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Identification of Functional Regions of Herpes Simplex Virus Glycoprotein gD by Using Linker-Insertion Mutagenesis

HSIEN-YUAN CHIANG,[1,2]† GARY H. COHEN,[1,2] AND ROSELYN J. EISENBERG[2,3]*

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

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Glycoprotein gD is a component of the herpes simplex virus (HSV) envelope essential for virus entry into susceptible cells. Previous studies using deletion and point mutations identified a functional domain of HSV-1 gD (gD-1) from residues 231 to 244. However, many of the deletion mutations had global effects on gD-1 structure, thus precluding assessment of the functional role of large portions of the protein. In this study, we constructed a large panel of linker-insertion mutants in the genes for gD-1 and HSV-2 gD (gD-2). The object was to create mutations which would have only localized effects on protein structure but might have profound effects on gD function. The mutant proteins were expressed in transiently transfected L cells. Monoclonal antibodies (MAbs) were used as probes of gD structure. We also examined protein aggregation and appearance of the mutant glycoproteins on the transfected cell surface. A complementation assay measured the ability of the mutant proteins to rescue the infectivity of the gD-null virus, FgDβ, in *trans*. Most of the mutants were recognized by one or more MAbs to discontinuous epitopes, were transported to the transfected cell surface, and rescued FgDβ virus infectivity. However, some mutants which retained structure were unable to complement FgDβ. These mutants were clustered in four regions of gD. Region III (amino acids 222 to 246) overlaps the region previously defined by gD-1 deletion mutants. The others, from 27 through 43 (region I), from 125 through 161 (region II), and from 277 to 310 (region IV), are newly described. Region IV, immediately upstream of the transmembrane anchor sequence, was previously postulated to be part of a putative stalk structure. However, residues 277 to 300 are directly involved in gD function. The linker-insertion mutants were useful for mapping MAb AP7, a previously ungrouped neutralizing MAb, and provided further information concerning other discontinuous epitopes. The mapping data suggest that regions I through IV are physically near each other in the folded structure of gD and may form a single functional domain.

Herpes simplex virus (HSV) is a human pathogen which causes a variety of diseases, including cold sores, eye and genital infections, neonatal infections, and encephalitis. The virion has a double-stranded DNA genome of 150 kb with at least 72 open reading frames, of which at least 11 code for glycoproteins (53, 54, 72). At the time of infection, several glycoproteins act singly or in concert to bind HSV to the susceptible cell and trigger direct fusion between the envelope and the plasma membrane (reviewed in reference 72).

Glycoprotein gD is found in the envelope of HSV-1 and HSV-2, and for both viruses, this protein is essential for entry into mammalian cells (25, 28, 39, 49). gD has been implicated in receptor binding, cell fusion, and neuroinvasiveness (24, 28, 34, 36, 37, 38, 55, 62). Recent studies suggest that gD binds to the mannose-6-phosphate receptor (35), but the significance of this binding to the role of gD in virus entry or fusion remains to be elucidated. Immunization of animals with gD stimulates the production of virus-neutralizing antibodies and protects them from lethal challenge with HSV and the establishment of latency (2, 3, 12, 45, 46, 56).

Experiments using deletion mutants attempted to define regions of gD which are needed for its function (22, 60). These studies showed that residues near the amino terminus, as well as residues 300 to 305 (located just prior to the transmembrane anchor) and the cytoplasmic domain (residues 338 to 369), could be removed with little effect on structure or function. In contrast, deletions which removed residues within the region 231 to 244 had a profound effect on gD function with little or no effect on gD structure (22, 60). These studies, as well as those on disulfide pairing, led to models of how gD might be folded (20, 51). Whether other regions of gD were also involved in its function could not be ascertained because deletions of many parts of gD caused global structural changes. This was judged by the failure of these altered molecules to bind monoclonal antibodies (MAbs) to discontinuous epitopes or to be properly processed and transported to the surface of transfected or infected cells (22, 60, 76).

In this study, linker-insertion mutagenesis was chosen as an alternative method for systematically creating mutations which would sometimes disrupt a functional site with minimal effects on protein structure (4, 5, 8, 31, 65, 67). A second goal was to compare the effects of mutations on both HSV-1 gD and HSV-2 gD (hereafter called gD-1 and gD-2), since all prior mutational studies had been carried out only on gD-1. Earlier findings suggest that the two glycoproteins are structurally very similar (9, 17, 47, 51, 52, 74, 75). Other studies imply that the two proteins are also functionally interchangeable. For example, studies with intertypic recombinants showed that gD-2 could be recombined into HSV-1 with no discernible effect on virus infection (48). Furthermore, gD-2 is able to complement gD-1-null mutants such as FgDβ (60).

This report shows that mutations in similar regions of gD-1 and gD-2 have similar effects on the two proteins. A panel of 50 linker-insertion and insertion-deletion mutants identified four functional regions within both proteins: region I (residues

* Corresponding author. Mailing address: Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St., Philadelphia, PA 19104-6008. Phone: (215) 898-6552. Fax: (215) 898-8385.

† Present address: Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan.

TABLE 1. gD linker-insertion and linker-insertion–deletion mutants

Sequential no.[a]	Plasmid[b]	Mutant name and location of insertion[c]	Amino acids inserted[d]	Sequential no.	Plasmid	Mutant name and location of insertion	Amino acids inserted
1	pRE4	Wild type (gD-1)		31	D1-N119	D1-∇187	GRSS
2	pWW65	Wild type (gD-2)		32	D2-R66	D2-∇191	WKIFH
3	D1-H28	D1-∇12	GKIFP	33	D2-H17	D2-∇207a	GKIFRKIFP
4	D2-H36	D2-∇12	GKIFP	34	D2-H17.1	D2-∇207b	GKIFP
5	D1-H110	D1-Δ12~77[e]	GKIFP	35	pHC235	D1-∇225	GRSS
6	D2-F54	D2-∇18	EDLP	36	D2-A21	D2-∇235	REDLP
7	D2-A56	D2-∇27	GRSS	37	D1-H24	D1-∇243	GRSS
8	D1-N29	D1-∇34	GKIFL	38	D2-H40	D2-∇243	EDLP
9	D1-H27	D1-∇43	EDLP	39	D1-H54	D1-∇246a	GKIFP
10	D2-R11	D2-∇58	WKIFH	40	D1-N1	D1-∇246b	EDLP
11	D2-R31	D2-Δ58~93[e]	WKIFRKIFH	41	D2-A5	D2-∇257	GRSS
12	D1-H78	D1-∇77a	GKIFP	42	D1-H26	D1-∇277	GKIFP
13	D1-N155	D1-∇77b	EDLP	43	D1-H75	D1-Δ277~310[e]	GKIFP
14	D2-H23	D2-∇77	GKIFP	44	D1-N2	D1-∇287	EDLP
15	D2-H38	D2-Δ77~88[e]	GKIFP	45	pHC236	D1-∇290	GKIFP
16	D1-N65	D1-∇83	GRSS	46	pHC237	D1-∇300	GKIFP
17	D1-H104	D1-∇84	GKIFP	47	D2-F92	D2-∇303	GKIFP
18	D2-H29	D2-∇88	GKIFP	48	D1-H15	D1-∇310	GKIFRKIFP
19	D2-R8	D2-∇93	WKIFH	49	D2-H131	D2-∇315	EDLP
20	D1-N22	D1-∇125	GRSS	50	D2-H20	D2-∇322	GKIFP
21	D1-H98	D1-∇126	GKIFP	51	D2-R6	D2-∇324	GRSS
22	D2-A32	D2-∇136	REDLP	52	D2-R56	D2-∇338	EDLP
23	D2-A36	D2-Δ136~140[e]	REDLP	53	pHC238	D1-Δ277~290[e]	GKIFP
24	D2-A46	D2-∇140	REDLP	54	pHC239	D1-Δ277~300[e]	GKIFP
25	D1-N37	D1-∇151	WKIFL	55	pHC240	D1-Δ290~300[e]	GKIFP
26	D2-F76	D2-∇161	GKIFP	56	pHC241	D1-Δ290~310[e]	GKIFP
27	D2-R15	D2-∇162	GRSS	57	pHC242	D1-Δ300~310[e]	GKIFP
28	D2-R1	D2-∇164	WKIFH	58	pHC233[f]	gD-gIV-gD	
29	D2-H108	D2-∇185	GKIFP	59	pHC234[g]	gD-gIV-gD	
30	D2-F45	D2-∇186	EDLP				

[a] Sequential numbers were given to each mutant to make graphs easier to follow. The mutants are numbered from N to C terminus (except for mutants 53 to 59).
[b] D1, gD-1 gene; D2, gD-2 gene. The letter after the dash refers to the restriction enzyme used (H, *Hae*III; A, *Alu*I; N, *Nla*IV; R, *Rsa*I; F, *Fnu*DII).
[c] D1, gD1; D2, gD2; ∇, insertion. The number after the symbol identifies the amino acid immediately preceding the insertion.
[d] Amino acids inserted at each site (single-letter code). When the insertion of the linker altered an amino acid at the mutation site of gD (always to glycine), it is indicated as a fifth or ninth amino acid.
[e] Residues following the symbol Δ were deleted, and amino acids in the adjacent column were inserted.
[f] Residues 277 to 310 (ALLEDPVGTVAPQIPPNWHIPSIQDAATPYHPPA) of gD-1 were deleted, and amino acids GKIFPDGESPTPEANGGAEGEPKPGP-SPDADRPEG were inserted.
[g] Residues 277 to 310 of gD-1 were deleted, and amino acids GKIFPAFGAVGVGAGAGFRLALGASVGFGGRTLAV were inserted.

27 through 43); region II (residues 126 through 161), region III (residues 225 through 246), and region IV (residues 277 through 310). Region III overlaps a portion of gD previously identified with deletion mutants as being important for function (22, 60). Identification of region IV (amino acids 277 to 310) was somewhat surprising. Previous studies suggested that these amino acids could form a stalk-like structure which would hold gD away from the virion envelope (33) and were not part of the functional domain of gD. A detailed mutational analysis of region IV indicates that although it may constitute a stalk-like structure, some amino acids (particularly residues between amino acids 277 and 300) are directly involved in gD function.

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MATERIALS AND METHODS

Cell culture and virus strain. Mouse L and COS-1 cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C. VD60 cells were grown in DMEM containing 5% FBS supplemented with 1 mM histidinol (39). FgDβ virus was propagated and titered in VD60 cells without histidinol (49).

Linker-insertion mutagenesis. (i) Mutagenesis using partial restriction enzyme digestion. Mutagenesis was performed as described by Seidel-Dugan et al. (67). Briefly, plasmids pRE4 (13) and pWW65 (60), which contain full-length gD of HSV-1 (Patton) and gD of HSV-2 (333), respectively, were partially digested with *Hae*III or with *Nla*IV for pRE4 or *Alu*I, *Fnu*DII, *Hae*III, or *Rsa*I for pWW65 (to create blunt-ended nicks at one of various places in the gD genes) and separated by electrophoresis on a 5% polyacrylamide gel. The full-length linear forms of plasmid DNA were purified by electroelution. Dodecameric *Bgl*II linkers (GGAAGATCTTCC) were ligated to the blunt-ended fragments to restore the original reading frame of gD-1 or gD-2. Each mutation was verified by supercoiled DNA sequencing (7).

Depending on the location of insertion, the four amino acids (single-letter code) inserted were either KIFP, EDLP, or GRSS (Table 1). GKIFP designated the replacement of an upstream residue with a G plus the four-amino-acid insertion. Plasmids D1-H15, D2-R31, and D2-H17 each contained two copies of linkers which resulted in an eight-amino-acid insertion. Plasmid D2-H17.1, which contained a single insert, was derived from plasmid D2-H17 by further restriction digestion

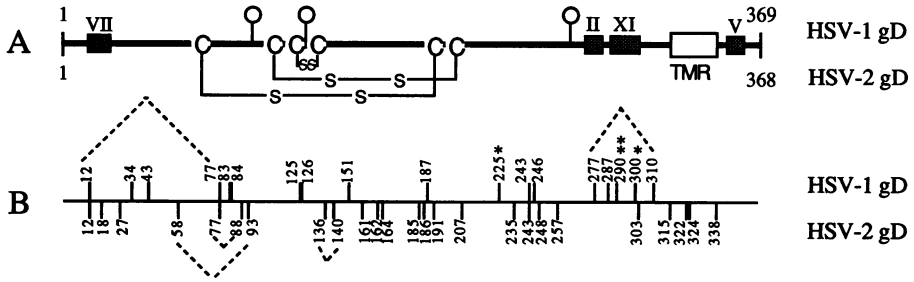

FIG. 1. Stick model of gD and locations of the linker-insertion mutations. (A) HSV-1 gD and HSV-2 gD contain 369 and 368 amino acids, respectively (74, 75). N-linked glycosylation sites (balloons) (52, 69) and cysteine (C) residues are indicated. A seventh cysteine which is found only in HSV-1 gD (not shown) is in the predicted TMR (empty box). Also indicated are the positions of the three disulfide bonds of gD (51) and the defined positions of four continuous antigenic sites, VII, II, XI (type common), and V (type 1 specific) (59). (B) Positions of 50 linker-insertion mutants constructed in this study are shown as vertical lines, and the residue numbers indicate the amino acid immediately preceding the site for insertion of the *Bgl*II linker. Dotted lines indicate the extent of in-frame deletion mutations obtained as a result of linker-insertion mutagenesis. A single asterisk indicates mutants which were constructed by PCR; double asterisks identify mutants constructed by oligonucleotide-directed mutagenesis. The positions of mutants 53 to 59 (Table 1) are not shown.

with *Bgl*II. Plasmids D1-H110, D2-R31, D2-H38, D2-A36, and D1-H75 each have a different in-frame deletion, along with a *Bgl*II linker inserted at the deletion site. The positions of the insertions are indicated in Fig. 1.

(ii) Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis (44) was used to insert a 12-base *Bgl*II linker at amino acid 290 of gD-1 (pHC236; Table 1). To do this, the *Hin*dIII fragment containing the entire coding region of gD-1 was excised from plasmid pWW78 (60) and subcloned into the *Hin*dIII site of M13 mp18. A 37-base oligonucleotide primer (5'TCGCGGCGTCCTGGAAGATCTTCCGGATCG ACGGGAT3') containing the 12-bp *Bgl*II linker (underlined) was inserted into this vector. The mutated gD-1 gene was excised from the replicative form of M13 mp18 and inserted into the expression vector pRSVnt EPA (13). The resulting protein has an amino acid G to replace I at residue 290 of gD-1 and four amino acids, KIFP, inserted after G.

(iii) Mutagenesis using PCR. Two mutants, pHC235 and pHC237, which have *Bgl*II linkers inserted at residue 225 (∇225) and 300 (∇300), respectively, were constructed by PCR (66). Five synthetic oligonucleotide primers were used to generate DNA fragments containing a *Bgl*II linker in the gD-1 gene. The oligonucleotide sequences were 5'CCC*AAGCT TATCCTTAAGGTCTCTTTT*3' (gD-1 upstream primer), 5'CCC*AAGCTT*CCCGCAGACCTGACCCCC3' (gD-1 downstream primer), 5'GAAGATCTTCCGAGAACCAGCGCA CCGTC3' (∇225 sense-strand primer), 5'GAAGATCTTC CGGGGATGAAGCGGGGC3' (∇225 antisense-strand primer), and 5'TCGCGGCGTCCTGGAAGATCTTCCGGATC GACGGGAT3' (∇300 antisense-strand primer). The nucleotides in italics are the recognition sequence for *Hin*dIII which was engineered into the mutant DNAs to facilitate subsequent insertion of either ∇225 or ∇300 DNA into pRSVnt EPA. The *Bgl*II linker sequence in each primer is underlined.

To construct pHC235, two segments (5' and 3') of mutant gD-1 were synthesized and amplified by PCR using *Taq* polymerase (Perkin-Elmer Cetus). The 5' segment of mutant DNA was synthesized by using the gD-1 upstream primer, the ∇225 antisense primer, and pRE4 (wild-type gD-1) as the template. The 3' segment was constructed by using the ∇225 sense primer, the gD-1 downstream primer, and pRE4. Twenty-five cycles of amplification were performed; in each cycle, template DNA was denatured at 94°C for 1 min, primers were annealed to the template at 55°C for 2 min, and bound primers were extended at 72°C for 3 min. Amplified DNA segments was digested with *Hin*dIII and *Bgl*II and ligated into pRSVnt EPA.

For pHC237, a two-step amplification was performed. The first involved synthesis of the 5' segment of ∇300 (using the gD-1 upstream primer, the ∇300 antisense-strand primer, and pRE4) and the 3' segment of ∇225. The second amplification used the gD-1 upstream and downstream primers, the 5' segment of ∇300, and the 3' segment of ∇225 (both as templates). The resulting fragment was digested with *Hin*dIII and ligated into pRSVnt EPA. The proper orientation was determined by restriction analysis, and mutations were verified by DNA sequencing (7).

(iv) Construction of deletion mutants containing the *Bgl*II linker at the deletion site. Plasmids pHC238, pHC239, pHC240, pHC241, and pHC242, lacking gD-1 residues 277 to 290, 277 to 300, 290 to 300, 290 to 310, and 300 to 310, respectively, were constructed by mixing and matching restriction fragments obtained from gD-1 linker-insertion mutants D1-H26 (∇277), pHC236 (∇290), pHC237 (∇300), and D1-H15 (∇310). For example, to construct pHC238 (Δ277–290), plasmids D1-H26 (∇277) and pHC236 (∇290) were each digested with *Bgl*II. The DNA fragment of D1-H26 containing the amino terminus of gD-1 and the fragment of pHC236 containing the carboxy terminus of gD-1 were religated. The resulting plasmid pHC238 lacked codons for residues 277 to 290 but contained a *Bgl*II linker at amino acid 277. The protein from this mutant as well as the other four deletion mutants, pHC239, pHC240, pHC241, and pHC242, contained the amino acids GKIFP at the site of the deletion.

(v) Construction of a chimeric protein consisting of HSV-1 gD and BHV-1 gD (gIV). A 90-bp DNA fragment coding for amino acids 309 to 338 of bovine herpesvirus 1 (BHV-1) gD (formerly known as BHV-1 gIV) (41) was constructed to replace the sequence coded for by amino acids 277 to 310 of gD-1. Four oligonucleotides each corresponding to a different part of the 90-bp BHV-1 DNA fragment and 11 bp of *Bgl*II linker (underlined sequences) were synthesized by W. Wunner of the Wistar Institute: 5' sense strand (5'GAAGATCTTC CGACGGCGAGAG TCCGACCCCCGAAGCCAACGGAG GCGCCGAGGGCGAGCCGAAACC3'), 3' sense strand (5'CGGCCCCAGCCCCGACGCCGACCGCCCCGAAGGC GGAAGATCTTC3'), 5' antisense strand (5'GGGCCGGGT TTCGGCTCGCCCTCGGCGCCTCCGTTGGCTTCGGG GGTCGGACTCTCGCCGTCGGAAGATCTTC3'), and 3'

antisense strand (5′GAAGATCTTCCGCCTTCGGGGCGG TCGGCGTCGGGGCTG3′).

After annealing and amplification, the resulting DNA fragment had a *Bgl*II linker attached to each end. The synthesized DNA fragment was digested with *Bgl*II and then ligated with *Bgl*II-linearized D1-H75 (Δ277–310). The plasmids contained the BHV-1 gD sequence either correctly oriented (pHC233) or reverse oriented (pHC234) in the gD-1 gene instead of the sequence coding for residues 277 to 310 of gD-1. In addition, both plasmids contained codons for GKIFP at the junction of the two glycoproteins. The sequences of the chimeric genes were verified by DNA sequencing.

DNA transfections. COS-1 or L cells were transfected by the calcium phosphate-DNA coprecipitation method (27) as modified (13). Cells were seeded at 8×10^5 cells per 60-mm-diameter dish or 1.6×10^5 cells per well in a 12-well plate the day before transfection. The cells were refed with fresh medium 1 h prior to transfection. For each 60-mm-diameter dish, 20 μg of precipitated supercoiled DNA (4 μg of DNA per well of each 12-well plate) was incubated with cells for 16 h at 37°C. For complementation assays, 15 to 30 μg of each mutant plasmid DNA was added to L cells in a 60-mm-diameter dish (3 to 6 μg of DNA per well for each 12-well plate) to give a level of expression similar to that obtained with a standard amount of pRE4 (wild-type gD-1) DNA, as judged by an immunoperoxidase assay (see below). The cells were then washed twice with serum-free DMEM, overlaid with fresh medium, and incubated for an additional 24 h at 37°C.

Preparation of cytoplasmic extracts. Cytoplasmic extracts were prepared from transfected COS-1 cells at 40 h posttransfection. Cells were washed twice with phosphate-buffered saline (PBS) and then scraped into 1 ml of ice-cold PBS. The cells were pelleted at 350 × *g* for 5 min, the supernatant fluid was removed, and 100 μl of ice-cold lysing buffer (0.02 M Tris [pH 7.5], 0.05 M NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate) containing 0.1 mM *N*-tosyl-L-lysine chloromethyl ketone (TLCK) and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was added to the cell pellets. Extracts were vortexed for 1 min, placed on ice for 10 min, and then centrifuged at 500 × *g* for 10 min to remove nuclei. The supernatant fluids were aliquoted and stored at −70°C.

Polyclonal and monoclonal antibodies. Rabbit anti-gD serum (R#7) (33) was used for Western immunoblotting and for immunoperoxidase assays (61). Rabbit anti-gB serum (R#68) (21) was used to determine the relative amount of gB in the envelope of complemented virus. ZC15 is a polyclonal rabbit serum prepared against a synthetic peptide mimicking residues 358 to 369 of gD-1, provided by M. Zweig. Seven MAbs belonging to groups Ia, Ib, II, III, and VII, and one other MAb, AP7 (55), were used to examine the antigenic conformation of gD mutant molecules (Fig. 8). Of these, DL6 (II) and DL11 (Ib) were isolated in our laboratories. HD1 (Ia) was provided by L. Pereira, 1D3 (VII) was provided by H. Friedman, ABD and VID (III) were provided by C. DesGranges, and AP7 was provided by A. Minson. HD1, DL11, ABD, VID, and AP7 recognize discontinuous epitopes on gD (11, 23, 55, 64, 68); 1D3 and DL6 recognize continuous epitopes of gD from residues 11 to 19 and 272 to 279, respectively (10, 33).

Antigenic analysis. Cytoplasmic extracts prepared from each transfection were analyzed for the ability to bind various MAbs, using both immuno-dot blot analysis and the native gel-Western blot procedure (11). Extracts were quantitated for the amount of gD expressed by using MAb DL6 or ID3 and a known amount of purified gD protein (69).

Immuno-dot blot analysis and quantitation of antigenic activity. Equal amounts of gD from each mutant were dotted onto nitrocellulose, washed with a blocking buffer (10), and incubated with a gD-specific antibody followed by iodinated *Staphylococcus aureus* protein A (ICN Biochemicals). Blots were dried and exposed to Kodak XAR-5 film. The films were scanned with a densitometer (Molecular Dynamics). Background was determined by measuring the intensity of a spot of extract prepared from cells transfected with vector alone. The percentage of binding of mutant gD to each test MAb compared with that of wild-type (WT) gD was calculated as [(amount of binding of mutant gD to the test MAb) − background/(amount of binding of wild-type gD to the test MAb) − background] × 100.

SDS-PAGE and Western blot analysis. Cytoplasmic extracts were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under denaturing or nondenaturing (native) conditions in 10% polyacrylamide gels (11). Following SDS-PAGE, proteins were transferred to nitrocellulose and reacted with antibodies as described above for dot blot analysis.

Immunoperoxidase assay. The immunoperoxidase assay was modified from that described earlier (29, 43, 61). Briefly, to examine gD on the cell surface, unfixed transfected cells were incubated with anti-gD-1 serum (R#7) in DMEM–5% FBS for 1 h at room temperature, washed twice, and then incubated for 1 h at room temperature with protein A-conjugated horseradish peroxidase (Boehringer Mannheim) in DMEM containing 1% FBS. Cells were washed twice with PBS and the substrate, 4-chloro-1-naphthol, was added for 15 min. Cells which were stained black or purple were considered positive for expressing gD on the plasma membrane. To detect the total amount of gD expressed (both intracellular and extracellular), cells in a duplicate plate were fixed with 5% methanol in PBS for 10 min, air dried, and then incubated with antibody and protein A-horseradish peroxidase. Transfection efficiency was determined by counting the gD-expressing cells (using the fixed cells) in the plate, using a Müller square eyepiece. The amount of cell surface expression was determined as a percentage of the total amount of gD expressed.

Complementation assay. The procedure was performed as described previously (61), with several modifications. Briefly, L cells were transfected for 20 h with an expression plasmid and then infected with 10^6 PFU of FgDβ virus in Tricine-buffered saline (4). After 1 h, DMEM containing 10% FBS was added, and plates were further incubated for 1 h at 37°C. The medium was removed, and extracellular virus was inactivated by the addition of glycine-saline buffer (pH 3.0) (4, 30, 61) for 1 min followed by two washes with tricine-buffered saline. The cells were overlaid with fresh medium and incubated for 24 h at 37°C. The culture supernatant containing extracellular virus was harvested and stored at −70°C. The intracellular virus was harvested by scraping the cells into DMEM containing 5% FBS. The cells were disrupted by one freeze-thaw cycle followed by sonication and low-speed centrifugation. The intracellular virus in the supernatant was stored at −70°C until titers were determined. Each mutant was tested from one to five times in separate transfections. To correct for differences in transfection efficiency, each complementation assay included both positive and negative controls, and transfection efficiencies for each plasmid were standardized against a positive control (pWW65 or pRE4 DNA) prior to the complementation assay. Virus titers were determined on VD60 monolayers (61). One hundred percent complementation is defined as the total amount of virus obtained (extracellular plus intracellular) after transfection with wild-type plasmid (either pRE4 or pWW65). Percent complementation with a mutant is

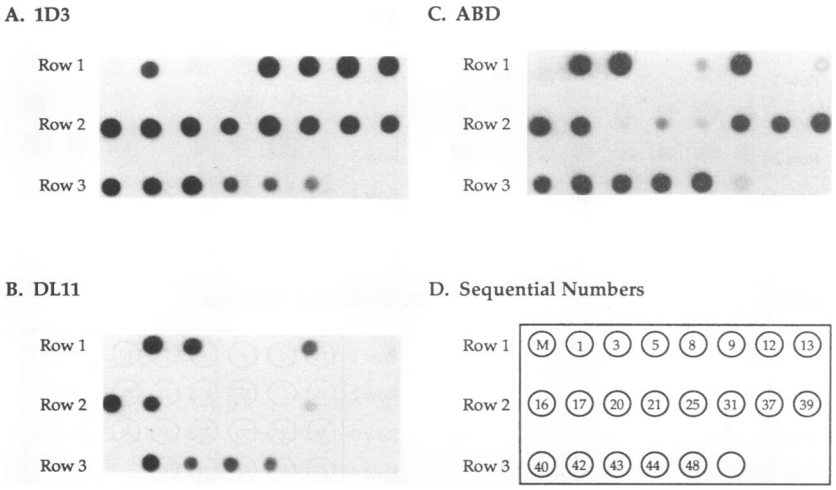

FIG. 2. Immuno-dot blot analysis of gD-1 linker-insertion mutant proteins. Cytoplasmic extracts prepared from COS-1 cells transfected with plasmids containing a wild-type or mutant gD-1 gene were spotted onto nitrocellulose. The membranes were incubated with MAb 1D3 (group VII) (A), DL11 (group Ib) (B), or ABD (group III) (C) and then with *S. aureus* ^{125}I-protein A. In each case, the proteins were spotted according to the number key in panel D (see Table 1 for the corresponding mutation). M, mock-infected extract spotted as a control on each blot.

defined as [PFU (mutant) − PFU (vector)]/PFU (wild type) − PFU (vector) × 100.

Identification of gD in the complemented viruses. Extracellular virions were obtained from the growth medium following complementation assays. Cell debris was removed by centrifugation at 13,000 × *g* for 10 min, and the supernatant was then pelleted through a 1-ml cushion of 5% sucrose at 100,000 × *g* for 2 h. The supernatant was aspirated, and the pellet was resuspended in denaturing electrophoresis sample buffer. The sample was boiled for 3 min, subjected to SDS-PAGE, and Western blotted. Anti-gD serum (R#7) was used to identify gD. The amount of gB was determined by using anti-gB serum (R#68). Since the amount of gB should be constant in each virus preparation, the relative amount of gD in each of the complemented virus preparations was expressed as the ratio of gD to gB.

RESULTS

Construction of in-frame linker-insertion mutations. A *Bgl*II linker was inserted at one of a number of different sites in the genes for gD-1 and gD-2. A total of 50 mutations, including 45 insertions and 5 in-frame insertion-deletion mutations (Table 1, mutants 5, 11, 15, 23, and 43), were constructed for this initial scan (Table 1). The two wild-type plasmids, pRE4 (gD-1) and pWW65 (gD-2), are numbered 1 and 2, respectively, then each plasmid (and mutant protein) is listed in the numerical order of the mutation from N to C terminus. Five mutations were constructed by PCR, to delete codons for various amino acids within residues 277 to 310 and to insert a *Bgl*II linker in that same site; these are listed sequentially as 53 through 57. Two mutants replacing residues 277 to 310 with a different sequence (see footnotes *f* and *g* to Table 1) are listed as 58 and 59. In addition, each name indicates whether it was derived from gD-1 or gD-2 (D1 or D2) and the position of the amino acid immediately upstream of the insertion (e.g., for D1-∇12, the linker was inserted after residue 12 of gD-1). In cases where there is also a deletion, e.g., D1-Δ12~77, residues 12 to 77 of gD-1 were deleted and there is an insertion of four amino acids. In some cases (e.g., D2-Δ58-93), eight amino acids were inserted. A stick figure of gD is shown (Fig. 1A) and the location of each mutation is indicated (Fig. 1B), except for mutants 53 through 59. As can be seen, the mutations are spaced fairly evenly across the proteins. Antibody ZC15 was used to confirm that mutant gD molecules contained the C terminus, and MAb 1D3 was used to confirm that the gD molecules contained the N terminus (data not shown).

Approaches used to study the mutants. Our working hypothesis is as follows: (i) if a mutant protein is both structurally and functionally intact, then the site altered by that particular mutation is not involved in gD function; (ii) if a mutant protein has lost its ability to function but shows global changes in conformation or transport, the functional importance of this 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 structural integrity of mutant gD proteins was assessed by (i) measuring the ability of each protein to bind MAbs to discontinuous epitopes as a reflection of conformation; (ii) studying cell surface expression as a reflection of proper transport; and (iii) measuring protein aggregation, as an indication of improper folding. The function of each mutant was determined by a complementation assay which measures the ability of the mutant protein to rescue the infectivity of the gD-null virus, FgDβ (39, 49, 61).

Binding of MAbs to gD mutants 3 through 52. Previous studies showed that MAbs to discontinuous epitopes can be used to assess the effect of mutations on gD structure (13, 22, 51, 58, 60, 71). Here, seven MAbs, five of which recognize discontinuous epitopes, were used to examine gD structure (33, 55, 59). Two MAbs, 1D3 and DL6, that recognize different continuous epitopes (10, 15, 19, 33) were used to assess the amount of gD in each extract.

Cytoplasmic extracts containing similar amounts of gD were spotted onto nitrocellulose and probed with a specific MAb. Representative results obtained with each mutant protein and three MAbs are shown (Fig. 2 and 3). In addition, extracts were subjected to native gel-Western blot analysis (Fig. 4 and 5).

The two methods for estimating binding of MAbs agreed in most cases (Table 2). For example, protein D1-∇83 (mutant

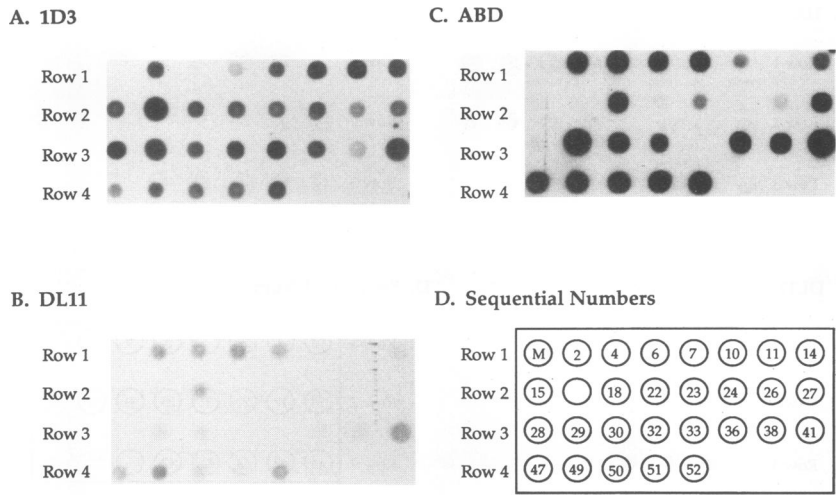

FIG. 3. Immuno-dot blot analysis of gD-2 linker insertion mutant proteins. Cytoplasmic extracts prepared from COS-1 cells transfected with plasmids containing a wild-type or mutant gD-2 gene were spotted onto nitrocellulose. The membranes were incubated with MAb 1D3 (group VII) (A), DL11 (group Ib) (B), or ABD (group III) (C) and then with *S. aureus* ^{125}I-protein A. In each case, the proteins were spotted according to the number key in panel D (see Table 1 for the corresponding mutation). M, mock-infected extract spotted as a control on each blot. The positive reaction seen in row 2 of panel A (rather than a blank) was due to spotting mutant 29 in that row as well as in row 3 by mistake.

16; Fig. 2D) bound DL11 well by dot blotting (Fig. 2B) or by Western blotting (Fig. 4A), and the level of binding was equivalent to that of wild-type gD by both techniques (mutant 1; Fig. 2D and 4A). Wild-type levels of DL11 binding were noted for eight D1 and seven D2 mutants, e.g., D2-∇27 (mutant 7). An even larger number bound ABD as well as the wild-type protein. Some mutants with diminished or abolished MAb binding in dot blots or Western blots, e.g., D1-∇34 (mutant 8; Fig. 2D), bound ABD less well than the wild-type protein (compare Fig. 2C and 4B). In contrast, for several mutants, e.g., D1-∇125 (mutant 20; Fig. 2D), there was little

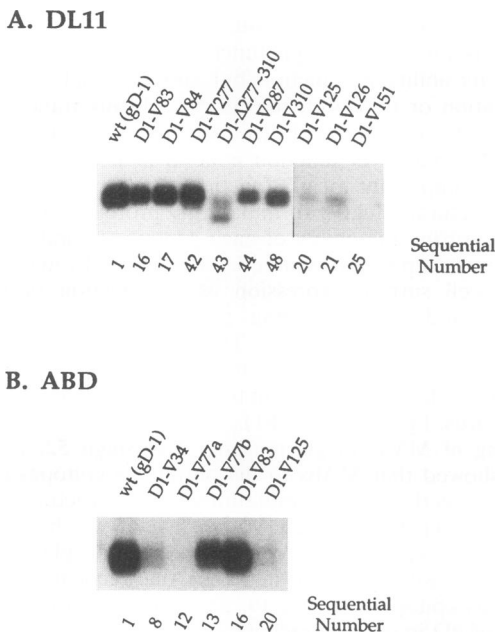

FIG. 4. Western blot analysis of wild-type and mutant gD-1 proteins. Cytoplasmic extracts prepared from COS-1 cells transfected with plasmids containing a wild-type (wt) or mutant gD-1 gene were electrophoresed under nondenaturing conditions, transferred to nitrocellulose, and reacted with DL11 (A) or ABD (B) and then with *S. aureus* ^{125}I-protein A. Protein names are indicated at the top, and sequential numbers of the mutants (Table 1) are indicated at the bottom.

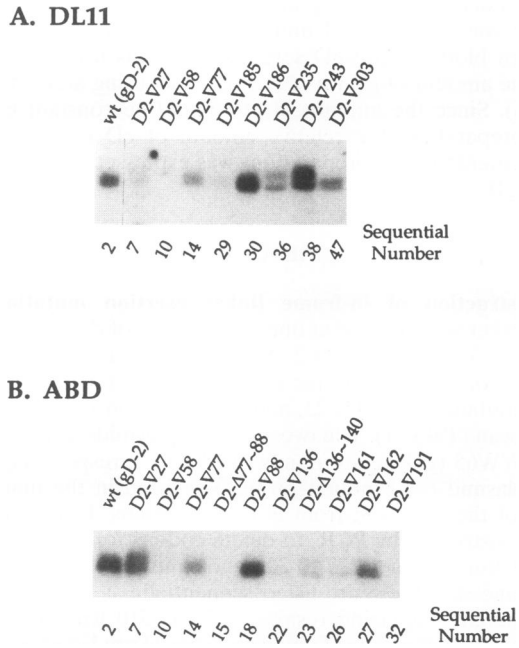

FIG. 5. Western blot analysis of wild-type and mutant gD-2 proteins. Cytoplasmic extracts prepared from COS-1 cells transfected with plasmids containing a wild-type (wt) or mutant gD-2 gene were electrophoresed under nondenaturing conditions, transferred to nitrocellulose, and reacted with DL11 (A) or ABD (B) and then with *S. aureus* ^{125}I-protein A. Protein names are indicated at the top, and sequential numbers of the mutants (Table 1) are indicated at the bottom.

TABLE 2. Properties of gD linker-insertion mutant proteins

Sequential no.	Mutant name and location of insertion[a]	MAb binding[b]							Cell surface expression[c]	Aggregate formation[d]
		1D3	DL6	HD1	DL11	ABD	VID	AP7		
1	Wild type (gD-1)	+	+	+	+	+	+	+	+	−
2	Wild type (gD-2)	+	+	+	+	+	+	+	+	−
3	D1-∇12	w	+	+	+	+	+	+	+	−
4	D2-∇12	w	+	+	+	+	+	+	+	−
5	D1-Δ12~77[e]	−	+	−	−	−	−	−	−	+
6	D2-∇18	w	+	+	+	+	+	+	+	−
7	D2-∇27	+	+	w	w	+	+	−	+	+
8	D1-∇34	+	+	−	−	w	w	−	+	+
9	D1-∇43	+	+	+	+	+	+	w	+	+
10	D2-∇58	+	+	−	−	−	−	−	+	+
11	D2-Δ58~93[e]	+	+	−	−	−	−	−	−	+
12	D1-∇77a	+	+	−	−	−	−	−	+	+
13	D1-∇77b	+	+	w	w	+	+	w	+	+
14	D2-∇77	+	+	+	+	+	+	w	+	+
15	D2-Δ77~88[e]	+	+	−	−	−	−	−	+	+
16	D1-∇83	+	+	+	+	+	+	+	+	−
17	D1-∇84	+	+	+	+	+	+	+	+	−
18	D2-∇88	+	+	+	+	+	+	+	+	−
19	D2-∇93	+	+	w	w	w	w	ND	+	ND
20	D1-∇125	+	+	w	w	w	w	−	+	+
21	D1-∇126	+	+	w	w	+	+	−	+	+
22	D2-∇136	+	+	−	−	w	w	−	+	+
23	D2-Δ136~140[e]	+	+	−	−	w	w	−	+	+
24	D2-∇140	+	+	−	−	w	w	−	+	+
25	D1-∇151	+	+	−	−	−	−	−	+	+
26	D2-∇161	+	+	−	−	w	w	−	+	+
27	D2-∇162	+	+	w	w	+	+	−	+	+
28	D2-∇164	+	+	−	−	−	−	−	−	+
29	D2-∇185	+	+	w	w	+	+	+	+	+
30	D2-∇186	+	+	+	+	+	+	+	+	+
31	D1-∇187	+	+	+	+	+	+	w	+	+
32	D2-∇191	+	+	−	−	−	−	−	+	+
33	D2-∇207a	+	+	−	−	w	w	−	−	+
34	D2-∇207b	+	+	−	−	w	w	−	−	+
35	D1-∇225	+	+	+	−	+	+	−	+	ND
36	D2-∇235	+	+	+	+	+	+	−	+	+
37	D1-∇243	+	+	+	+	+	+	−	+	+
38	D2-∇243	+	+	+	+	+	+	−	+	+
39	D1-∇246a	+	+	+	+	+	+	−	+	+
40	D1-∇246b	+	+	+	+	+	+	−	+	+
41	D2-∇257	+	+	+	+	+	+	+	+	−
42	D1-∇277	+	−	+	+	+	+	+	+	−
43	D1-Δ277~310[e]	+	−	+	+	+	+	−	+	+
44	D1-∇287	+	+	+	+	+	+	+	+	ND
45	D1-∇290	+	+	+	+	+	+	+	+	ND
46	D1-∇300	+	+	+	+	+	+	+	+	−
47	D2-∇303	+	+	+	+	+	+	+	+	−
48	DI-∇310	+	+	+	+	+	+	+	+	−
49	D2-∇315	+	+	+	+	+	+	+	+	−
50	D2-∇322	+	+	+	+	+	+	+	−	−
51	D2-∇324	+	+	+	+	+	+	+	−	−
52	D2-∇338	+	+	+	+	+	+	+	+	−

[a] D1, gD-1 gene; D2, gD-2 gene. The number after the dash refers to the residue number of the amino acid immediately preceding the insert. Insertions are indicated by ∇.

[b] Determined by both dot blot and Western blot analysis. In those cases where Western blot analysis gave a stronger positive result, that result is recorded. w, weakly positive reaction with the MAb by both analyses (compared with the wild type); −, no reaction by either Western blotting or dot blotting; ND, not done.

[c] Determined by immunoperoxidase assay using polyclonal anti-gD serum.

[d] Determined on nondenaturing SDS-PAGE using polyclonal anti-gD serum.

[e] Deletion mutant. In each case, the residues indicated by Δ were deleted.

evidence of binding of ABD and DL11 by dot blotting (Fig. 2B and C), but distinct bands were seen by Western analysis (Fig. 4A and B). The latter results might be attributed to the larger amount of extract used for Western blotting than for dot blotting. Alternatively, small amounts of SDS present during electrophoresis might have exposed a discontinuous epitope that was present but inaccessible for binding MAb by dot blotting. In a few cases, mutant proteins bound MAbs by dot blotting but not on Western blots. For example, D2-∇191 (mutant 32; Fig. 3D) bound ABD strongly by dot blotting (Fig. 3C), but no band was detected by Western blotting (Fig. 5B). It is possible that SDS present during electrophoresis disrupted

the epitope. In addition, the dot blot assay is more prone to giving false-positive results as a result of high levels of nonspecific binding.

The dot blot data were quantitated by densitometry, and the relative binding of MAbs by mutant proteins was compared with binding of the wild-type protein (Table 2). Three categories were observed: no binding; weak binding (less than 50% of the wild-type level), and normal binding (50% or more of the wild-type level). Among mutants 3 through 50, eight were negative for all MAbs to discontinuous epitopes. Thus, these proteins suffer from global structural changes and cannot be used to assess the effect of the mutation. They include two insertion mutants of gD-1 at residues 77 (mutant 12) and 151 (mutant 25), three of gD-2 at residues 58 (mutant 10), 164 (mutant 28), and 191 (mutant 32), and three deletion mutants, D1-Δ12~77 (mutant 5), D2-Δ58~93 (mutant 11), and D2-Δ77~88 (mutant 15). Since the three disulfide bonds are located within residues 66 to 202 (51), it is possible that mutations which caused global effects disrupted one or more disulfide bond.

Forty-two mutants bound some or all MAbs to discontinuous epitopes and were useful in carrying out functional studies. These results support the conclusion that gD-1 and gD-2 are structurally very similar (9, 17, 18, 47, 50–52, 74, 75). Furthermore, although the insertions made in gD-1 and gD-2 were not precisely at the same sites, the MAb binding patterns were similar. Thus, insertions in some regions had little effect on either protein (e.g., compare mutants 17 and 18, which have insertions at residues 84 and 88 of gD-1 and gD-2, respectively). Some insertions, again in similar regions, had global effects on MAb binding, e.g., mutants 25 and 26, at residue 151 of gD-1 and residue 161 of gD-2. Consequentially, we combined the mutants into one group (Table 2). Subsequently, the effects of mutations in one protein will be considered relevant to both proteins.

Cell surface expression of gD by mutants 3 to 52. When membrane proteins fail to fold properly or associate into proper oligomeric complexes, they tend to be trapped in the endoplasmic reticulum (16). Transport to the cell surface can therefore be used as an indicator of the level of structural damage of the protein. Each mutant was tested for gD expression on the transfected cell surface (Fig. 6 and Table 2). Ten to twenty percent of cells transfected with plasmids for wild-type gD-1 (Fig. 6A) and gD-2 (Fig. 6C) expressed gD on the cell surface. Figure 6 shows two examples of mutants which were transported, mutant 5, D1-Δ12~77 (Fig. 6B), and mutant 4, D2-∇12 (Fig. 6D), and one example of a mutant which was not transported, mutant 33, D2-∇207a (Fig. 6E).

Among the 50 gD mutants, only 7, D1-Δ12-77 (mutant 5), D2-Δ58-93 (mutant 11), D2-∇164 (mutant 28), D2-∇207a (mutant 33), D2-∇207b (mutant 34), D2-∇322 (mutant 50), and D2-∇324 (mutant 51), were not detected on the plasma membrane, though the protein was synthesized (Fig. 7). Among these, three (D1-Δ12-77, D2-Δ58-93, and D2-∇164) showed global changes in structure, whereas D2-∇207a and D2-∇207b retained some secondary structure (Table 2). From these and other data, we believe that residues just downstream of Cys 6 (residue 202) are critical for structure. A second situation was found for D2-∇322 and D2-∇324. In these mutants, the membrane anchoring sequence was disrupted by the insertion. Both proteins were detected in the growth medium, indicating that they were secreted.

Detection of aggregates of mutants 3 to 52 by nondenaturing SDS-PAGE. Proteins with altered native conformation tend to aggregate during synthesis, and this condition is most likely due to aberrant intermolecular disulfide bonding and/or abnormal hydrophobic interactions (16, 50, 69, 70, 76).

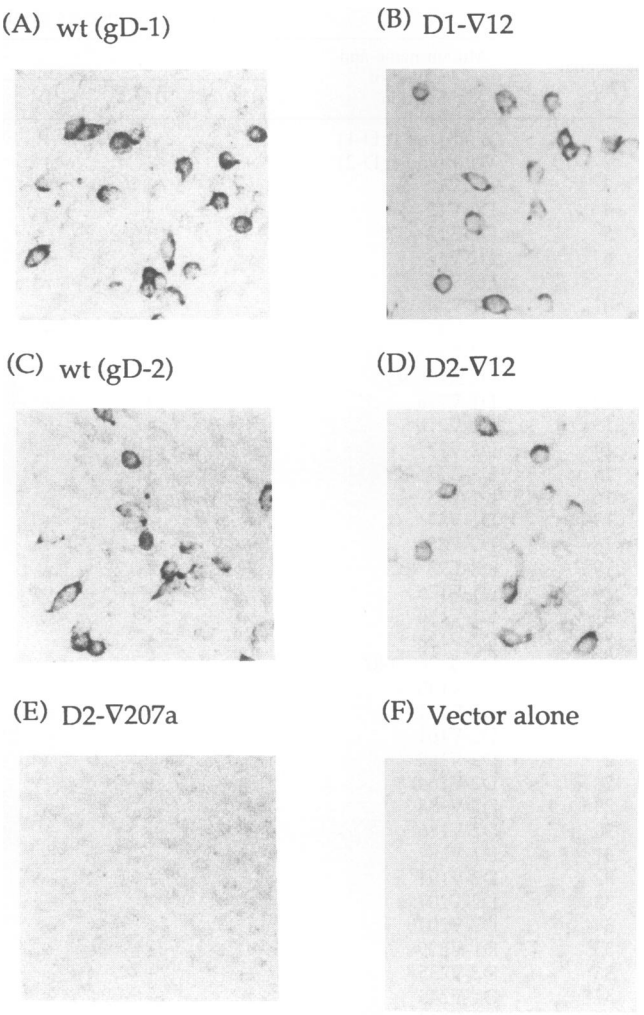

FIG. 6. Cell surface expression of wild-type and mutant gD proteins. Mouse L cells were transfected with plasmids containing either a wild-type (wt) or mutant form of gD-1 or gD-2. Unfixed transfected cells were tested for gD expression in an immunoperoxidase assay. The cells were incubated sequentially with polyclonal anti-gD serum, *S. aureus* protein A (conjugated to horseradish peroxidase), and substrate (4-chloro-1-naphthol). The black stain indicates the presence of gD on the cell surface.

To detect aggregation, cytoplasmic extracts were subjected to native SDS-PAGE, transferred to nitrocellulose, and probed with a polyclonal antibody. The various mutants exhibited different degrees of aggregation. Several examples are shown (Fig. 7 and 8A). Under denaturing conditions, each mutant protein migrated to the expected position (Fig. 8B). As expected, the wild-type forms of gD-1 and gD-2 did not aggregate.

In some cases, there was agreement of all three assays: antigenic conformation, cell surface expression, and aggregation. For example; D1-Δ12~77 (mutant 5) showed global conformational changes, aggregated, and was not found on the cell surface, whereas D2-∇322 (mutant 50), with a mutation in the transmembrane region (TMR), bound all five MAbs to discontinuous epitopes, did not aggregate, and was transported

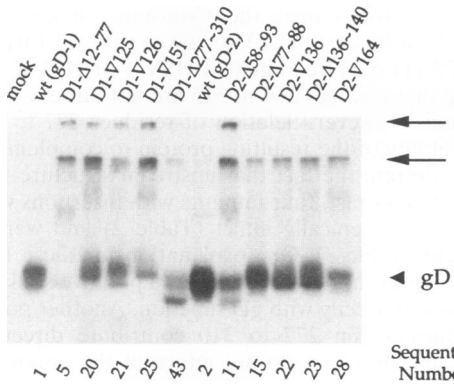

FIG. 7. Western blot analysis of wild-type and mutant gD-1 and gD-2 proteins electrophoresed on nondenaturing (native) gels. Cytoplasmic extracts prepared from COS-1 cells transfected with plasmids containing a wild-type (wt) or mutant gD-2 gene were electrophoresed under nondenaturing conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gD serum followed by *S. aureus* ^{125}I-protein A. As a control, cells were infected with a plasmid containing no insert (mock). The arrows point to aggregates found at the stacking gel-resolving gel interface and also at the top of gel (in the loading wells).

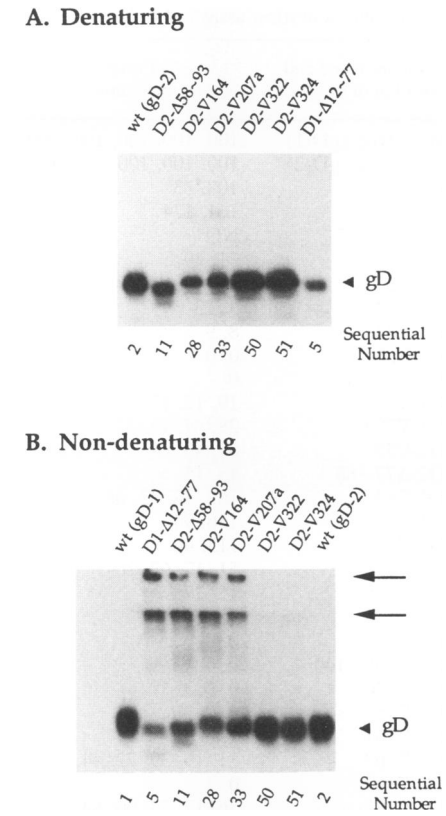

FIG. 8. Western blot analysis of wild-type and mutant gD-1 and gD-2 proteins not expressed on the surface of transfected cells. Cytoplasmic extracts prepared from COS-1 cells transfected with plasmids containing a wild-type (wt) or mutant gD-2 gene were electrophoresed under denaturing (A) or nondenaturing (B) conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gD serum followed by *S. aureus* ^{125}I-protein A. The expected positions of monomeric gD are indicated in both panels. The arrows in panel B point to aggregates found at the stacking gel-resolving gel interface and also at the top of gel (in the loading wells).

and secreted. In other cases, aggregation did not correlate with transport or with global changes in structure. For example, D1-∇125 (mutant 20) aggregated and exhibited conformational changes but was still detected on the cell surface. In general, the tendency of mutant forms of gD to aggregate correlated better with a reduction in antigenic activity than with defects in transport.

Functional analysis of gD linker-insertion mutants 3 to 52. We used a complementation assay to test whether the mutant proteins were functional in vivo (61). This assay uses the gD-null virus FgDβ. The virus replicates in and forms plaques on VD60 cells. These cells contain the integrated gD gene under the control of its own promoter (49). L cells were first transiently transfected with plasmids containing the gD gene and then superinfected with FgDβ. Pseudotype particles were harvested and titered on VD60 cells. The number of plaques measures the extent to which the mutant gD rescued the infectivity of FgDβ. When infectivity was rescued with the wild-type gD gene, the yields were typically 2×10^6 PFU of progeny extracellular virus and 10^6 PFU of intracellular virus. Virus yields using wild-type gD were considered to be 100% (Table 3). Mutants whose complementation activity was 25% or more of the wild-type level were considered functional; those that complemented 3% or less were considered nonfunctional; mutants whose complementation activity was between 3 and 24% were considered to be reduced in function. Of the 50 linker-insertion mutants, 19 were functional, 21 were nonfunctional, and the rest exhibited reduced function.

The effects of the mutations on gD structure or function fit into four categories (Fig. 9): (i) mutations with little effect on gD structure, transport, or function (21 mutants), representing sites which are not critical for gD function; (ii) mutations which caused global conformational changes (8 mutants) that did not allow a conclusion to be drawn about that particular site; (iii) mutations which interfered with expression of gD on the cell surface, which could reflect the absence of gD in the virion envelope (4 mutants, including 2 in the TMR which caused the protein to be secreted); and (iv) mutations which had little effect on protein conformation or transport but did impair gD function (17 mutants). The mutation sites of the last group are considered to be important for function and are mostly clustered in four different regions of gD: region I (residues 27 through 43), region II (residues 125 through 161), region III (residues 225 through 246), and region IV (residues 277 to 310) (Fig. 9). Two previously studied deletion mutants (Δ234–244 and Δ231–235) (22, 60) are located within region III. Region IV is interesting, since deletion of residues 277 to 310 impaired gD function, but insertion at 277, 287, 303, or 310 had no such effect. Experiments below address region IV in more detail. A single insertion site, D2-∇186, was also important for function, since this mutant retained antigenic structure but had low complementation activity. Interestingly, mutations on either side of this residue, D2-∇185 and D1-∇187, had no effect on gD function. Additional mutations between 161 and 186 will be needed to determine whether region II constitutes a fifth region.

Is gD present in the envelope of virions that exhibit no complementation activity? Although most of the mutant proteins obtained from cell extracts were structurally intact and were transported to the transfected cell surface, it was not clear whether gD was always incorporated into the virion envelope after superinfection with FgDβ. We purified several pseudotype virus preparations, including some that were non-

TABLE 3. Complementation assays of linker-insertion mutants

Sequential no. of mutant	Mutant name and location of insertion	% Complementation[a]	Avg % complementation
1	Wild type (gD-1)	100, 100, 100, 100, 100	100
2	Wild type (gD-2)	100, 100, 100, 100, 100	100
3	D1-∇12	108, 83	96
4	D2-∇12	161, 124	143
5	D1-Δ12~77	ND[b]	ND
6	D2-∇18	31, 49	40
7	D2-∇27	38, 14, 14, 13	20
8	D1-∇34	3, 13	2
9	D1-∇43	8, 9	9
10	D2-∇58	0, 0	0
11	D2-Δ58~93	0	0
12	D1-∇77a	19, 12, 17	16
13	D1-∇77b	28, 34, 18, 37	29
14	D2-∇77	67, 43	55
15	D2-Δ77~88	13, 15	14
16	D1-∇83	162, 38, 43, 66	77
17	D1-∇84	136, 94	115
18	D2-∇88	115, 46, 66	76
19	D2-∇93	51, 17, 62, 20	38
20	D1-∇125	15, 27	21
21	D1-∇126	12, 21	17
22	D2-∇136	0, 0	0
23	D2-Δ136~140	0, 0	0
24	D2-∇140	0, 0	0
25	D1-∇151	0, 0	0
26	D2-∇161	1, 0	1
27	D2-∇162	31, 19, 39, 72, 44	41
28	D2-∇164	0	0
29	D2-∇185	36, 17, 33, 90, 84	52
30	D2-∇186	3, 25, 1	10
31	D1-∇187	72, 86	79
32	D2-∇191	0, 2	1
33	D2-∇207a	0	0
34	D2-∇207b	0	0
35	D1-∇225	0	0
36	D2-∇235	0, 0	0
37	D1-∇243	3, 3	3
38	D2-∇243	0, 4	2
39	D1-∇246a	0, 1	1
40	D1-∇246b	0, 1	1
41	D2-∇257	41, 78	60
42	D1-∇277	26, 40, 26, 83	44
43	D1-Δ277~310	0, 0, 0	0
44	D1-∇287	37, 90, 51	59
47	D2-∇303	28, 87, 45, 78	60
48	D1-∇310	43, 158, 113	105
49	D2-∇315	12, 54, 48, 59	43
50	D2-∇322	14, 30	22
51	D2-∇324	2, 1	2
52	D2-∇338	33, 34	34

[a] Each mutant was tested against the appropriate wild-type control. In most cases, experiments were repeated twice. If results did not agree, the experiment was repeated several more times. Percent complementation = [PFU (mutant) − PFU (vector)]/[PFU (wild type) − PFU (vector)] × 100, (PFU represents the total of intracellular and extracellular virus). Results of individual experiments are presented.

[b] ND, not determined.

functional in the complementation assay. The virus preparations were electrophoresed under denaturing conditions, Western blotted, and probed for gD and gB with polyclonal sera. The amount of gD present relative to the amount of gB was approximately the same in each of the seven preparations that were examined.

Fine mapping of functional region IV. Earlier, we localized continuous epitopes near the C-terminal one-third of the protein (33). Johnson et al. (36) showed that gD-1 truncated at residue 275 (1) inhibited entry of HSV-1 or HSV-2 into cells, suggesting that residues 264 to 315 are not directly involved in gD function. However, deletion of residues 277 to 310 abolished the ability of the resulting protein to complement FgDβ (Table 3), despite the fact that upstream structure was intact (Table 2). In contrast, four mutants with insertions within this region were antigenically intact (Table 2) and were able to complement (Table 3). One explanation for these findings is that the deletion shortens or removes a putative stalk structure or interferes indirectly with gD function. Another possibility is that residues within 277 to 310 contribute directly to gD function, and insertions do not interrupt the important functional element.

(i) Effect of BHV-1 gIV replacement sequence on gD structure and function. To determine whether region IV in gD served only as a spacer region, residues 277 to 310 of gD were replaced with residues 309 to 338 of the homologous protein BHV-1 gD (also called gIV). This sequence was chosen because in naturally isolated strains of BHV-1, it can be present from one to four times without altering gIV function (41), and the amino acids are in the same general region of gIV as residues 277 to 310 of HSV gD. Moreover, the replacement sequence is quite different from that of residues 277 to 310 of gD, regardless of the orientation of the gene sequence. Two mutants were isolated; one (gD-gIV-gD) contained the gIV sequence in the correct orientation, and the other (gD-gIVr-gD) contained the sequence which results from reverse orientation of the gIV gene.

Neither chimeric proteins bound MAb DL6, because part of antigenic site II (residues 272 to 279) was missing (33). Both proteins still bound five of the other six MAbs (Table 4). Thus, replacement of amino acids 277 to 310 of gD with part of the gIV sequence did not grossly affect upstream gD structure. Interestingly, neither of the chimeric proteins reacted with MAb AP7. Since amino acid 25 is associated with AP7 binding (55), the data suggest that some residues within region IV of gD may be in close proximity with amino acids close to the N terminus of the native protein. gD-gIV-gD was detected on the cell surface, but gD-gIVr-gD was not (Table 4), suggesting that the insertion affected proper transport. In complementation tests, both proteins exhibited low activity (7 to 12%) (Fig. 10 and Table 5), suggesting that region IV does more in terms of function than simply serve as a stem structure.

(ii) Effects of small deletions in region IV. To further localize the functional domain within region IV, we constructed five in-frame insertion-deletion mutants, D1-Δ277~290, D1-Δ277~300, D1-Δ290~300, D1-Δ290-310, and D1-Δ300~310 (Table 1). Each mutant was tested for its effect on gD structure and function. D1-Δ277~290, D1-Δ277~300, and D1-Δ277~310 each had part of antigenic site II deleted and, as expected, were not recognized by MAb DL6 (Table 4). All six mutants were recognized by four MAbs to discontinuous epitopes, but only two mutants, D1-Δ277~290 and D1-Δ300~310, reacted with AP7, implicating residues between 290 and 300 in the AP7 epitope. All of the mutants were expressed on the surface of transfected cells.

Each mutant was tested for gD function (Fig. 10 and Table 5). D1~Δ277-310 did not complement FgDβ, but D1-Δ300~310, on the other hand, had 57% complementation activity and is therefore considered functional. All other deletions within region IV showed low functional activity (7 to 14% of the wild-type gD level) (Table 5 and Fig. 10). These results suggest that residues 277 to 300 within region IV may be specifically needed for gD to function in virus entry.

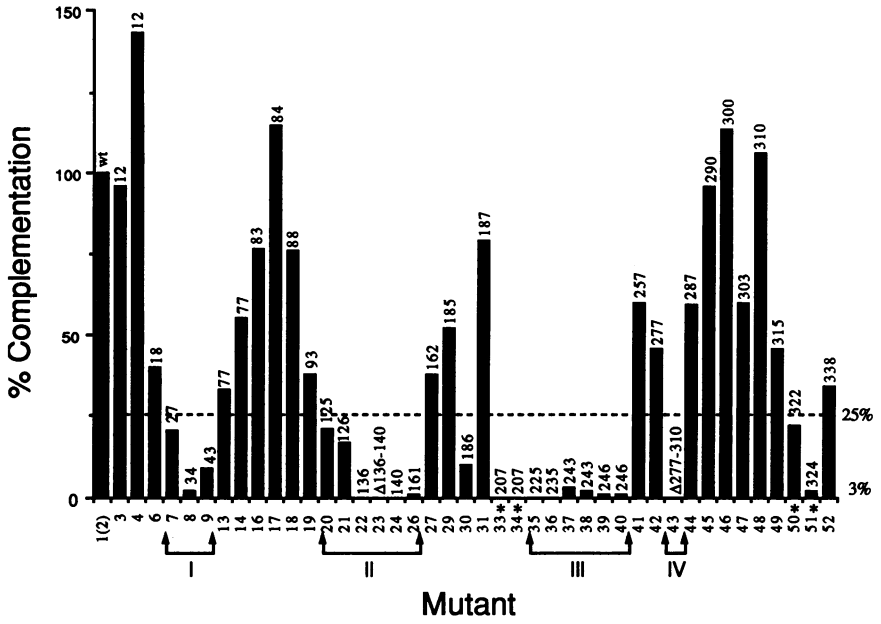

FIG. 9. Functional analysis of gD-1 and gD-2 linker-insertion mutants in a complementation assay. Each mutant was tested for its ability to complement the infectivity of FgDβ. Numbers above the data bars indicate mutation sites in the gD molecule. The data plotted represent the average complementation activities (see Table 3) of mutants that retained part or all native protein conformation. (Those omitted from the graph were mutants 5, 10, 11, 12, 15, 25, 28, and 32). As expected, none of these mutants exhibited any complementation activity (Table 3) as a result of gross protein malfolding (Table 2). Mutants with 25% or more complementation activity were interpreted as functionally intact. For mutants with complementation activities between 3 to 24%, gD function was considered positive but reduced. Mutants that provided 3% or less complementation activity compared with wild-type gD were considered nonfunctional. A dotted line separates the last two categories from the first. Mutants in the last two categories, with reduced or no gD function, are mainly clustered in four regions of gD (designated I to IV). In some cases, mutants were not detected on the surface of transfected cells (indicated by asterisks). In those cases, low complementation activity could be due to the absence of gD from the virion envelope; therefore, they were not included in the functional regions.

Moreover functional region IV (277 to 300) overlaps a portion of the AP7 epitope (residues 290 to 300).

DISCUSSION

Linker-insertion mutagenesis has proven to be a useful approach in scanning proteins systematically for functional regions. In this method, an oligonucleotide containing the recognition sequence for a particular restriction enzyme is inserted at known locations through the length of the gene, and the effects of each insertion on protein structure and function are studied (26). The goal is to disrupt a functional site without causing global structural changes in the protein (5, 31, 63, 65, 67, 73).

TABLE 4. Antigenic properties and cell surface expression of mutants in region IV

Type of mutation	Sequential no.	Mutant name and mutation[a]	MAb binding[b]							Cell surface expression[c]
			1D3	DL6	HD1	DL11	ABD	VID	AP7	
Linker-insertion	1	Wild type (gD-1)	+	+	+	+	+	+	+	+
	42	D1-∇277	+	−	+	+	+	+	+	+
	44	D1-∇287	+	+	+	+	+	+	+	+
	45	D1-∇290	+	+	+	+	+	+	+	+
	46	D1-∇300	+	+	+	+	+	+	+	+
	47	D2-∇303	+	+	+	+	+	+	+	+
	48	D1-∇310	+	+	+	+	+	+	+	+
Linker-insertion + deletion	43	D1-Δ277~310	+	−	+	+	+	+	−	+
	52	D1-Δ277~290	+	−	+	+	+	+	+	+
	54	D1-Δ277~300	+	−	+	+	+	+	−	+
	55	D1-Δ290~300	+	+	+	+	+	+	−	+
	56	D1-Δ290~310	+	+	+	+	+	+	−	+
	57	D1-Δ300~310	+	+	+	+	+	+	+	+
Amino acid replacement	58	gD-gIV-gD[d]	+	−	+	+	+	+	−	+
	59	gD-gIVr-gD[e]	+	−	+	+	+	+	−	−

[a] Data for mutants 1, 42, 44, 45, 46, 47, and 48 are from Table 2, shown for comparison with the other mutants.
[b] Determined by immuno-dot blot analysis.
[c] Determined by immunoperoxidase assay using polyclonal anti-gD serum.
[d] The substituted sequence represents the correct reading frame of BHV-1 gIV amino acids 309 to 338.
[e] The substituted sequence represents the reverse reading frame of the gIV sequence.

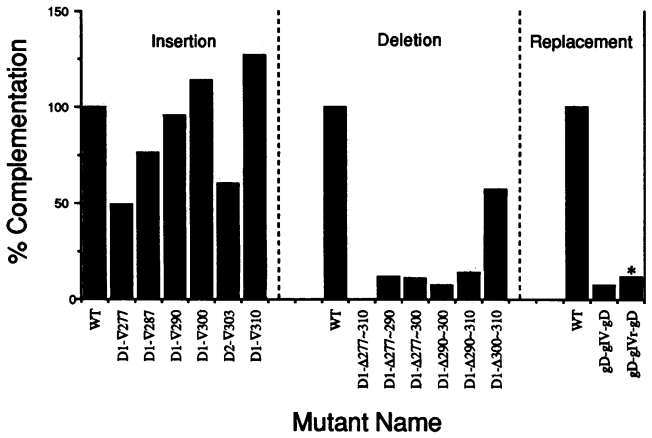

FIG. 10. Effects of mutations in region IV of gD on the ability of gD to complement FgDβ virus. Fourteen mutant gD molecules from three different types of mutations in region IV of gD were tested for the ability to complement the gD-null virus FgDβ. Except for gD-gIVr-gD, which was tested only once, the results shown represent averages of three to five independent assays (Table 5). Mutants with 25% or more complementation activity were interpreted as functionally intact. For mutants with complementation activities between 3 to 24%, gD function was considered positive but reduced. Mutants that provided 3% or less complementation activity compared with wild-type gD were considered nonfunctional. An asterisk indicates that the mutant protein was not expressed on the cell surface; therefore, the absence of complementation activity could be due to the absence of the mutant protein from the virion envelope.

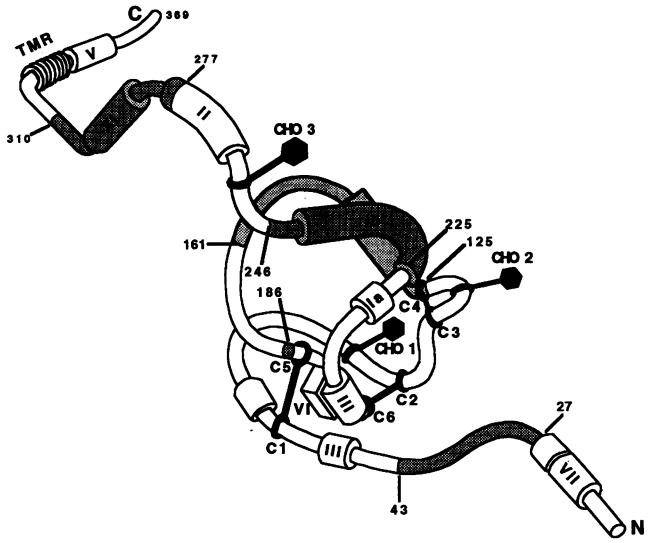

FIG. 11. Model of gD structure showing the four functional regions. This model of gD structure, adapted from that of Long et al. (51), depicts our current concept of the folded form of gD. Both the N and C termini are depicted as extended structures and contain continuous antigenic sites at amino acids 11 to 19 (site VII), 264 to 279 (site II), 284 to 301 (site XI), and 340 to 356 (site V). Cysteines are disulfide bonded according to Long et al. (51). Shaded portions represent functional regions I (amino acids 27 to 43), II (amino acids 126 to 161), III (amino acids 222 to 251), and IV (amino acids 277 to 310). A single amino acid (at position 186) may also be involved in gD function.

The results presented above show that linker-insertion mutations in similar regions of gD-1 and gD-2 have equivalent effects on the structure and function of these two proteins. Furthermore, a large number of mutants generated by this technique retained structure and therefore could be assessed for function. These mutants identified four functional regions within gD-1 and gD-2. Region III (residues 225 through 246) overlaps a previously identified functional site (22, 60). The other three regions, from 27 through 43 (region I), from 125 through 161 (region II), and from 277 to 310 (region IV), are newly described here. These regions are indicated on a model of gD (Fig. 11) similar to one previously described (51).

Region I is close to or overlaps residues that have been implicated in gD restriction of HSV entry (6, 14). In both studies, gD-expressing cell lines were refractory to HSV entry.

TABLE 5. Complementation assays of region IV mutants

Type of mutation	Sequential no.	Mutant name	% Complementation[a]				Avg[b]
			Expt 1	Expt 2	Expt 3	Previous expt[c]	
Linker-insertion	1	Wild type (gD-1)	100	100	100	100	100
	42	D1-∇277	68			26, 40, 26, 83	49
	44	D1-∇287	126			37, 90, 51	76
	45	D1-∇290	122	80	87		96
	46	D1-∇300	106	95	140		114
	47	D2-∇303				28, 87, 45, 78	60
	48	D1-∇310	192			43, 158, 113	127
Linker-insertion + deletion	43	D1-Δ277~310	0			0, 0, 0	0
	52	D1-Δ277~290		15	9		12
	54	D1-Δ277~300	13	9	11		11
	55	D1-Δ290~300	8	7	7		7
	56	D1-Δ290~310	23	10	8		14
	57	D1-Δ300~310	71	55	45		57
Amino acid replacement	58	gD-gIV-gD[d]	6	8	6		7
	59	gD-gIVr-gD[e]	12				12

[a] Defined as presented in Table 3, footnote *a*.
[b] The data from these and all previous experiments (Table 3) were averaged.
[c] Data shown are from Table 3.
[d] The sequence represents the correct reading frame of residues of BHV-1 gIV.
[e] The sequence represents the reverse reading frame of the gIV sequence.

HSV mutants which overcame this restriction had mutations at amino acid 25 or 27. In one case, an HSV-1 mutant with a leucine-to-proline change at position 25 in gD, was able to overcome the restriction and infect gD-expressing cells. This same amino acid is part of the epitope for MAb AP7 (55). When gD-expressing cells were treated with AP7, the cells became susceptible to infection (6). One interpretation of the result is that gD in the cell membrane interacts with gD on the virion, thereby preventing virion gD from functioning. Another interpretation is that the cells expressing gD sequester a cell receptor for gD which is required for entry (36, 40).

Region II encompasses a loop structure that is stabilized by the disulfide bond between cysteines 3 (amino acid 118) and 4 (amino acid 127) (51). It is interesting that several MAb-resistant (*mar*) mutations selected with group Ia and Ib antibodies lie within or just adjacent to region II (58, 60). Insertion at amino acid 125 or 126 had some local effects on gD structure, e.g., eliminating binding by MAb AP7, and a large effect on gD function (complementation values were 21 and 17%, respectively). In addition, a gD construct that contained several *mar* mutations (125, 140, and 216) was unable to function in a complementation assay (60), stressing the importance of region II.

Six mutants helped to define region III (225 through 246). In all cases, the proteins exhibited no complementation activity. The structural changes appeared to be minimal, since all of the mutant proteins bound at least three MAbs to discontinuous epitopes and in most cases bound four. However, MAb DL11 (group Ib) failed to bind to D1-∇225 but did bind to the other five mutants, and MAb AP7 did not bind to any of the mutants that define region III. Fine mapping of this region by using small-deletion mutations is now under way (57).

Region IV was initially pinpointed with a single insertion-deletion mutation, D1-Δ277~310. This mutant protein was able to bind all MAbs to discontinuous epitopes except AP7 but had no complementation activity. In addition, this mutant was expressed on the cell surface. However, the deletion apparently had some effect on gD structure because the processing of the protein was impaired (Fig. 7). Accumulation of precursor forms of glycoproteins is generally indicative of retention in the endoplasmic reticulum due to improper folding. Similar processing defects were also seen with smaller deletions of region IV (data not shown). Thus, the protein could be misfolded in regions not detected by most of the MAbs. Interestingly, insertions at amino acids 277, 287, 300, 303, and 310 had no effect on structure (all MAbs, including AP7, bound) and little or no effect on function. It was originally proposed that the region from amino acid 260 up to the TMR at amino acid 319 could act as a putative stalk structure or spacer region (22, 33). One interpretation of the linker-insertion data was that deletion of 34 amino acids adversely affected this putative stalk structure and therefore interfered with gD function.

Two approaches were taken to address this issue. First, the entire region from residues 277 to 310 of gD was replaced with unrelated amino acids. The sequence chosen for the replacement represents a portion of the BHV-1 gD (or gIV) protein which has been shown to be duplicated in some strains of the BHV-1 (42). The resulting chimera, gD-gIV-gD, was able to bind all MAbs except AP7 and was transported to the cell surface, but still failed to complement FgDβ. This result suggests that simple replacement of the gD sequence was not sufficient to restore function. A second approach was to create smaller deletions within the region 277 to 310. In all cases, the smaller-deletion mutants were able to bind four out of five MAbs to discontinuous epitopes. Only two, D1-277~290 and D1-300~310, were able to bind AP7. Interestingly, only one mutant of the five, D1-300~310, was able to complement FgDβ. These data suggest that specific residues within amino acids 290 to 300 were involved in AP7 binding but that a larger region, within amino acids 277 to 300, was important for gD function. Thus, AP7 binding requires the presence of gD residues well downstream of any MAbs to discontinuous epitopes that have been mapped in previous studies (28, 33, 51, 55, 59). It is noteworthy that one of the human vaccines employing truncated forms of gD uses a construct that ends at residue 281 of gD-2 (46). Since this protein protects animals from virus challenge, it does not appear to lack efficacy due to the absence of region IV.

Use of linker-insertion mutants for epitope mapping. Several mutants bound some MAbs weakly or not at all but bound other MAbs at wild-type levels. This finding suggests that some of the mutations disrupted specific epitopes, rather than having global structural effects, and can therefore provide information which will help localize residues involved in the binding of specific MAbs. A good example is D1-∇277 (mutant 42), which bound all MAbs except DL6. Previous mapping showed that the continuous epitope for this MAb is within residues 272 to 279 (33).

(i) Mapping of MAb AP7. Previous attempts to group MAb AP7 by using blocking studies were unsuccessful (32, 59). Further experiments will be needed to complete the grouping of AP7, but we now consider it to be in a new group, XII, which may partially overlap group Ib. The data supporting this concept are as follows: (i) it is likely that the insertion at residue 27 (D2-∇27 [mutant 7]) specifically disrupted a portion of the epitope for MAb AP7 binding, since this insertion had a local rather than a global effect; (ii) Minson et al. (55) isolated and sequenced an AP7 neutralization-resistant mutant with a change at residue 25; (iii) the specific lack of binding of AP7 to insertion mutants between residues 235 and 246 implies that the AP7 site overlaps antigenic site Ib; (iv) AP7 did not bind to D1-Δ277~310, showing that residues downstream of amino acid 259 are important for binding of AP7 but not Ib MAbs; and (v) other mutations suggest that a portion of the AP7 epitope is located within amino acids 290 to 300, since the MAb did not react with D1-Δ277~300, D1-Δ290~300, or D1-Δ290~310. AP7 did not bind either gD-gIV-gD chimera, and both of these lacked gD amino acids 290 to 300. The results suggest that the region of gD downstream of residue 277 is more complex than previously thought. Furthermore, downstream regions may be physically closer to upstream positions of gD than depicted in the gD model (Fig. 11).

(ii) Implications of AP7 mapping for gD structure. Epitope mapping of MAb AP7 by using the mutants in region IV has led to the idea that region IV is physically close to the N terminus of the protein near functional region I, and these two regions are somehow associated with region III. It is tempting to speculate that the four functional regions of gD are folded into a single functional domain. Our future modeling efforts take this possibility into consideration. Because of the complexity of gD structure, a major goal is to obtain enough gD for crystallization trials.

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