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Structure-Function Analysis of Soluble Forms of Herpes Simplex Virus Glycoprotein D

ANTHONY V. NICOLA,[1,2]* SHARON H. WILLIS,[1,2] NIRINJINI N. NAIDOO,[1,2]†
ROSELYN J. EISENBERG,[2,3] AND GARY H. COHEN[1,2]

Department of Microbiology[1] and Center for Oral Health Research,[2] School of Dental Medicine, and Department of Pathobiology, School of Veterinary Medicine,[3] University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Glycoprotein D (gD) of herpes simplex virus (HSV) is essential for virus entry. Truncated forms of gD lacking the transmembrane and cytoplasmic tail regions have been shown to bind to cells and block plaque formation. Using complementation analysis and a panel of gD mutants, we previously identified four regions of gD (regions I to IV) which are important for virus entry. Here, we used baculovirus vectors to overexpress truncated forms of wild-type gD from HSV type 1 (HSV-1) [gD-1(306t)] and HSV-2 [gD-2(306t)] and four mutants, gD-1(∇34t), gD-1(∇126t), gD-1(∇243t), and gD-1(Δ290-299t), each having a mutation in one of the four functional regions. We used an enzyme-linked immunosorbent assay and circular dichroism to analyze the structure of these proteins, and we used functional assays to study the role of gD in binding, penetration, and cell-to-cell spread. gD-1 and gD-2 are similar in antigenic structure and thermal stability but vary in secondary structure. Mutant proteins with insertions in region I or II were most altered in structure and stability, while mutants with insertions in region III or IV were less altered. gD-1(306t) and gD-2(306t) inhibited both plaque formation and cell-to-cell transmission of HSV-1. In spite of obvious structural differences, all of the mutant proteins bound to cells, confirming that binding is not the only function of gD. The region I mutant did not inhibit HSV plaque formation or cell-to-cell spread, suggesting that this region is necessary for the function of gD in these processes. Surprisingly, the other three mutant proteins functioned in all of the in vitro assays, indicating that the ability of gD to bind to cells and inhibit infection does not correlate with its ability to initiate infection as measured by the complementation assay. The region IV mutant, gD-1(Δ290-299t), had an unexpected enhanced inhibitory effect on HSV infection. Taken together, the results argue against a single functional domain in gD. It is likely that different gD structural elements are involved in successive steps of infection.

Herpes simplex virus (HSV) encodes at least 11 glycoproteins (50). The initial attachment of HSV with cell surface heparan sulfate is mediated by glycoprotein C (gC) and/or gB (12, 22, 23, 61). This is followed by interaction of gD with cellular receptors (27, 28, 32, 36). Then gD and gB, gH, and gL act alone or in combination to trigger pH-independent fusion of the viral envelope and the host cell plasma membrane (50). gD is essential for entry into mammalian cells (33) and has been implicated in cell fusion (50), superinfection restriction (5, 29), and neuroinvasiveness (26). At the amino acid level, gD from HSV type 1 (HSV-1) (gD-1) is 85% identical to its homolog in HSV-2, gD-2 (31, 56, 57). The two proteins are functionally interchangeable (40, 44) and give rise to type-common and type-specific monoclonal antibodies (MAbs) (39). Immunization of animals with purified gD-1 or gD-2 stimulates the production of neutralizing antibodies and a cross-protective immune response to lethal virus challenge (11). Human subunit vaccines containing gD are currently in phase III clinical trials (4, 30).

Antigenic, biochemical, and mutational analyses have led to a current model of gD structure (8, 17). gD structure is critical for HSV infection and for its efficacy as a vaccine (4, 17). The three disulfide bonds in the extracellular portion of gD are necessary for stability of the structure of the protein as well as the function of the virus (34). The three N-linked oligosaccharides (9) are dispensable for gD function in vitro and in vivo (48, 49, 53) but critical for maintenance of antigenic structure (47, 48). However, X-ray crystallography is necessary to solve the true three-dimensional structure of gD (60).

We previously used linker insertion mutagenesis to define functional regions of gD (8). Our working hypothesis is as follows: (i) if a variant is both structurally and functionally intact, then the site altered by that particular mutation is not involved in gD function; (ii) if a variant has lost its ability to function but shows global changes in conformation, processing, or transport, the functional importance of the site cannot be assessed; and (iii) if the mutation does minimal structural damage yet function is abolished, then the site is considered important for gD function. The properties of the insertion variants indicated that four separate regions of gD are important for function. These were defined as region I (residues 27 through 43), region II (residues 126 through 161), region III (residues 225 through 246), and region IV (residues 277 through 310) (Fig. 1). These results confirmed and extended studies that demonstrated the functional importance of residues 234 to 244 (contained in region III) (19, 40). It was hypothesized that the four functional regions may form a single functional domain.

* Corresponding author. Mailing address: Department of Microbiology, School of Dental Medicine, University of Pennsylvania, 4010 Locust St., Philadelphia, PA 19104-6002. Phone: (215) 898-6558. Fax: (215) 898-8385. Electronic mail address: anicola@biochem.dental.upenn.edu.

† Present address: Department of Biochemistry, University of Witwatersand, Johannesburg, South Africa.

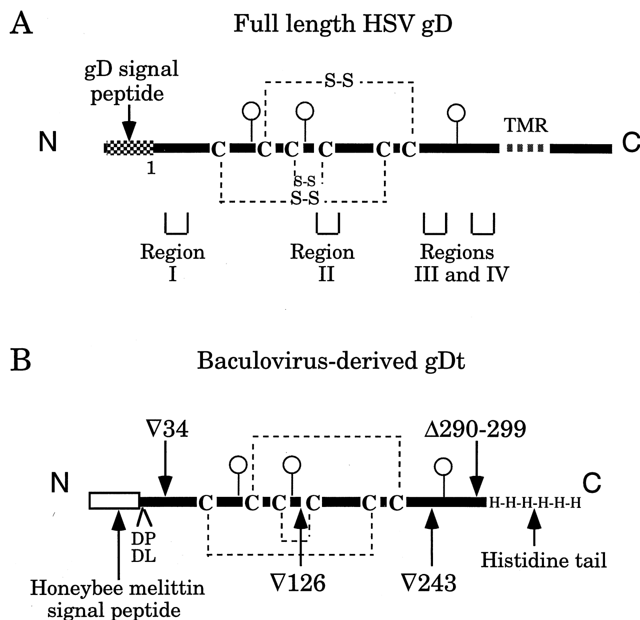

FIG. 1. Schematic representations of full-length and truncated HSV gD. (A) Full-length gD expressed on HSV virion and infected cell surfaces. gD-1 is 369 amino acids (57). gD-2 is 368 amino acids (58). Each has a signal sequence that is cleaved from the mature protein (15). gD has three N-linked sugars (balloons) (9), three disulfide bonds (S-S) (34), and a hydrophobic transmembrane region (TMR). Mutations in gD associated with function are clustered in four regions (I to IV) (8). (B) Schematic representations of baculovirus-derived gD-1(306t), gD-2(306t), gD-1(∇34t), gD-1(∇126t), gD-1(∇243t), and gD-1(Δ290-299t). These gD constructs were cloned into baculovirus as described in the text. These ectodomain forms of gD are truncated at residue 306 and lack the TMR and cytoplasmic tail regions yet retain native conformation (46; this report). The honeybee melittin signal peptide is used in place of the wild-type gD signal, and the proteins have two extra amino acids at the N terminus: DP for gD-1(306t) and variants and DL for gD-2(306t). A histidine tag is at the C terminus of each. The locations of the four insertion mutations (one representing each functional region) are indicated.

Previous studies showed that a mammalian truncated form of gD (gDt) blocked HSV infection in vitro and in vivo (27, 35). Moreover, gDt bound to cell receptors, including the mannose-6-phosphate receptor (3, 27). The baculovirus expression system has proven useful for analysis of soluble HSV glycoproteins (46, 54, 59). To further study the gD structure-function relationship, we used baculovirus vectors to overexpress wild-type gD-1(306t) (46), wild-type gD-2(306t), and four gD-1 mutants: gD-1(∇34t), gD-1(∇126t), gD-1(∇243t), and gD-1(Δ290-299t), each having a mutation in one of the previously characterized functional regions (8, 19, 40). Each protein was truncated prior to the hydrophobic transmembrane region at amino acid 306. We examined the antigenic and biochemical properties of these proteins. To address why these gD mutants do not function in infection and to examine which regions are involved in various aspects of gD function, we tested the two wild-type and four "nonfunctional" forms of baculovirus-derived gDt in binding (27, 54), plaque formation (27, 54), and cell-to-cell spread assays.

Our studies show that despite changes in structure and stability, all mutant proteins bound to cells, suggesting that binding is not the only function of gD. The region I mutant failed to inhibit HSV plaque formation and cell-to-cell spread. Unexpectedly, the region II, III, and IV mutants inhibited these processes. Thus, the ability of gD to bind to cells and inhibit infection does not correlate with its ability to initiate infection, suggesting that gD has more than a single functional domain.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal calf serum at 37°C. Baby hamster kidney (BHK) cells were grown in Eagle's minimal essential medium supplemented with 5% fetal calf serum. *Spodoptera frugiperda* Sf9 cells (GIBCO BRL) used for producing recombinant baculoviruses and recombinant glycoproteins were propagated in Sf900II medium (GIBCO BRL). HSV-1 strain NS (20) was grown and titered on Vero cells.

Construction of recombinant baculoviruses. (i) Baculovirus expressing wild-type gD-2(306t). Plasmid pWW65 (40) was used as a PCR template to generate DNA fragments containing the gD-2 gene as previously described (46). The 5′ primer was GAAAGATCTAAATACGCCTTAGCAGACC, and the 3′ primer was TGCAGCGCGGCGTGGTGGTAGTAGTAGTAAATCTTAAGGC. This strategy was designed to produce gD-2 (from HSV-2 strain 333) truncated at amino acid 306 prior to the hydrophobic transmembrane region and to add four histidine residues to the two histidines already present at residues 305 and 306 of gD-2. The transfer vector pVTBac (55) was digested with *Bam*HI and *Eco*RI, and the gD-2 PCR product was digested with *Bgl*II and *Eco*RI. The fragment was ligated into pVTBac for 15 h at 15°C with T4 DNA ligase (New England Biolabs). The ligated plasmid was used to transform *Escherichia coli* XL-1 Blue competent cells (Stratagene). Plasmid pAN243 was isolated from ampicillin-resistant colonies after screening by restriction enzyme analysis. pAN243 was recombined into baculovirus (*Autographa californica* nuclear polyhedrosis virus) with Baculogold DNA (Pharmingen). Plaques were picked and amplified. Culture supernatants were screened for gD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. The resultant recombinant virus is designated bac-gD-2(306t). The protein product is designated gD-2(306t). The cloning of bac-gD-1(306t), which expresses gD-1(306t) (from HSV-1, Patton strain), was described elsewhere (46).

(ii) Baculoviruses expressing mutant forms of gD-1(306t). DNA fragments were generated by PCR using plasmids D1-N29, D1-H98, D1-H24, and pHC240 (8) as templates and the primers used to construct bac-gD-1(306t) (46). Each PCR product encoded a gD-1 variant bearing an insertion in functional regions I to IV, respectively, which would be truncated at amino acid 306 before the transmembrane region and would contain a six-histidine C-terminal tail. The PCR products were each ligated into the transfer vector pVTBac, as described above, to produce plasmids pAN254, pAN253, pAN251, and pAN258, respectively. Each of these was recombined into baculovirus as described above and resulted in viruses designated bac-gD-1(∇34t), bac-gD-1(∇126t), bac-gD-1(∇243t), and bac-gD-1(Δ290-299t). The protein products are designated gD-1(∇34t), gD-1(∇126t), gD-1(∇243t), and gD-1(Δ290-299t). gD-1(∇34t) has G replacing V at residue 34 and amino acids KIFL inserted after G; gD-1(∇126t) has G replacing A at residue 126 and amino acids KIFP inserted after G; gD-1(∇243t) has amino acids GRSS inserted after residue 243; and gD-1(Δ290-299t) has amino acids 290 to 299 deleted, R replacing I at residue 290, and amino acids KIFL inserted after R.

Production and purification of gDt. Detailed protocols exist elsewhere for purification of gDt (46, 59). In brief, Sf9 cells were grown in 1-liter 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 DL6 MAb-Sepharose column. The protein was eluted with freshly prepared 0.1 M ethanolamine (pH 11 [Sigma]), then neutralized with 2.5 M Tris-HCl, concentrated with a PM10 membrane (Amicon), and finally dialyzed against phosphate-buffered saline (PBS).

Polyclonal and monoclonal antibodies. Rabbit anti-gD serum R7 (25) was used for Western blotting. Rabbit anti-gB (R69) and anti-gC (R46) sera (18) were used in immunoperoxidase assays (54). Anti-gD MAb DL6 (group II), which recognizes a continuous epitope from residues 272 to 279 (16, 25), was used for immunoaffinity purification and for analysis of antigenic activity, thermal stability, and binding. Anti-gD MAbs HD1 (group Ia) (38, 43), DL11 (group Ib) (10, 38), ABD (group III) (45), DL2 (group VI) (10), and AP7 (group XII) (8, 37) recognize discontinuous epitopes and were used for analysis of antigenic structure and thermal stability.

SDS-PAGE analysis. Purified glycoproteins were separated by SDS-PAGE under denaturing conditions in 10% polyacrylamide gels. Following SDS-PAGE, proteins were either stained with Coomassie blue or transferred to nitrocellulose and reacted with MAb DL6.

Antigenic analysis by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (Corning) were coated with proteins at a concentration of 8 μg/ml in PBS. Nonspecific binding was blocked by addition of blocking buffer (PBS plus 1% bovine serum albumin [BSA] and 1% chicken ovalbumin). Twofold serial dilutions (in blocking buffer) of ascitic fluids of anti-gD MAb DL11, HD1, DL2, ABD, DL6, or AP7 were added and incubated for 1 h. Plates were washed thrice with PBS, and then protein A-horseradish peroxidase (Boehringer Mannheim) was added and incubated for 1 h. Plates were washed thrice 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 added, and the A_{405} was read with a microtiter plate reader (Dynatech).

Secondary-structure analysis. Circular dichroism (CD) spectra were recorded on an AVIV 62DS spectropolarimeter. Spectra were routinely recorded from 260 to 180 nm every 0.5 nm with a time constant of 2 s. Cells with path lengths of 1

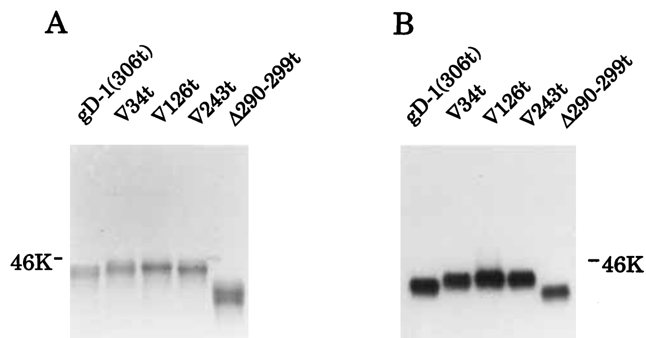

FIG. 2. SDS-PAGE analysis of gD-1 mutants. Purified proteins were electrophoresed under denaturing conditions on 10% polyacrylamide gels and either Coomassie blue stained (A) or Western blotted and probed with rabbit polyclonal antiserum R7 (B). Protein names are indicated at the top.

or 0.02 mm were used where appropriate. Protein concentrations were typically 0.3 mM in 25 mM KP_i buffer (pH 7.2). A blank spectrum of buffer alone was subtracted from the sample spectra. All data are expressed in terms of mean residue ellipticity (θ), using a mean residue weight of 116. The data were normalized with the Softsec software package (Softwood Co.). Secondary structure analysis was carried out with the SELCON program (51) supplied in the Softsec package.

Thermal denaturation analysis. (i) CD. The temperature at which half the protein molecules are denatured (T_m) was determined at 210 nm. This is the wavelength at which there was the largest difference in signal when spectra recorded at 25 and 95°C were compared. The T_m for each protein was determined by raising the temperature of the thermostatted cuvette in 2°C steps from 25 to 85°C with a 5-min equilibration time.

(ii) ELISA. Eighty micrograms of each protein per ml in PBS was brought to 37°C and then to various temperatures from 37 to 100°C for 5 min with a DNA thermal cycler (Perkin-Elmer Cetus). The samples were placed on ice, diluted to 8 μg/ml in PBS, and used to coat 96-well plates. The ELISA was then performed as described above with MAb ascitic fluid dilutions ranging from 1:400 to 1:10,000.

Cell binding assay. Binding of purified glycoproteins to the surface of uninfected cells was assayed essentially as described previously (54). Vero cell monolayers in 96-well plates were fixed with 1% paraformaldehyde in PBS for 30 min. To block nonspecific binding sites, cells were incubated with blocking buffer for 30 min. Proteins were serially diluted in dilution buffer (PBS plus 0.5% BSA and 0.5% chicken ovalbumin) and added to fixed cells for 1 h. Cells were washed thrice with washing buffer (PBS plus 0.1% Tween 20). Then, MAb DL6 ascitic fluid in dilution buffer was added for 30 min. Cells were washed three times, and then protein A-horseradish peroxidase and ABTS were added as described above.

Plaque inhibition assay. The effect of purified forms of gDt on HSV plaque production was assayed essentially as described previously (27, 54). Briefly, Vero cell monolayers in 48-well plates were treated with one of the purified glycoproteins diluted in 5% DMEM for 1.5 h at 4°C. HSV was added (50 PFU per well) and incubated for 1.5 h at 4°C. The cells were overlaid with 5% DMEM containing the competing proteins at the appropriate concentrations. After 24 h at 37°C, the medium was removed. The cells were fixed with methanol-acetone solution (2:1 ratio) for 20 min at −20°C and air dried. Virus titers were determined by an immunoperoxidase assay (54) using anti-gC or anti-gB rabbit polyclonal antisera.

Inhibition of cell-to-cell spread assay. Fifty PFU of HSV per well was added to Vero cells in 48-well plates for 1.5 h at 4°C. After 3 h at 37°C, cells were overlaid with 5% DMEM containing the competing proteins at the appropriate concentrations. At 24 h postinfection the plates were treated as described above, and spread from single infected cells was scored. When at least three adjacent cells were stained, this group of cells was counted as positive for cell-to-cell spread. Cases in which only single cells were stained were not included in the count.

RESULTS

Production of gDt proteins. Schematic representations of constructs used in this study are shown in Fig. 1. Each protein has the honeybee melittin signal peptide in place of the wild-type gD signal for efficient translocation into the endoplasmic reticulum lumen (55). Each protein is truncated before the transmembrane region at amino acid 306 and has a six-histidine tail at the C terminus. gD-1(306t), gD-2(306t), gD-1(∇34t), gD-1(∇126t), gD-1(∇243t), and gD-1(Δ290-299t) were purified from supernatants of baculovirus-infected cells by immunoaffinity chromatography (46, 59). Purified proteins migrated as a single band on SDS-PAGE as detected by protein staining or Western blot (Fig. 2). gD-1(306t) migrated at an approximate molecular size of 45 kDa. gD-1(∇34t), gD-1(∇126t), and gD-1(∇243t) migrated more slowly because of a 4-amino-acid insertion in each. gD-1(Δ290-299t), a deletion-insertion mutant, migrated faster than the wild type. The yield of purified proteins ranged from 15 to 20 mg/liter of infected cell supernatant, except for gD-1(∇126t), which yielded 2 mg/liter.

Antigenic analysis of gDt. We previously used electrophoresis on nondenaturing "native" gels followed by Western blotting (10) to show that gD-1(306t) reacted with gD conformation-dependent MAbs and therefore retained its wild-type structure (46). Western and dot blots have also been used to assess the effect of mutation on gD antigenic structure (8, 34, 40, 41, 47, 48). In these studies, assessment of MAb binding was described qualitatively as positive, negative, or weak. As a more quantitative approach, we used an ELISA to examine the antigenic conformation of gD-1(306t) and to compare it with gD-2(306t) and the gD-1 mutant proteins. Reactivity of each protein with six MAbs that recognize different epitopes across gD was quantitated relative to that of gD-1(306t) (Table 1). Each protein reacted equally well with DL6, which binds to a linear epitope (16, 25). gD-1 and gD-2 have common structural features, as is evidenced by reactivity with many type-common MAbs (39), yet they have sequence differences. Moreover, type-specific MAbs have been isolated, indicating that gD-1 and gD-2 are not identical. By quantitative ELISA, gD-1(306t) and gD-2(306t) reacted equally well with HD1 and ABD. However, relative to gD-1(306t), gD-2(306t) had 132 and 139% reactivity with type-common MAbs DL11 and AP7, respectively. Thus, the antigenic structures of gD-1 and gD-2 are measurably different.

To quantitate the effect of mutations on gD structure, the four mutant proteins were subjected to ELISA. The antigenic structure of each form of baculovirus-derived gD mirrored that of its full-length, mammalian counterpart as determined by native Western and dot blot (8). Each variant failed to react with AP7 yet retained wild-type levels of reactivity with ABD and reacted with HD1 and DL2; therefore, the variants were antigenically divergent but not globally altered in structure. By quantitative ELISA, the region I and II mutants were more divergent from the wild type than were the region III and IV mutants.

CD analysis of gDt. The antigenic analysis summarized in Table 1 is a measure of primary, secondary, and tertiary structures. CD examines protein secondary structure by measuring the difference in absorbance of right and left circularly polarized light (7). The CD signal in the far-UV region (180 to 260

TABLE 1. Analysis of gDt by ELISA

Protein	Binding[a] of MAb:					
	DL6	HD1	DL11	ABD	DL2	AP7
gD-1(306t)	1.00	1.00	1.00	1.00	1.00	1.00
gD-2(306t)	1.00	1.03	1.32	1.00	0.00	1.39
gD-1(∇34t)	1.00	0.17	0.06	1.00	0.55	0.00
gD-1(∇126t)	1.00	0.43	0.26	1.00	0.59	0.00
gD-1(∇243t)	1.00	1.00	0.63	1.00	0.53	0.00
gD-1(Δ290-299t)	1.00	1.27	1.07	1.00	0.67	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.

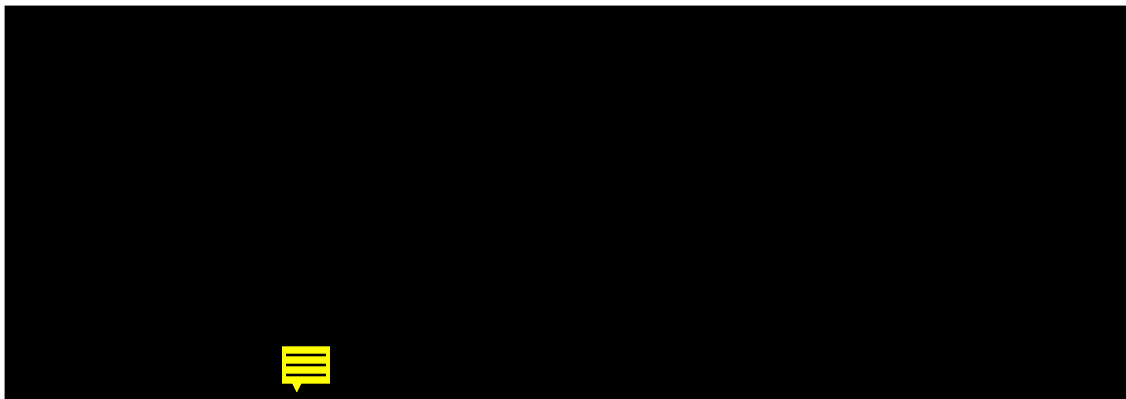

FIG. 3. CD spectroscopy of gDt. CD spectra were recorded for the indicated proteins on an AVIV 62DS spectropolarimeter from 260 to 180 nm every 0.5 nm with a time constant of 2 s and a path length of 1 or 0.02 mm as appropriate. Protein concentrations were typically 0.3 mM in 25 mM KP_i, pH 7.2. Blank spectra of buffer alone were subtracted from the sample spectra. The spectra shown are the average of multiple scans recorded at 25°C. MRW, mean residue weight.

nm) is used to probe the secondary structure of proteins. To estimate the quantity of secondary structural elements in gD-1(306t) and to compare it with that in gD-2(306t), the CD spectra (Fig. 3) were analyzed (51). gD-1(306t) had a β-sheet content of 34% and an α-helix content of 10% (Table 2). gD-2(306t) had a β-sheet content of 59% and, surprisingly, no detectable α-helix. Thus, the ectodomains of gD-1 and gD-2 are primarily β-sheet yet differ in overall secondary structure.

The effect of insertions in functional regions on secondary structure was also assessed. The CD spectroscopic analysis is shown in Fig. 3B and C. gD-1(∇34t) was 59% β-sheet and had no detectable α-helical content. gD-1(∇126t) diverged slightly from gD-1(306t) in β-sheet and turn content (Table 2). Thus, insertions in region I or II had measurable effects on secondary structure. In contrast, gD-1(∇243t) and gD-1(Δ290-299t) had the same secondary structure content as gD-1(306t), suggesting that these mutations in region III or IV had no detectable effect on secondary structure. These results agree in broad terms with the MAb binding data, suggesting that mutations affected elements of both the secondary and tertiary structures of gD.

Thermal denaturation of gDt. As another measure of structure, we determined the thermal denaturation profile of each form of gDt using both an ELISA and CD. T_m, the temperature at which each protein is 50% reactive with the indicated MAb, is a good indicator of protein stability. The ELISA data for two MAbs, HD1 and DL6, are shown in Fig. 4. Each protein when heated to 37°C reacted with HD1 (Fig. 4A). However, when each form of gDt was heated to higher temperatures, there was a decline in HD1 reactivity (Fig. 4A). DL6 was used as a control because it recognizes a linear epitope (16, 25). As expected, heating each protein had no effect on DL6 binding (Fig. 4B). Thermal denaturation results with HD1 and three other conformation-dependent MAbs are summarized in Table 3. gD-1(306t) and gD-2(306t) both had T_m values of 58°C.

We were interested to determine if the T_m of the mutant proteins would correlate with their antigenic and secondary-structure profiles. gD-1(∇34t) and gD-1(∇126t), which showed the greatest antigenic changes, had T_m values of approximately 50°C and therefore were more thermally labile than wild-type gD (Table 3). gD-1(∇243t) and gD-1(Δ290-299t), which were antigenically more similar to gD-1(306t), had T_m values of 57 and 56°C, respectively (Table 3), and therefore were also similar in stability to the wild type. Thus, the antigenic, secondary-structure, and thermal denaturation profiles of the mutant proteins agreed in broad terms, and the last two approaches provide additional ways to examine the effect of mutation on gD structure. T_m values determined by CD (Table 3) were similar to those obtained by ELISA, indicating that heating had similar effects on both secondary and tertiary structures of each protein.

Binding of gDt to cell surface. Johnson et al. (27) reported that gDt produced in Chinese hamster ovary (CHO) cells bound to cells with a specificity ranging from 30 to 80%. gD-1(306t) and gD-2(306t) produced from baculovirus-infected insect cells bound to Vero cells in a dose-dependent manner with similar specificity (data not shown). When gD-1(306t) was heated to 65°C for 5 min, it lost its native conformation (Fig. 4A). However, heat-denatured gD-1(306t) bound to cells as well as the wild type did, suggesting that conformation is not critical for binding. Surprisingly, all four mutant proteins exhibited an enhanced ability to bind to the cell surface compared with that of gD-1(306t). Furthermore, each mutant competed with gD-1(306t) for binding by 50 to 60% (data not shown). Thus, the failure of these gD variants to complement the infectivity of a gD null virus (8) does not correlate with binding and could be due to their inability to function at a postbinding step. These results support the notion that gD has more than one function in virus infection.

Effect of gDt on HSV plaque formation. Truncated forms of wild-type gDt were previously shown to inhibit plaque production by HSV (27, 54). Here, gD-1(306t) and gD-2(306t) inhibited plaque formation on Vero cells in a dose-dependent manner (Fig. 5A). The protein concentration that inhibited HSV-1 plaque production by 50% (IC_{50}) was 1.6 μM for gD-1(306t)

TABLE 2. Secondary-structure content of gDt[a]

Protein	% α-Helix	% β-Sheet	% Turns	% Random coil
gD-1(306t)	10	37	22	31
gD-2(306t)	0	59	10	31
gD-1(∇34t)	0	59	10	31
gD-1(∇126t)	10	34	20	36
gD-1(∇243t)	10	37	22	31
gD-1(Δ290-299t)	10	37	22	31

[a] CD data were analyzed with the program SELCON (51). All data are expressed in terms of mean residue ellipticity (θ), using a mean residue weight of 116.

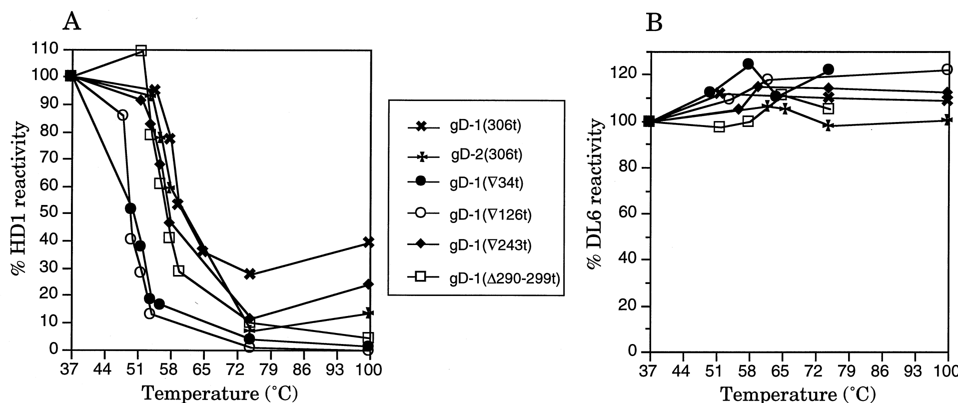

FIG. 4. Effect of temperature on antigenic conformation of gDt. Proteins were heated for 5 min at temperatures ranging from 37 to 100°C, chilled on ice, used to coat 96-well plates, and subjected to ELISA with MAb HD1, which recognizes a discontinuous epitope (A), or MAb DL6, which recognizes a continuous epitope (B). One hundred percent corresponds to the reactivity (A_{405}) of protein heated to 37°C. Each point is the average of duplicates. Several experiments yielded similar results.

and 0.41 μM for gD-2(306t) (Fig. 5A and Table 4). Equivalent concentrations of BSA caused no inhibition (Fig. 5A). Heat-denatured gD-1(306t) failed to inhibit even when present at 2.8 μM (Fig. 5A). This indicates that conformation is critical for the inhibitory effect of gDt.

Since full-length gD bearing a mutation in any one of four regions fails to function in HSV entry (8), the four truncated mutants were tested for their ability to inhibit HSV plaque formation. If these two assays were measuring the same aspect of gD function, we anticipated that none of the mutants would block infection. As expected, the region I mutant, gD-1(∇34t), did not inhibit plaque production; however, the region II and III mutants showed only a slightly reduced ability to block plaque formation compared with that of the wild type (Fig. 5B). The IC_{50}s of gD-1(∇126t) and gD-1(∇243t) were 2.4 and 4.2 μM, respectively. Surprisingly, the region IV mutant, gD-1 (Δ290-299t), blocked plaque formation 400-fold better than did gD-1(306t). The IC_{50} of gD-1(Δ290-299t) was 4 nM. Similar results were obtained with each form of gDt with BHK cells, although in each case two- to threefold more gDt was required for inhibition (data not shown). Thus, Vero cells were more sensitive to the inhibitory effect of gDt. A strain sensitivity to inhibition was also observed. Tenfold less gD-1(306t) was required to inhibit HSV-1 strains KOS and HFEM (data not shown) than to inhibit strain NS (Fig. 5). Since soluble forms of region II, III, and IV variants still inhibit HSV infection of cultured cells, the complementation and inhibition of plaque formation assays appear to be measuring different aspects of gD function. Also, our data suggest that gD has more than one functional domain.

Effect of gDt on cell-to-cell spread of HSV. Since gDt is present in the plaque assay during the entire period of plaque formation (27, 54; this report), it is not clear whether its inhibitory effect is exerted at the level of initial virus penetration or postinfection at the level of cell-to-cell spread or both. To measure the effect of gDt on HSV spread, the assay was modified. Here, infection was carried out for 3 h at 37°C, and then gDt was added. Thus, any reduction in plaque development was due to inhibition by gDt of virus spread from a single infected cell. gD-1(306t) and gD-2(306t) both inhibited cell-to-cell spread of HSV-1 with IC_{50}s of 3.6 and 5.6 μM, respectively (Fig. 6A; Table 4). Heat-denatured gD-1(306t) failed to inhibit cell spread, stressing the importance of gD conformation in this process (Fig. 6A). gD-1(∇34t) also did not inhibit HSV spread (Fig. 6B). In contrast, gD-1(∇126t) and gD-1 (∇243t) both blocked this process, although the region III mutant inhibited it to a lesser extent than did the wild type (Fig. 6B). The IC_{50}s for gD-1(∇126t) and gD-1(∇243t) were 3.8 and >5.6 μM, respectively (Table 4). gD-1(Δ290-299t) had an IC_{50} of 0.19 μM, indicating that it blocked cell-to-cell transmission of HSV 20-fold better than did gD-1(306t) (Fig. 6B; Table 4).

DISCUSSION

Immunological, biochemical, and genetic approaches have been used to study the structure-function relationship of gD (17). gD-1 and gD-2 have high structural and functional homology (8, 31, 40, 44, 56). Both proteins have identical disulfide bond patterns (34), and analogous mutations in gD-1 or gD-2 have similar effects on antigenicity and function of both proteins (8). Extensive mapping of type-common and type-specific epitopes has been used to predict which regions are on the exterior of the protein and which are in proximity to each other (17). Antigenic studies have also proven useful for understanding function since epitopes recognized by neutralizing MAbs overlap functional regions (8, 39, 40). Recently, we used linker insertion mutagenesis to define four functional regions in gD. We hypothesized that they might fold together to form a single functional domain (8).

In this study, we used baculovirus recombinants to produce truncated forms of HSV gD to further investigate the structure

TABLE 3. Effect of temperature on structure of gDt

Protein	T_m (°C) determined by:				
	ELISA[a] with MAb (group):				CD[b]
	HD1 (Ia)	DL11 (Ib)	DL2 (VI)	AP7 (XII)	
gD-1(306t)	59	58	57	58	58
gD-2(306t)	59	57	NA	58	58
gD-1(∇34t)	50	NA	50	NA	50
gD-1(∇126t)	50	NA	49	NA	53
gD-1(∇243t)	58	56	58	NA	58
gD-1(Δ290-299t)	57	55	56	NA	57

[a] See legend to Fig. 4 for details. NA, not applicable.

[b] The temperature of a thermostatted cuvette was raised from 25 to 85°C in 5°C steps with a 3-min equilibration time. T_m values were determined at 210 nm, the wavelength at which the greatest difference was observed by comparing gDt spectra at 25 and 95°C.

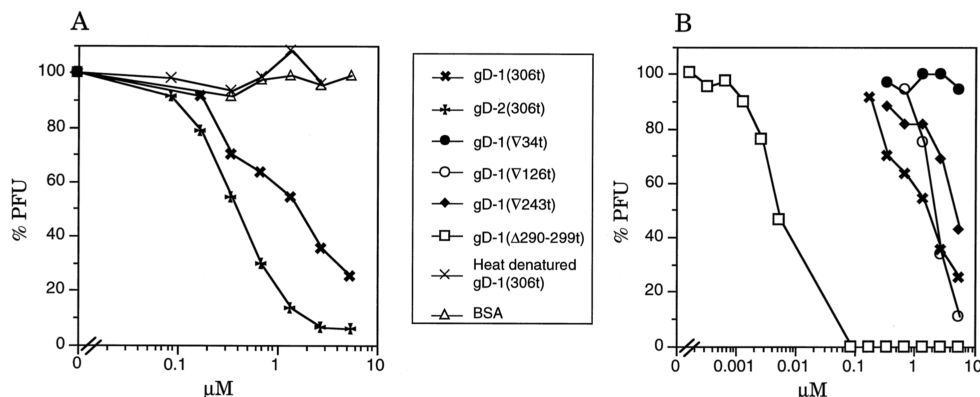

FIG. 5. Effect of gDt on HSV plaque formation. gDt was added to Vero cell monolayers in 48-well plates for 90 min at 4°C. HSV-1 (50 PFU) was added for 90 min at 4°C. Cells were overlaid with medium containing appropriate concentrations of gD and shifted to 37°C for 24 h. Cells were fixed, and plaques were visualized by immunoperoxidase staining. Each point represents the average from quadruplicate wells. 1 µM gDt = 36 µg/ml. The experiment was repeated several times. Data shown are from one representative experiment.

and function of gD. First, we examined the antigenic and biochemical properties of gD using an ELISA and CD. We compared the structure of wild-type forms of gD from both HSV serotypes and then assessed the effect of mutation on gD-1 using four linker insertion mutants. Second, we used these proteins in functional assays to better understand why these mutants fail to mediate viral entry.

Structural analysis of gD. Our studies confirm that gD-1 and gD-2 are structurally similar but that differences can be demonstrated. First, using a quantitative ELISA we found differences in the reactivity of gD-1 and gD-2 with type-common MAbs, suggesting that shared epitopes are presented somewhat differently by the two proteins. Second, we found that both proteins are primarily β-sheet in secondary structure. However, gD-1 had more α-helical content than gD-2, and gD-2 had more β-sheet content than gD-1. Despite the differences in antigenic and secondary structure, both proteins have the same T_m of 58°C, indicating that both are fairly thermally stable.

In the case of the four gD mutants we found that insertions had various effects on structure. In terms of antigenicity, secondary structure, and thermal stability, gD-1(∇34t) and gD-1(∇126t) were divergent from the wild type whereas gD-1(∇243t), and gD-1(Δ290-299t) were more similar. Since full-length versions of these mutants failed to mediate virus entry in the complementation assay (8), it was possible that this was due to a general instability of gD. Although gD-1(∇34t) and gD-1(∇126t) had reduced T_m values, all of the mutant proteins retained their structure at the physiologic temperature of 37°C, so thermal lability does not explain the failure of any of the four mutants to complement infectivity of a gD null virus. Although gD-1(∇34t) and gD-1(∇126t) are conformationally divergent from the wild type, the latter was capable of inhibiting infection; thus, the altered structure of these mutants is not the sole explanation for their inability to function.

Functional analysis of gD. Functional studies have presented gD in three different contexts, each implicating gD in inhibition of infectivity. (i) UV-inactivated wild-type but not gD-null virus (28, 32), (ii) soluble gD added to cells (21, 27, 54; this report), and (iii) gD expressed in cells (5, 29) all block HSV entry. Is the ability of gD to inhibit infection related to its role in initiation of infection as in the complementation assay? We addressed this by using soluble forms of gD mutants that failed to mediate entry. We predicted that each mutant would fail in at least one functional assay and this would help determine why these forms of gD were nonfunctional in entry.

Binding to cells. gD-1(306t) and gD-2(306t) bound to Vero cells with 30 to 40% specificity. Binding of gCt (52), but not gDt (27, 52), was blocked by heparin competition and heparatinase treatment of cells, suggesting that the fraction of gDt that bound specifically interacts with a nonheparan sulfate receptor. Heat-denatured gDt and each of the mutant forms of gDt also bound to cells. At face value, this suggests that native conformation is not critical for gD binding and that complemented viruses containing these mutants fail to function at a postbinding step in entry. Alternatively, the enhanced binding ability of the four mutants may be due to a site that is more exposed in structurally altered gD and may account for the inability of these complemented viruses to complete the entry process. However, conclusions about gD function using this assay need to be drawn carefully because of high levels of nonspecific binding and difficulty in achieving saturation with gDt (27, 42, 52). We speculate that the binding of gDt in isolation is not analogous to the binding of gD in the context of other viral glycoproteins; indeed, gDt may bind to cell surface molecules not involved in HSV infection.

Inhibition of plaque production. To date, gD is unique among HSV glycoproteins in its inhibitory activity, since gBt and gCt fail to block plaque production (27, 54). Here, we show that baculovirus-derived forms of gDt from both HSV serotypes inhibited plaque formation on Vero cells. Since gD-1(∇34t) failed to inhibit infection and cell spread of HSV and its full-length counterpart failed to complement infectivity (8), it is tempting to speculate that this mutation disrupts a common domain necessary for viral entry and inhibition. However, more region I mutants need to be examined before a firm

TABLE 4. Inhibition of HSV infection by gDt

Inhibition assay	IC_{50}[a] (µM) of:					
	gD-1 (306t)	gD-2 (306t)	gD-1 (∇34t)	gD-1 (∇126t)	gD-1 (∇243t)	gD-1 (Δ290-299t)
Plaque formation	1.6	0.41	NE	2.4	4.2	0.004
Cell-to-cell spread	3.6	5.6	NE	3.8	>5.6	0.19

[a] Concentration necessary for 50% inhibition of 50 PFU of HSV-1 (NS) as measured by plaque formation on Vero cells. NE, no effect.

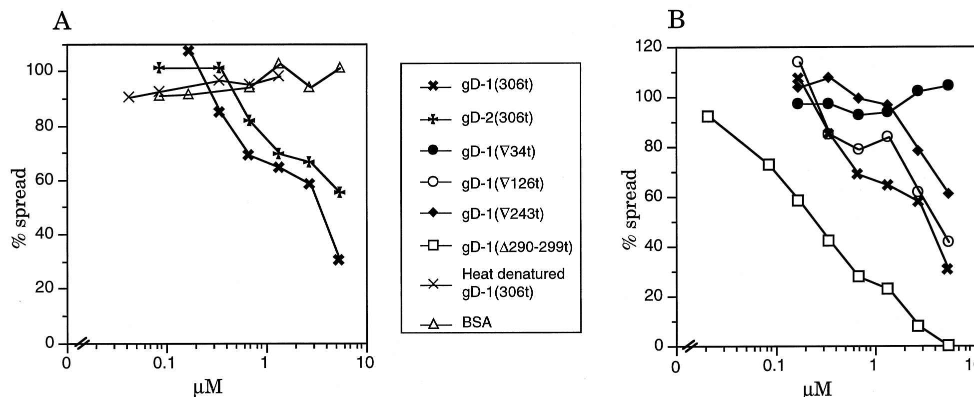

FIG. 6. Effect of gDt on HSV cell-to-cell spread. HSV-1 (50 PFU) was added to Vero cell monolayers in 48-well plates for 90 min at 4°C. Plates were shifted to 37°C for 3 h, and then cells were overlaid with medium containing appropriate concentrations of gDt. At 24 h postinfection cells were fixed, and spread from singly infected cells was visualized by immunoperoxidase staining. Each point represents the average from quadruplicate wells.

conclusion can be drawn. In contrast, soluble forms of three HSV gD mutants that do not function in entry (8), namely, gD-1(∇126t), gD-1(∇243t), and gD-1(Δ290-299t), were capable of inhibiting virus infection.

This suggests that gD entry domains do not correlate perfectly with domains responsible for inhibition and that the complementation and inhibition assays are measuring different aspects of gD function. Interestingly, there was not a strict correlation between gD regions involved in superinfection interference and those involved in infectivity (1, 6, 8, 13), so it will be of interest to determine if this restriction phenomenon which involves gD expressed on the cell surface is analogous to inhibition by gDt added to cells. HSV entry is a multistep process (12), with gD thought to play a role in binding, fusion, and penetration events (50). Results presented here are consistent with the idea that gD is a multifunctional protein with more than a single domain responsible for function, which contrasts with what we previously hypothesized. We speculate that a mutation in gD may disable one but not all functions of virion gD.

Inhibition of cell-to-cell spread. Glycoproteins essential for penetration, gB, gD, gH, and gL, are also required for cell-to-cell spread (36). However, these two processes are not identical since gE, gI, and mannose-6-phosphate residues on gD are involved primarily in spread but not penetration of HSV (2, 14). Johnson et al. (27) demonstrated that gDt inhibited virus penetration as measured by immediate-early gene expression, but it has not been determined if gDt inhibits spread.

Here, to look specifically at spread, gDt was added after virus penetration but prior to spread. gD-1(306t) and gD-2(306t) directly inhibited cell-to-cell transmission of HSV-1, indicating that gDt inhibits plaque production, at least in part, at the level of virus spread. Since wild-type and mutant forms of gDt had the same inhibitory phenotype in both assays [e.g., gD-1(Δ290-299t) was the best inhibitor of plaque production and spread, and gD-1(∇34t) was not effective], this supports the notion that gD has a similar role in HSV entry and cell-to-cell spread (24, 36, 37). Also, heat-denatured gDt failed to block plaque production and spread, stressing the importance of gD conformation in both of these processes.

To study the effect of gDt specifically on virus entry, we plan to use HSV mutants that can be detected within several hours of entry. Efforts are under way to determine the mechanism of virus inhibition by gDt. We are also investigating the unexpected and pronounced inhibitory effect of gD-1(Δ290-299t) as well as the effect of gDt on other herpesviruses, HSV-2, and different strains of HSV-1.

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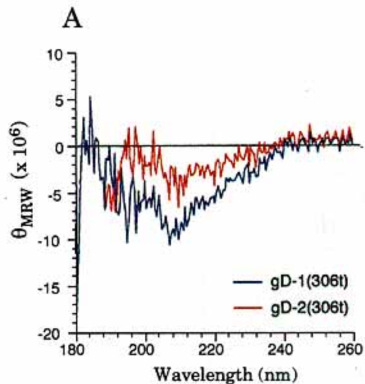
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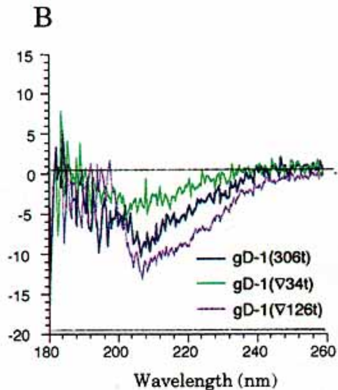
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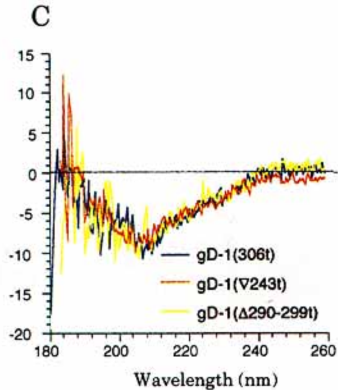
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0
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-20
180
200
220
240
260
Wavelength (nm)
gD-1(306t)
gD-1(∇243t)
gD-1(Δ290-299t)