean et al. (11) constructed a recombinant HSV, ANG/KOS, that contains ANG gD in a KOS background to demonstrate that gD is an important determinant for the resistance of HSV ANG to gD-mediated interference. We found that neither gD-1(306t) nor gD-1(Δ290–299t) inhibited ANG/KOS (Fig. 3). Thus, by virtue of changes in their gD alleles, ANG and rid strains overcome inhibition by both endogenous and exogenous wild-type gD.

Effect of gD-1(rid1t) and gD-1(ANGt) on HSV plaque formation. Since the gD molecules from rid and ANG viruses were associated with resistance to gD-mediated inhibition, we decided to examine soluble forms of these proteins. We constructed baculovirus vectors that express truncated, ectodomain forms of gD from HSV rid1 and ANG (Fig. 1C and D); they were designated gD-1(rid1t) and gD-1(ANGt). Compared to wild-type gD-1(306t), gD-1(rid1t) has a Gln-27–to–Pro substitution and gD-1(ANGt) has Leu-25–to–Pro, Gln-27–to–Arg, and Thr-230–to–Ile substitutions (Fig. 1). Both proteins were immunoaffinity purified (Fig. 1E and F) and tested for their ability to inhibit HSV plaque formation (Fig. 4; Table 1). gD-1(rid1t) and gD-1(ANGt) effectively inhibited HSV-1 KOS, HFEM syn, Patton, and 17 and HSV-2 333 and G. Surprisingly, these proteins were less effective at inhibiting HSV-1 rid1 or rid2 and were not effective at inhibiting HSV-1 ANG, ANG path, or ANG/KOS. Thus, gD-1(rid1t) and gD-1(ANGt) inhibited heterologous wild-type strains more effectively than they inhibited the viruses from which they were derived. In fact, HSV-1 ANG, ANG path, and ANG/KOS were highly resistant to inhibition by all forms of gDt tested. Unexpectedly, HSV strains bearing wild-type forms of gD were inhibited 7 to 53 times more effectively by gD-1(rid1t) and gD-1(ANGt) than by wild-type gD-1(306t) (Table 1). It is also interesting that the IC_{50} for rid1 inhibition by gD-1(rid1t) is similar to the IC_{50} for wild-type HSV inhibition by gD-1(306t).

A truncated form of HSV-2 gD, gD-2(306t), differs from gD-1(306t) at 35 residues yet had an equivalent inhibitory activity to that of gD-1(306t) against HSV-1 NS (42). Furthermore, despite these many changes, gD-2(306t) inhibited wild-type HSV-1 and HSV-2 strains and failed to inhibit rid and ANG viruses (data not shown) in a manner similar to gD-1(306t) (Table 1). Thus, the seemingly minor amino acid substitutions in gD-1(rid1t) and gD-1(ANGt) have a specific effect on functional activity. Interestingly, the amino acids at which gD-1(rid1t) and gD-1(ANGt) differ from gD-1(306t) are conserved between HSV-1 and HSV-2. This enhanced blocking activity is shared by gD-1(Δ290–299t). Since gD-1(Δ290–299t) has an insertion-deletion mutation near the C terminus of the ectodomain and both gD-1(rid1t) and gD-1(ANGt) have sequence changes primarily at the N terminus, we asked whether the functional similarity of these three molecules reflected a structural similarity.

Structural analysis of forms of gDt that have potent inhibitory activity. Mutations in either the N- or C-terminal end of the gD ectodomain disrupt the AP7 epitope (antigenic site XII), suggesting that these two areas are in contact (7). Several prior observations related the loss of AP7 reactivity to changes at the N terminus of gD. Minson et al. (36) characterized an AP7 antibody-resistant virus which had a Pro substitution at Leu-25 in gD. Subsequently, Campadelli-Fiume et al. (5) selected an HSV-1 mutant (U-10) with an identical mutation that infected gD-expressing cells. Immunoprecipitation analysis of U-10 gD revealed that it failed to react with AP7 yet reacted with several MAbs, including two that recognized antigenic sites Ia and Ib (5). Also, rid and ANG viruses were resistant to neutralization by AP7, and rid viruses were partially resistant to the ungrouped MAb I-99-1 (10). gD with a more C-terminal mutation (Δ290–299) also lacked the AP7 epitope (7, 42). Furthermore, gD-1(Δ290–299t) had wild-type-like levels of reactivity with MAbs that recognize antigenic sites Ia, Ib, IIb, and IIIb (42). To determine whether gD-1(rid1t), gD-1(ANGt), and gD-1(Δ290–299t) had other common antigenic alterations, we used quantitative ELISA (42) with MAbs that recognize 10 different epitopes across gD.

ELISA reactivity was quantitated relative to wild type gD-1(306t) (Table 2). As expected, each protein reacted equally well with DL6 and 1D3, which bind linear epitopes (12, 17, 24), and the three variants failed to react with AP7. gD-1(rid1t) and gD-1(ANGt) had wild-type or enhanced reactivity with conformation-dependent MAbs DL11, VID, and ABD and decreased reactivity with MAbs HD1, 45S, DL2, and D4. Thus, the overall antigenic structures of gD-1(rid1t) and gD-1(ANGt) are

TABLE 2. Analysis of variant gDt by ELISA

Protein	MAb binding[a] at antigenic site:									
	Ia (HD1)	Ib (DL11)	IIb (DL6)[b]	IIIa (VID)	IIIb (ABD)	IV (45S)	VI (DL2)	VII (1D3)[b]	IX (D4)	XII (AP7)
gD-1(306t)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
gD-1(Δ290–299t)[c]	1.07	1.07	1.00	0.83	1.00	0.84	0.67	1.00	0.58	0.00
gD-1(rid1t)	0.73	1.05	1.00	1.00	1.00	0.57	0.81	1.00	0.52	0.00
gD-1(ANGt)	0.70	1.15	1.00	1.18	1.17	0.35	0.79	1.00	0.34	0.00

[a] Values shown represent the 50% saturation point for MAb binding relative to gD-1(306t) reactivity, which is normalized to 1.00. Each value represents the mean of at least two independent experiments done in duplicate. MAbs are shown in parentheses.
[b] These two MAbs recognize continuous epitopes. All others recognize discontinuous (conformation-dependent) epitopes.
[c] ELISA reactivity of gD-1(Δ290–299t) with HD1, DL11, DL6, ABD, DL2, and AP7 was reported previously (42) and is shown here for comparison.

similar to each other yet divergent from that of wild-type gD-1(306t) in several distinct discontinuous epitopes. Like gD-1(rid1t) and gD-1(ANGt), gD-1(Δ290–299t) had decreased reactivity with 45S, DL2, and D4 (Table 2). Thus, common antigenic changes in gD, most notably the loss of the AP7 epitope, correlate with the resistance of rid and ANG viruses to inhibition mediated by both exogenous and endogenous gD. Also, the antigenic alterations shared by gD-1(rid1t), gD-1(ANGt), and gD-1(Δ290–299t) correlate with enhanced inhibitory activity.

The pI of a protein represents the overall surface charge of a molecule and is based, in part, on the presence of charged amino acids on the exterior of a protein. Therefore, it is an indirect measure of protein conformation. To determine the pI of the gD variants, they were resolved on IEF gels and then stained with silver nitrate (Fig. 5). The average pI of the three major species of gD-1(306t) was 5.9. In contrast, gD-1(Δ290–299t) had an average pI of 6.3, and gD-1(rid1t) and gD-1(ANGt) had average pI values of 5.5. The different pI values of the variant proteins cannot be explained solely by a change in the total number of charged residues in gD (Fig. 1) but may be due to changes in protein structure induced by the amino acid alterations. The pI values of gD-1(rid1t) and gD-1(ANGt) were divergent from that of gD-1(Δ290–299t), suggesting structural differences that are not readily apparent from the antigenic analysis.

DISCUSSION

The involvement of gD in inhibition of HSV infection is well documented. First, pretreatment of cells with UV-inactivated HSV blocks penetration of untreated HSV, and this is dependent on the presence of gD in the blocking virions (27, 31). Second, gD expressed in cells restricts the entry of many strains of HSV (4, 6, 11, 28), supporting the notion that gD plays a role in HSV superinfection restriction. Lastly, soluble gD blocks HSV infectivity in vitro and in vivo (18, 26, 35, 42, 50). One common explanation for gD-mediated inhibition is that the blocking gD competes with virion gD for binding to a cellular receptor. We previously showed that baculovirus-derived wild-type gD-1(306t) inhibited HSV-1 NS (42). In the present study, we extend this characterization to include 11 other strains of HSV. We demonstrate that inhibition of virus infection by gDt is influenced by the antigenic conformation of the blocking gDt and that of gD in the target virus.

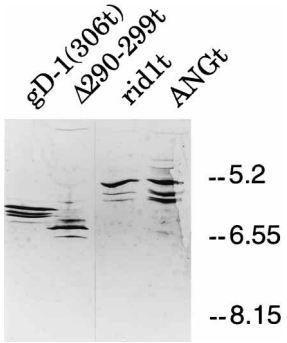

FIG. 5. Isoelectric point determination of gDt. Purified proteins in PBS were resolved on polyacrylamide IEF gels (pH gradient from 3 to 9) and then stained with silver nitrate. Values for standards are indicated on the right.

Inhibition by gDt is similar, but not identical, to gD-mediated interference. Although the two HSV serotypes have sequence differences (52), most strains of HSV-1 and both strains of HSV-2 tested were inhibited by gD-1(306t). However, HSV-1 ANG, ANG path, rid1, and rid2, which are very similar in sequence to wild-type HSV-1, were resistant to inhibition by gD-1(306t). The rid viruses and ANG were previously reported to overcome inhibition by gD-expressing cells (10). Taken together, these results suggest that exogenously added gDt and endogenously produced gD share a similar mechanism of inhibition. Furthermore, gD is the major viral determinant for susceptibility to inhibition by gDt as well as to interference by gD-expressing cells (2, 5, 10, 11). An advantage of using purified forms of gDt to study gD-mediated inhibition is that it allows for quantitation of the amount and effect of blocking gD added.

There are differences, however, between inhibition by exogenously added gD and endogenously produced gD. First, Dean et al. (11) noted an increased resistance of HSV-1 17 to inhibition by gD-expressing cells relative to other strains, yet we found that strain 17 was as sensitive to inhibition by gDt as were other strains. Second, HSV-1 ANG was highly resistant to inhibition by all forms of gDt, whereas rid1 was only partially resistant. In contrast, Dean et al. (10, 11) did not note a difference in the susceptibility to gD-mediated interference for these viruses. These discrepancies may be explained by cell type differences or the possibility that the fixed amount of gD produced in gD-expressing cells is insufficient in some cases. Alternatively, in gD-expressing cells, full-length gD may be acting during synthesis and/or transport to the cell surface. Although the majority of gDt added to cells remains at the cell surface (41), it is not clear whether some fraction of gDt is internalized.

Implications for gD structure. gD-1(Δ290–299t), gD-1(rid1t), and gD-1(ANGt) fail to react with MAb AP7 and are measurably reduced in reactivity with 45S, DL2, and D4. Thus, gD molecules from three different sources have common antigenic alterations that correlate with enhanced inhibitory activity. However, HSV with the Δ290–299 mutation in full-length gD is nonfunctional for penetration (7) whereas the rid1

(10) and ANG strains of HSV are infectious. Thus, the Δ290–299 mutation must have some unidentified effect on gD function that the rid1 and ANG amino acid changes do not. It is interesting that a mutation (Δ290–299) in functional region IV of gD had an effect on antigenic structure similar to that of a point mutation at residue 27 (the rid1 mutation). This supports the hypothesis of Chiang et al. (7) that the N terminus is in contact with the C-terminal end of the ectodomain in the native structure of gD. Maintenance of this structure may be critical for AP7 binding. Alternatively, a mutation in either end of the ectodomain may result in a similar overall alteration of gD.

Although all three variant proteins are divergent from the wild type in terms of overall surface charge, gD-1(rid1t) and gD-1(ANGt) are similar to each other yet different from gD-1(Δ290–299t). Furthermore, gD-1(Δ290–299t) (42) and gD-1(rid1t) (54) are similar to the wild type in terms of secondary-structure content as determined by circular dichroism analysis whereas gD-1(ANGt) (54) is divergent. Although the antigenic profile of gD-1(Δ290–299t) differs slightly from those of gD-1(rid1t) and gD-1(ANGt) at antigenic sites Ia and IIIa (Table 2), we conclude that antigenic structure is the best corollary to inhibitory function. The different results from the three structural approaches emphasize the need to solve the three-dimensional structure of gD (44).

Implications for cellular receptors. Inhibition of plaque formation by gDt occurs at the level of both initial virus entry (26) and subsequent spread from infected to uninfected cells (cell-to-cell spread) (42). To specifically measure the effect of gDt on virus entry, we used an HSV-1 KOS mutant, *hr*R3 (19), which has the *Escherichia coli lacZ* gene under the control of the HSV ICP6 immediate-early gene promoter. β-Galactosidase activity was assayed at 5 h postinfection, prior to virus spread. Wild type gD-1(306t) inhibited HSV-1 *hr*R3 entry in a dose-dependent manner (data not shown), which is consistent with a report that gDt inhibited immediate-early gene expression (26). Relative to the wild-type form, gD-1(Δ290–299t), gD-1(rid1t), and gD-1(ANGt) had a 10- to 60-fold-enhanced inhibitory effect on virus entry (data not shown), providing direct evidence that each form of gDt blocks an early step in infection, possibly the receptor-binding step. These results agree with the corresponding plaque inhibition results, suggesting that the mechanism of inhibition by each form of gD is similar in both assays.

Based on susceptibility to inhibition of infection by gDt, there are at least three different categories of HSV, (i) sensitive, (ii) partially resistant, and (iii) highly resistant, represented by the KOS, rid, and ANG viral strains, respectively. This observation fits the hypothesis that certain viruses, by virtue of changes in their gD molecules, may utilize different receptors and/or the same receptors with different affinity (2, 10). Our results can be explained by the latter model in which the three categories of virus have different affinities for a common gD-specific Vero cell receptor. KOS and other wild-type virus strains may have the weakest affinity; rid viruses may have a stronger affinity; and ANG viruses may have the strongest affinity. This would explain the inability of wild-type gDt to inhibit rid and ANG viruses and the ability of gD-1(rid1t) and gD-1(ANGt) to inhibit wild-type and rid viruses. The resistance of HSV-1 ANG to inhibition by all forms of gDt including gD-1(ANGt) may be due to an even higher affinity of full-length ANG virion gD for receptor than that of the truncated protein.

There is much evidence to suggest that gD binds to cellular receptors (3, 4, 18, 23, 26, 27, 30, 31, 42). Two different receptors which bind gD, the mannose-6-phosphate receptor (3) and an unidentified 60-kDa protein (23), have been implicated in cell-to-cell spread and entry of HSV. Recently, using expression cloning, Montgomery et al. (37) isolated and characterized a novel member of the tumor necrosis factor/nerve growth factor receptor family, designated HVEM, which mediates the entry of many HSV-1 strains including KOS into normally nonpermissive Chinese hamster ovary (CHO) cells. Interestingly, ANG and rid viruses were not able to infect HVEM-expressing CHO cells, supporting the notion that these viruses can use alternate entry mediators. However, no direct experiments were presented to determine which viral glycoprotein(s) interacts with HVEM. It is not yet clear whether HVEM is present and functional on the Vero cell surface; however, these results (37) suggest that changes in virion gD may affect Vero cell receptor utilization. To clarify these issues, efforts to determine the ability of the panel of gD molecules to bind to HVEM are under way.

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