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Identification of a Site on Herpes Simplex Virus Type 1 Glycoprotein D That Is Essential for Infectivity

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Herpes simplex virus glycoprotein D (gD) plays an essential role during penetration of the virus into cells. There is evidence that it recognizes a specific receptor after initial attachment of virions to cell surface heparan sulfate and also that gD-1, gD-2, and gp50 (the pseudorabies virus gD homolog) bind to the same receptor. Although the antigenic structure of gD has been studied intensively, little is known about functional regions of the protein. Antigenic site I is a major target for neutralizing antibodies and has been partially mapped by using deletion mutants and neutralization-resistant viruses. Working on the assumption that such a site may overlap with a functional region of gD, we showed previously that combining two or more amino acid substitutions within site I prevents gD-1 from functioning and is therefore lethal. We have now used a complementation assay to measure the functional activity of a panel of deletion mutants and compared the results with an antigenic analysis. Several mutations cause gross changes in protein folding and destroy functional activity, whereas deletions at the N and C termini have little or no effect on either. In contrast, deletion of residues 234 to 244 has only localized effects on antigenicity but completely abolishes functional activity. This region, which is part of antigenic site Ib, is therefore essential for gD-1 function. The complementation assay was also used to show that a gD-negative type 1 virus can be rescued by gD-2 and by two gD-1–gD-2 hybrids but not by gp50, providing some support for the existence of a common receptor for herpes simplex virus types 1 and 2 but not pseudorabies virus. Alternatively, gp50 may lack a signal for incorporation into herpes simplex virions.

Herpes simplex virus (HSV) has at least seven glycoproteins, of which gB, gD, and gH are essential for virus replication in tissue culture. All three are involved in penetration. Thus, viruses with temperature-sensitive (*ts*) mutations in gB (27, 40, 53) and mutants lacking gB entirely (4) can bind to cells but not initiate infection. In both cases, the defect is overcome by polyethylene glycol-induced fusion between virus and cell membranes. F-gDβ, a gD-negative mutant with a β-galactosidase gene replacing the gD gene, has the same properties (39), and a *ts* gH mutant grown at the nonpermissive temperature produces extracellular virions that lack gH and are noninfectious (15). Additional evidence for the participation of these proteins in penetration comes from studies with monoclonal antibodies (MAbs). Virus entry can be prevented by some anti-gB and anti-gD MAbs even when the virus has been preadsorbed to the cell surface (28, 29), and electron microscopy demonstrated that virions neutralized with anti-gD or anti-gH MAbs can attach to cells but not penetrate (23, 24). The role of each protein in penetration is unclear. Cell lines expressing large amounts of gD are resistant to infection (1, 7, 35), leading to speculation that gD in virions binds to a cell receptor after their initial attachment to cells and that this receptor is sequestered in gD-expressing cell lines. Infection can also be prevented by prior exposure of cells to UV-inactivated wild-type virus but not by exposure to gD-negative virus (34), supporting the concept of a receptor for gD.

The expression of viral proteins in cell lines has allowed the isolation of HSV mutants lacking the genes for essential proteins, such as gD (5, 6, 14, 34, 39, 43, 52, 55). Mutant virus stocks grown in these cells can penetrate normal cells but cannot then produce infectious virions. This has enabled the development of complementation assays, in which the missing protein is provided by prior transfection of cells with an expression vector containing the appropriate viral gene. If the gene encodes a functional protein, growth of infectious mutant virus will be complemented, and its titer can be determined by plaquing on the permissive cell line. If the gene encodes a mutant protein, the virus titer provides a quantitation of the effect of the mutation on protein function. The first HSV glycoprotein to be studied in this way was gB (5). We subsequently described a complementation assay for analysis of gD mutants and showed that gD function can be abolished by a combination of point mutations within antigenic I, even though each mutation is found singly in viable neutralization-resistant viruses (42). The assay works even though there is gD in the plasma membrane at the time the cells are infected; presumably, the amount is insufficient to prevent penetration by incoming virus.

This assay has now been used to study a number of gD-1 deletion mutants as well as gD-2, two hybrid proteins, and gp50, the gD homolog of pseudorabies virus (PRV). Several deletions in gD abolished its activity, and antigenic analysis showed that in some cases this was due to gross effects on protein folding. Other mutants were folded normally apart from localized changes, thus identifying a region that appears to be crucial for the functional activity of gD.

MATERIALS AND METHODS

Cells and virus strains. Vero cells were grown in Dulbecco modified minimal essential medium (DME) containing 5% fetal bovine serum (FBS). VD60 cells were grown in DME containing 5% FBS and 1 mM histidinol (39). Isolation of the gD-negative virus, F-gDβ, was described previously (39); F-gDβ was propagated in VD60 cells.

Antibodies. Anti-gD MAbs have been divided into several groups, each recognizing a distinct antigenic site (for a review, see M. I. Muggeridge, S. R. Roberts, V. J. Isola,

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G. H. Cohen, and R. J. Eisenberg, *in* M. V. H. Van Regenmortel and A. R. Neurath, ed., *Immunochemistry of Viruses II*, in press). Each antigenic site consists of a number of overlapping epitopes. MAbs HD1 and DL11 belong to groups Ia and Ib, respectively (12, 41, 45), and ABD is a human MAb that belongs to group III (54; V. J. Isola and C. Desgranges, unpublished data). II-436 is a type 1-specific MAb that belong to group VI (44; V. J. Isola, Ph.D. thesis, University of Pennsylvania, 1988). DL6 is a group II MAb that recognizes a continuous epitope of gD-1 (residues 272 to 279 of the mature protein) (20, 33). 1D3 is a group VII MAb that recognizes residues 1 to 23 (16, 22). MAb 3A-4 recognizes PRV gp50 (48).

Construction of PRV gp50 expression vector pWW117. Plasmid pN50, provided by Erik Petrovskis and Leonard Post, consists of pSV2neo with a 1.3-kilobase (kb) *Bam*HI-to-*Eco*RI insert containing the PRV gp50 gene (48). The *Bam*HI-to-*Eco*RI insert was excised, and the 5′ overhangs were filled in with Klenow fragment, followed by the addition of *Hin*dIII linkers and cloning into the *Hin*dIII site of pRSVnt EPA (8). The resulting plasmid, pWW117, expresses gp50 from the Rous sarcoma virus long terminal repeat promoter in the vector.

Construction of gD-2 expression vector (pWW65). A 2.0-kb *Spe*I-to-*Xho*I fragment containing the gD gene from HSV type 2 (HSV-2) strain 333 was isolated from plasmid pDG310, which contains the HSV-2 *Bgl*II L fragment (25). The 5′ overhangs were filled in with Klenow fragment, followed by the addition of *Hin*dIII linkers and cloning into the *Hin*dIII site of pRSVnt EPA (8). The resulting plasmid, pWW65, expresses gD-2 from the Rous sarcoma virus long terminal repeat promoter in the vector and is the type 2 equivalent of plasmid pRE4, which expresses gD-1 (Patton strain) and has been described previously (13).

Construction of mutants. (i) Plasmid pRE112 (amino acids 7 to 21 deleted, Δ7–21). pRE112 was derived from plasmid pSS117-1 (kindly provided by David Johnson), which contains an HSV-1 *Bam*HI J fragment lacking the coding sequences for amino acids 7 to 21 of the mature form of gD. A 978-base-pair *Afl*II-to-*Bst*XI fragment, which spans the deletion, was removed from pSS117-1 and substituted for the corresponding fragment in pRE4, generating pRE112.

(ii) Plasmid pWW126 (amino acids 28 to 63 deleted, Δ28–63). Plasmid pRE4 was digested with *Pvu*II and *Bss*HII. The *Bss*HII 5′ overhang was filled in with Klenow fragment, and the blunt ends were then ligated.

(iii) Plasmid pWW63 (truncation after amino acid 337, Δ338–369). The codon for amino acid 338 of gD-1 was changed to a stop codon by site-directed mutagenesis with the oligonucleotide 5′ CGGTGCATCTAGTACACAAT 3′.

(iv) Plasmids pWW78 and pWW79 (creation of *Sph*I restriction sites within the genes for gD-1 and gD-2, respectively). *Sph*I sites were introduced into the gD genes within pRE4 and pWW65 by using site-directed mutagenesis with mismatched oligonucleotides (37). The sites were within the first cysteine codons of the respective genes, at residue 66, and neither mutation altered the amino acid sequence.

(v) Plasmids pWW97 and pWW107 (gD hybrids). The gD-1 and gD-2 genes both contain a unique *Fsp*I site within the codon for residue 196. The pRSVnt EPA vector also contains one *Fsp*I site. Therefore, cleavage of pWW78 or pWW79 generated two fragments (Fig. 1A), one containing the N-terminal coding sequence for gD and the other containing the C-terminal sequence. The 2.6-kb fragment from pWW79 and the 1.7-kb fragment from pWW78 were isolated and ligated. In the orientation shown (plasmid pWW97), a complete gD gene was reformed, encoding residues 1 to 196 of gD-2 and 197 to 369 of gD-1 (there are no amino acid differences between gD-1 and gD-2 between residues 188 and 199). A second hybrid, encoding residues 1 to 65 and 197 to 369 of gD-1 and 66 to 196 of gD-2, was then constructed (Fig. 1B). First, pWW78 and pWW97 were both digested with *Sph*I. The 0.6-kb fragment of pWW97, including the N-terminal coding sequence for gD-2, was discarded, and the corresponding fragment from pWW78 was ligated to the 3.7-kb fragment of pWW97 to form pWW107.

DNA sequencing and oligonucleotide-directed mutagenesis. Double-stranded plasmid DNA was sequenced by the procedure of Chen and Seeburg (9) by using a set of oligonucleotide primers spaced at intervals along the gD gene. Mutagenesis of the gD gene cloned into the *Hin*dIII site of M13mp18 was performed by the method of Zoller and Smith (64), as modified by Kunkel et al. (38). *Hin*dIII fragments containing mutated gD genes were excised from replicative-form phage DNA and inserted into pRSVnt EPA, a eucaryotic expression vector (8).

Antigenic analysis of mutants. Transfection of Vero cells for the preparation of cytoplasmic extracts was performed by the same procedure as for the complementation assay. The extracts were prepared as previously described (41) and analyzed on native and denaturing gels, followed by Western immunoblotting (12).

Immunoperoxidase assay. This procedure, which was performed as previously described (42), is a modification of that of Holland et al. (31) and Kousoulas et al. (36). Surface staining of transfected cells was studied with unfixed cells; for detection of intracellular antigens, the cells were fixed with 5% methanol in phosphate-buffered saline before incubation with MAbs and protein A-horseradish peroxidase.

Complementation assay. The complementation assay was performed essentially as previously described (42). Briefly, petri dishes were seeded with 8×10^5 Vero cells and transfected with DNA-calcium phosphate precipitates the next day. The cells were incubated with the precipitates for 5 h at 37°C, subjected to a glycerol shock, washed once, and incubated in DME–5% FBS for 16 h at 37°C. Each dish of cells was subsequently infected at room temperature with 10^6 PFU of F-gDβ virus in tricine-buffered saline (5), followed by the addition of 5 ml of DME–5% FBS and incubation for 1 h at 37°C. The medium was then removed, and extracellular virus was inactivated by incubating the monolayer for 1 min in glycine-saline (pH 3.0) (5), followed by two washes with tricine-buffered saline. After 24 h in DME–5% FBS at 37°C, the cells were lysed by freeze-thawing and sonication with a Microson cell disruptor; nuclei were pelleted by low-speed centrifugation, and the supernatant was stored at −70°C for subsequent determination of virus titers on VD60 cells. We have found that the inclusion of carrier DNA (such as the vector with no gD insert, or salmon sperm DNA) in the negative control transfection results in greater consistency in the background number of plaques, and this has been adopted for the calculation of complementation. One hundred percent complementation is defined as the titer obtained after transfection with plasmid pRE4, which expresses wild-type gD. Complementation with a mutant is then defined by the formula: % complementation = 100 × [(titer with mutant plasmid − titer with carrier DNA)/(titer with pRE4 − titer with carrier DNA)].

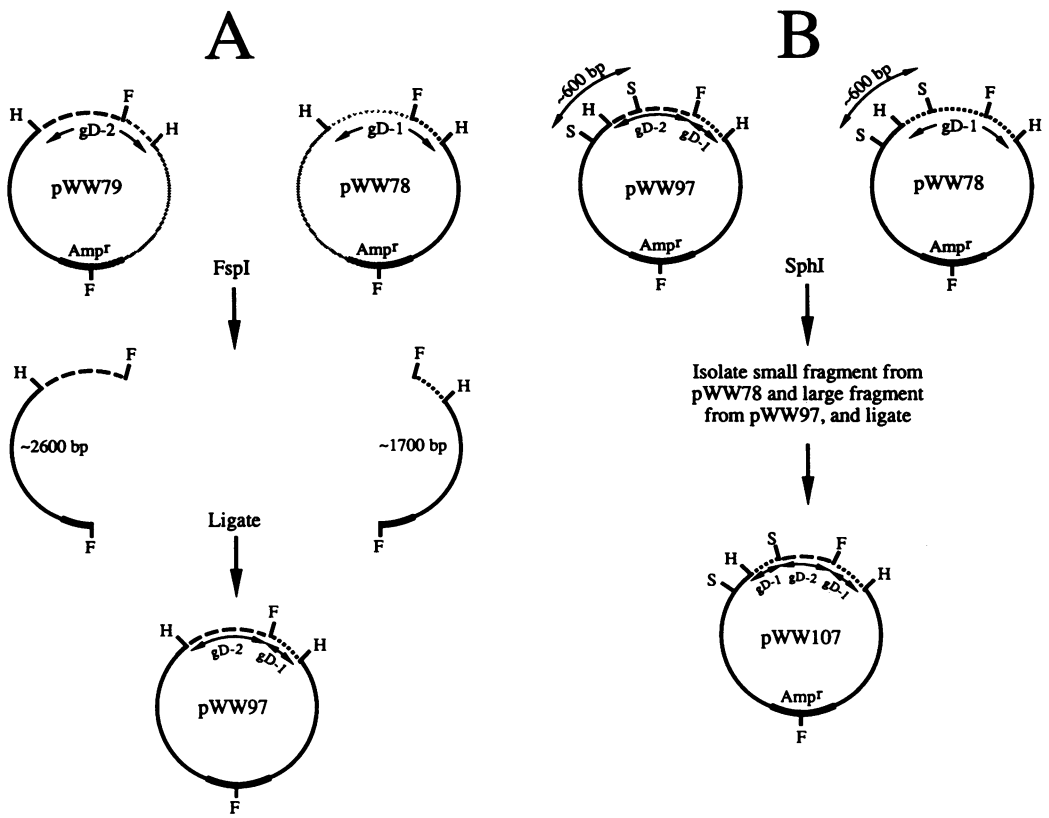

FIG. 1. Construction of plasmids expressing gD hybrids. (A) pWW78 and pWW79 contain the genes for gD-1 and gD-2, respectively, each with an *Sph*I site introduced by site-directed mutagenesis. By digesting these plasmids with *Fsp*I and ligating the indicated fragments, we constructed pWW97, which encodes a gD-2/1 hybrid. (B) A 600-base-pair *Sph*I fragment of pWW97 was then excised and replaced with the corresponding fragment from pWW78 to produce pWW107, which encodes a gD-1/2/1 hybrid. F, *Fsp*I; H, *Hin*dIII; S, *Sph*I.

RESULTS

gD-1 deletion mutants. (i) Complementation assay. To determine whether the mutants retained functional activity, they were tested for the ability to complement the production of infectious virus by the gD-negative mutant F-gDβ (34, 39). When grown in VD60 cells, which synthesize wild-type gD-1 from an integrated copy of the gene, F-gDβ virions contain gD and are infectious. Virions produced in Vero cells lack gD and are noninfectious. We have previously shown that prior transfection of Vero cells with plasmids expressing wild-type gD-1 complements the F-gDβ defect, enabling infectious virus to be produced (42). The virus titer after transfection with each gD mutant (which was determined in three independent experiments) is expressed as a percentage of the titer obtained after transfection with wild-type gD-1 (plasmid pRE4) in the same three experiments. The results for the deletion mutants are shown in Table 1. Note that the number of cells expressing wild-type or mutant gD after transfection was in all cases about 10%, as measured by phase-contrast microscopy after immunoperoxidase staining; this uniformity was achieved by varying the quantities of plasmid DNA used in the transfections. Initially, we examined mutants that had previously been used to map antigenic sites on gD-1 (13), and we found that deletion of residues −1 to 82, 196 to 207, 234 to 244, or 243 to 286 abolished the functional activity of gD-1 in the complementation assay. Subsequently, mutants with smaller deletions near the N terminus were analyzed, and the results indicated that residues 7 to 21 are completely dispensable for activity, whereas residues 28 to 63 are required. Deletion of residues 338 to 369, which results in truncation of gD-1 at the predicted junction between the transmembrane region and cytoplasmic tail, reduced complementation about twofold; since we have previously shown that 30% complementation is sufficient to sustain virus viability (42), presumably this

TABLE 1. Properties of gD-1 deletion mutants

Plasmid	Deletion[a]	PFU[b]		Complementation (%)	Cell surface expression[c]
		Mutant	pRE4		
pRE4	None	198,350[d]	198,350[d]	100	Yes
pWW11	Δ−1–82	2,840	172,200	2	No
pRE112	Δ7–21	582,000	382,000	152	Yes
pWW126	Δ28–63	130	110,280	0	No
pWW13	Δ196–207	50	136,570	0	No
pWW17	Δ234–244	100	90,150	0	Yes
pDL24	Δ243–286	900	316,130	0	No
pWW63	Δ338–369	91,120	177,790	51	Yes

[a] Numbering of residues excludes the 25-amino-acid signal peptide.

[b] The value for each mutant is an average from three independent experiments, together with the average obtained for pRE4 in the same three experiments. These are the values after subtraction of the background (generally about 2,000 PFU).

[c] Determined by immunoperoxidase staining of unfixed cells. MAb DL6 was used for all the mutants except pDL24, for which MAb 1D3 was used instead.

[d] Average from 11 independent experiments.

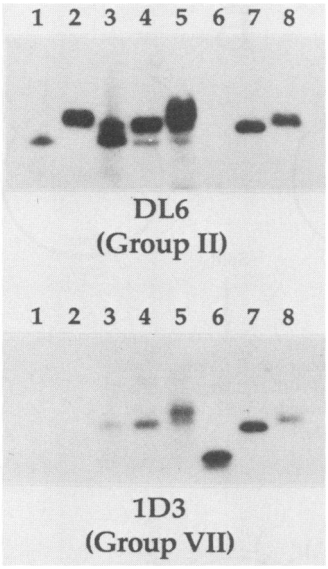

FIG. 2. Reaction of MAbs with gD deletion mutants electrophoresed under denaturing conditions. Cytoplasmic extracts of transfected Vero cells were electrophoresed on denaturing polyacrylamide gels, Western blotted, and then probed with MAb DL6 or 1D3 followed by ^{125}I-labeled protein A. Lanes: 1, pWW11, Δ−1–82; 2, pRE112, Δ7–21; 3, pWW126, Δ28–63; 4, pWW13, Δ196–207; 5, pWW17, Δ234–244; 6, pDL24, Δ243–286; 7, pWW63, Δ338–369; 8, pRE4, wild-type (Patton) gD-1.

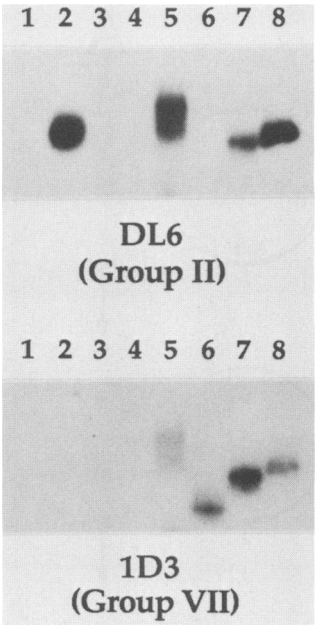

FIG. 3. Reaction of MAbs with gD deletion mutants electrophoresed under nondenaturing conditions. Cytoplasmic extracts of transfected Vero cells were electrophoresed on native polyacrylamide gels, Western blotted, and then probed with MAb DL6 or 1D3 followed by ^{125}I-labeled protein A. Lanes: 1, pWW11, Δ−1–82; 2, pRE112, Δ7–21; 3, pWW126, Δ28–63; 4, pWW13, Δ196–207; 5, pWW17, Δ234–244; 6, pDL24, Δ243–286; 7, pWW63, Δ338–369; 8, pRE4, wild-type (Patton) gD-1.

would not be a lethal mutation. The cytoplasmic tail of gD is therefore dispensable.

(ii) Antigenic analysis. An antigenic analysis of some of the mutants used in the present study, using extracts of transfected COS cells, has been published previously (13, 41). Because of the use of Vero cells for the complementation assay, those mutants have now been reexamined in Vero extracts, together with the new mutants described here. Cells were transfected with plasmid DNA, and cytoplasmic extracts were prepared 24 h later. To quantitate relative gD expression, equal volumes of each extract were electrophoresed on denaturing polyacrylamide gels, followed by transfer to nitrocellulose and probing with MAbs DL6 and 1D3, which recognize continuous epitopes (data not shown). Since some mutants were expressed at higher levels than others, the volumes loaded on subsequent gels were adjusted to give approximately equal signals with these MAbs (Fig. 2). MAb DL6 recognizes residues 272 to 279 and so bound all of the mutants except Δ243–286 (lane 6). MAb 1D3 recognizes an epitope within residues 1 to 23, so it bound all of the mutants except Δ−1–82 and Δ7–21 (lanes 1 and 2). The fastest-migrating bands obtained with Δ28–63, Δ196–207, and Δ234–244 (lanes 3, 4, and 5, respectively) reacted with DL6 but not with 1D3 and so possibly represent proteolytic breakdown products missing the N terminus rather than precursors. The mobilities of Δ−1–82, Δ196–207, Δ234–244, and Δ243–286 were as previously found for the same proteins made in COS cells (13); note that the reduced mobility of Δ234–244 (lanes 5) relative to wild-type gD-1 (lanes 8) is due to increased O-linked glycosylation (13). Of the remaining mutants, Δ7–21 (lane 2) migrated at the same rate as the wild-type, and Δ28–63 and Δ338–369 (lanes 3 and 7, respectively) ran slightly faster.

These quantities of each extract were then electrophoresed on native polyacrylamide gels and probed with DL6 and 1D3 (Fig. 3). In agreement with our previous results with COS cell extracts, Δ−1–82 (lanes 1) or Δ196–207 (lanes 4) did not enter the separating gel; this was also found with Δ28–63 (lanes 3) and is presumably due to gross effects on protein folding. These three deletions would therefore be expected to prevent the correct functioning of gD, as was indeed the case (Table 1). In contrast, the other four mutants were able to enter the separating gel and showed the expected reactions with DL6 and 1D3. Thus, Δ7–21 (lanes 2) reacted with DL6 but not 1D3, Δ234–244 (lanes 5) reacted with both, Δ243–286 (lanes 6) reacted with 1D3 but not DL6, and Δ338–369 (lanes 7) reacted with both. Subsequently, folding of these four mutants was examined by probing native Western blots with three MAbs that recognize nonoverlapping discontinuous epitopes (Fig. 4). Since these antibodies do not bind denatured gD, their recognition of mutant forms of gD on native gels indicates a degree of correct folding (12, 19, 20, 41; Muggeridge et al., in press). None of the deletions affected the binding of MAb ABD, which binds to discontinuous antigenic site III, and deletion of residues 7 to 21 (lanes 1) also had no effect on MAb HD1 (site Ia) or DL11 (site Ib). Deletion of residues 338 to 369, which constitute the cytoplasmic tail, surprisingly reduced the binding of HD1 and DL11, whose epitopes are in the external domain of gD (lanes 4), suggesting that deletions outside site I can affect its stability in Vero cell extracts. This may explain why the binding of HD1 to Δ234–244 and Δ243–286 (lanes 2 and 3, respectively) was reduced compared with binding to wild-type gD (lane 5), in contrast to previous results obtained with transfected COS cells (41). Binding of DL11 was essentially abolished by the deletion of residues 234 to 244 and reduced by the deletion of residues

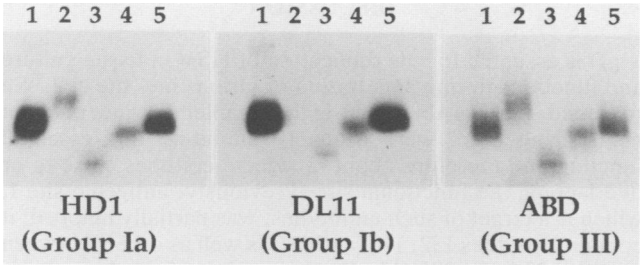

FIG. 4. Retention of discontinuous epitopes in deletion mutants. Cytoplasmic extracts of transfected Vero cells were electrophoresed on native polyacrylamide gels, Western blotted, and then probed with MAb HD1, DL11, or ABD followed by ^{125}I-labeled protein A. Lanes: 1, pRE112, Δ7–21; 2, pWW17, Δ234–244; 3, pDL24, Δ243–286; 4, pWW63, Δ338–369; 5, pRE4, wild-type (Patton) gD-1.

TABLE 2. Properties of gD hybrids and of the PRV homolog gp50

Plasmid	Protein	PFU[a]		Complementation (%)	Cell surface expression[b]
		Mutant	pRE4		
pRE4	gD-1	99,520[c]	99,520[c]	100	Yes
pWW65	gD-2	69,270	136,600	51	Yes
pWW97	gD-2/1	71,900	87,900	82	Yes
pWW107	gD-1/2/1	91,900	87,900	105	Yes
pWW117	PRV gp50	270	102,600	0	Yes

[a] Each value is an average from three independent experiments, together with the average obtained for pRE4 in the same three experiments. These are the values after subtraction of the background (generally about 2,000 PFU).

[b] Cell surface expression, determined by immunoperoxidase staining of unfixed cells, was positive for all plasmids. MAb DL6 was used for all the gD-expressing plasmids, and MAb 3A-4 was used for pWW117.

[c] Average from six independent experiments.

243 to 286 (even compared with Δ338–369), as found previously (13, 41).

These results show that deletion of residues 234 to 244 or 243 to 286 does not prevent gD from folding and therefore support the conclusion that residues between 234 and 286 are important for the correct functioning of gD. However, the Δ234–244 and Δ243–286 proteins were present at three- to fourfold lower levels than wild-type gD in cytoplasmic extracts, when the number of gD-expressing cells was similar (by immunoperoxidase staining). This suggested the possibility that failure to complement could be due to insufficient gD per cell. To address this possibility, cells were transfected with larger amounts of these mutant DNAs (50 μg instead of the usual 10 to 20 μg). Immunoperoxidase staining showed that this did not increase the number of expressing cells, which had presumably reached a plateau at the lower DNA concentration, but it did raise the quantity of the mutant proteins in cytoplasmic extracts to a level similar to that found for wild-type gD (data not shown). However, Vero cells transfected with 50 μg of mutant DNA were still unable to complement the growth of infectious F-gDβ (data not shown).

(iii) Cell surface expression. As a preliminary part of the complementation assay, immunoperoxidase staining was used to determine the number of cells expressing each mutant; by adjusting the quantity of plasmid DNA, expression was equalized at about 10% of the transfected cells. We did not observe a gradation in the intensity of staining, as might have been expected from the variable expression detected by Western blotting; presumably, this reflects the sensitivity of the immunoperoxidase assay. However, some mutants gave positive staining with unfixed cells, indicative of transport to the cell surface, whereas other mutants were accessible to MAbs only after cell fixation and permeabilization with 5% methanol (Table 1). Deletions that had global effects on protein folding (Δ−1–82, Δ28–63, and Δ196–207) prevented transport to the cell surface. With the exception of Δ243–286, mutants that retained discontinuous epitopes (Δ7–21, Δ234–244, and Δ338–369) were transported. The deletion of residues 243 to 286, which prevents transport, is less disruptive for discontinuous epitopes than is the deletion of residues 234 to 244 (Fig. 4), which does not prevent transport. This suggests that the structure of Δ243–286 is altered in some way that is not detected by MAb binding.

gD-2 and gD hybrids. With the above results we have located a site (amino acids 234 to 244) that is important for the correct functioning of gD-1, but these results provide no information about what that function might be. Recent data suggest that gD interacts with a cell receptor after initial attachment of virions to the cell (7, 34, 35). Moreover, the evidence indicates that gD-1 and gD-2 interact with the same receptor, whereas previous reports have suggested that HSV-1 and HSV-2 may use different cell receptors (57, 58). To address the question of whether gD-2 can function in an exclusively type 1 background, we tested its ability to complement the growth of F-gDβ, an HSV-1 mutant. The gD gene from HSV-2 (strain 333) was subcloned from plasmid pDG310 (25) into the pRSVnt EPA expression vector to generate pWW65. Transfection of pWW65 into Vero cells resulted in expression of gD-2 at the cell surface, as detected by immunoperoxidase staining with the type-common MAb DL6, and also in complementation of F-gDβ (albeit at a lower level than with gD-1) (Table 2). Therefore, if gD-1 and gD-2 do bind to different receptors, the choice of receptor does not affect subsequent steps in penetration. In addition, we examined two gD hybrids for complementation. The objective in constructing these hybrids was to replace the central cysteine-containing section of gD-1 with the corresponding section of gD-2 (the first cysteine is at position 66 and the sixth is at position 202, for both gD-1 and gD-2). Construction of the hybrids is described in Materials and Methods and shown in Fig. 1. pWW97 encodes a protein with amino acids 1 to 196 of gD-2 and 197 to 369 of gD-1 (gD-2/1); pWW107 encodes a protein with amino acids 1 to 65 and 197 to 369 of gD-1 and 66 to 196 of gD-2 (gD-1/2/1). Since residues 197 to 203 of gD-1 and gD-2 are identical except for a Ser-to-Ala change at residue 200, the gD-1/2/1 hybrid encoded by pWW107 essentially fits our objective. Both hybrid proteins were expressed at the surface of transfected Vero cells and were able to complement the growth of F-gDβ (Table 2).

Given this result, it was expected that both proteins would be correctly folded. To test this, cytoplasmic extracts of transfected Vero cells were electrophoresed on native gels, Western blotted, and probed with MAbs followed by ^{125}I-labeled protein A (Fig. 5). Probing with DL6 showed that each extract contained a similar amount of gD. The apparent increase in mobility of gD-1 (lane 4) compared with that of the hybrids (lanes 2 and 3) was due to incomplete processing of N-linked sugars from the high-mannose form to the complex form. The greater mobility of gD-2 has been observed previously with protein made in infected cells (21, 51). To study antigenicity and folding, the hybrids were probed with representative MAbs chosen from groups Ia, Ib, and III, all of which recognize type-common discontinuous epitopes, and group VI, which recognizes a type 1-specific

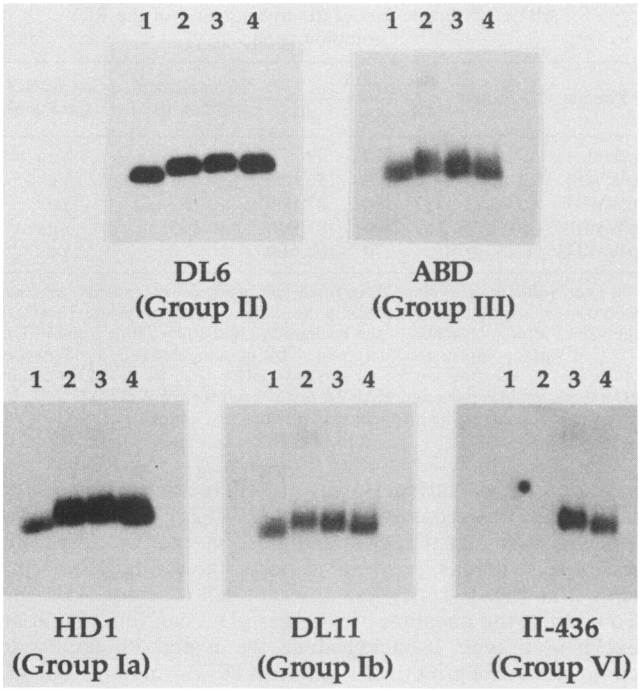

FIG. 5. Reaction of MAbs with gD hybrids. Cytoplasmic extracts of transfected Vero cells were electrophoresed on native polyacrylamide gels, Western blotted, and then probed with MAb DL6, ABD, HD1, DL11, or II-436 followed by ^{125}I-labeled protein A. Lanes: 1, pWW65, gD-2; 2, pWW97, gD-2/1 hybrid; 3, pWW107, gD-1/2/1 hybrid; 4, pRE4, gD-1.

discontinuous epitope (12, 19, 20, 41). As mentioned above, these epitopes do not overlap, and because they are conformation dependent their presence is indicative of correct folding (Muggeridge et al., in press). The three type-common MAbs (ABD, HD1, and DL11) bound to gD-1 (lane 4), gD-2 (lane 1), and both hybrids (lanes 2 and 3), indicating that the latter are correctly folded. HD1 binding to gD-2 (lane 1) was weaker than binding to gD-1 and the hybrids, suggesting that its epitope contains type-specific residues between 197 and 369. The type 1-specific MAb (II-436) bound to gD-1 (lane 4) and the 1/2/1 hybrid (lane 3) but not to gD-2 (lane 1) or the 2/1 hybrid (lane 2), and the relevance of this result to epitope mapping is discussed below.

PRV glycoprotein gp50. The receptor used by gD-1, and possibly by gD-2, is also implicated in the penetration of cells by PRV virions (47). Cell lines expressing gp50, the PRV homolog of gD, produce lower yields of PRV than do comparable nonexpressing cells (47), a situation analogous to that found with HSV and cells expressing large amounts of gD (1, 7, 35). Expression of gp50 also reduces the yield of HSV-1, suggesting a common pathway of virus entry (47). We therefore wondered whether gp50 would be able to substitute for gD in the production of infectious HSV-1 virions. The gp50 gene was subcloned from plasmid pN50 into pRSVnt EPA, as described in Materials and Methods, to give plasmid pWW117. This was transfected into Vero cells, and cell surface expression of gp50 was demonstrated by immunoperoxidase staining with MAb 3A-4. Similarly transfected cells were then tested for their ability to complement the growth of F-gDβ virus (Table 2). No infectious virus was produced, so gp50 did not substitute for gD in this assay. There are several possible explanations, and these are discussed below.

DISCUSSION

gD is essential for the replication of HSV in tissue culture and functions during penetration of virions into the cell. We reasoned that MAbs with high complement-independent neutralizing titers were probably inhibiting this essential function and therefore might recognize epitopes close to or overlapping the functional site. Previously, antigenic site I, which is a target of such antibodies, was partially mapped; it contains residues 132, 140, and 216 as well as others between residues 234 and 275 (13, 41). In support of our hypothesis that site I is close to a functional site, we found that when a gD gene containing point mutations in codons 132, 140, and 216 is recombined into the viral genome, only one mutation is retained in viable recombinants, suggesting that the combination is lethal (42). In addition, transient expression of a gD protein containing substitutions at residues 132, 140, and 216 was not able to complement the growth of a gD-negative virus in Vero cells (42). The wild-type protein and mutants with single amino acid substitutions at these positions did complement (42).

We have now used the complementation assay to examine gD deletion mutants for functional activity. The construction and antigenic analysis of several of these mutants have been reported previously (13, 63); of particular relevance to this study is that deletions involving any of the cysteine residues resulted in loss of all discontinuous epitopes, altered carbohydrate processing, and aggregation, indicating gross effects on protein folding. We therefore carried out an antigenic analysis in parallel with the complementation assays, using Vero cells for both. Some of the mutants that failed to complement growth of the gD-negative F-gDβ virus had grossly altered folding, which accounted for the lack of function.

To recap briefly, deletion of residues −1 to 82 (including a cysteine at 66) or 196 to 207 (including a cysteine at 202) causes aberrant folding in COS and Vero cells. This effect is not confined to deletions involving cysteines, as shown by the malfolding and lack of complementation after deletion of residues 28 to 63, which are upstream of the first cysteine. The other four deletion mutants we have examined (Δ7–21, Δ234–244, Δ243–286, and Δ338–369) all appear to fold similarly to wild-type gD in that they can be electrophoresed on a native gel and retain most, or all, of the discontinuous epitopes. Sites Ia and III are present in each mutant, whereas site Ib is present in Δ7–21 and Δ338–369 but missing or greatly reduced in Δ234–344 and Δ243–286. However, since all of the discontinuous epitopes map within the first 275 amino acids of gD (Muggeridge et al., in press), we cannot be certain whether the membrane-proximal part of the protein is folded correctly. This is especially true for Δ243–286, as discussed below.

The Δ338–369 mutant complements the growth of F-gDβ, a gD-negative virus, indicating that the cytoplasmic tail of gD is not essential for its incorporation into virions and for its function. In fact, Ligas et al. (M. W. Ligas, V. Feenstra, G. H. Cohen, R. J. Eisenberg, and D. C. Johnson, Abstr. 14th International Herpesvirus Workshop, 1989, p. 109) have isolated a viable recombinant virus in which residues 339 to 369 of gD have been deleted. Similarly, the cytoplasmic tail of HSV gC-1 is not required for cell surface expression or incorporation into virions (30). Huff et al. (32) showed that removal of the C-terminal 41 amino acids from the 109-amino-acid cytoplasmic tail of gB-1 had no effect on function, whereas a more recent analysis showed that removal of the entire cytoplasmic tail had no effect on trans-

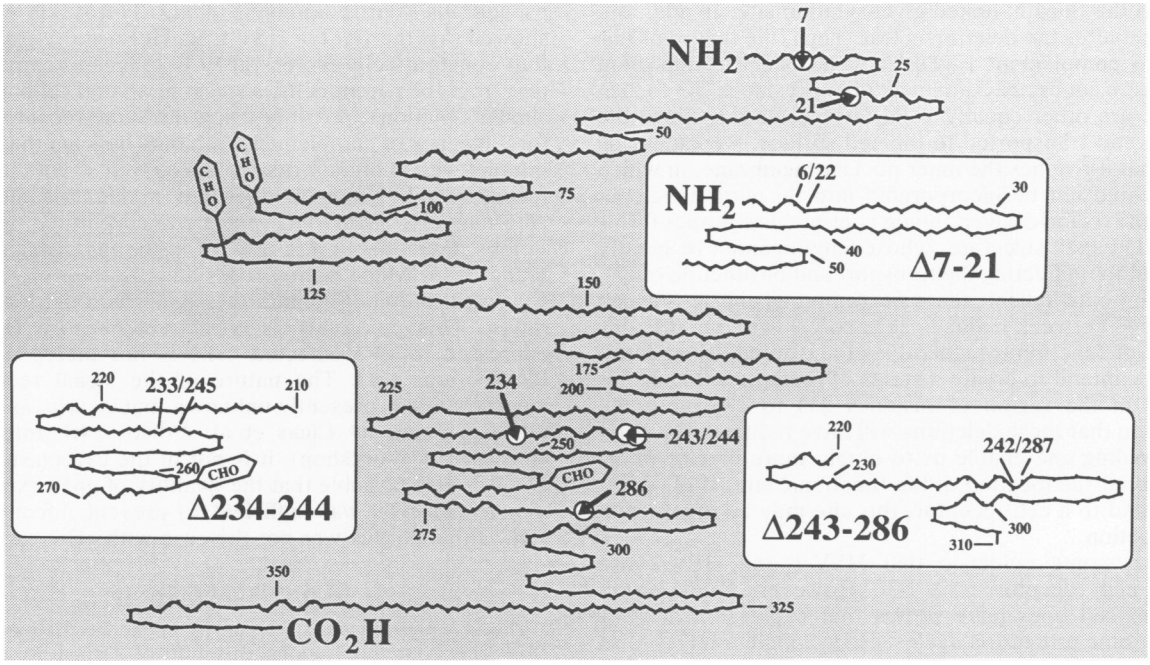

FIG. 6. Secondary structure predictions for wild-type gD-1 (shown in full) and for mutants with deletions in residues 7 to 21, 234 to 244, and 243 to 286 (partial structures shown in inserts). The predictions were produced by the ProtPlot program (50), available from Intelligenetics, based on the rules of Chou and Fasman (10, 11).

port to the nuclear membrane but abolished transport to the plasma membrane; removal of the C-terminal 66 residues reduced the rate of transport (49). However, in the latter study no data were reported concerning incorporation into virions or functional activity. Results with glycoproteins of other viruses seem to be equally varied. Thus, removal of the cytoplasmic tail of the Rous sarcoma virus envelope glycoprotein does not impair its transport to the cell surface, incorporation into virions, or viral infectivity (46). In contrast, cell surface expression of the vesicular stomatitis virus G protein is reduced 10- to 20-fold when the cytoplasmic tail is removed (26), and Doms et al. (17) speculated that it normally accelerates transport from the endoplasmic reticulum. Moreover, a mutant retaining 9 of the 29 amino acids in the cytoplasmic tail is incorporated into virions and can rescue a temperature-sensitive G protein mutant of vesicular stomatitis virus, whereas a mutant with only three residual residues cannot (62).

Deletion of residues 7 to 21 of gD-1 has no apparent effect on epitopes in discontinuous antigenic sites, nor does it affect complementation. In addition, Ligas et al. (Abstr. 14th International Herpesvirus Workshop) reported the successful recombination of this mutation into the viral genome. The effect of the deletion on the predicted secondary structure of gD-1 (using the rules of Chou and Fasman) is shown in Fig. 6, with the wild-type structure drawn in full and a partial structure for the mutant shown as an insert. Note that if the algorithm predicts the occurrence of α-helix or β-sheet on the N-terminal side of a deletion, that assignment cannot be overridden; therefore, most changes will occur on the C-terminal side. The deletion is predicted to remove two β-turns as well as to destroy the short α-helix after residue 21 and to prematurely terminate the next length of β-sheet. However, the only observed effect of the mutation is to destroy the continuous epitope of MAb 1D3, which lies within residues 1 to 23 (antigenic site VII) (16). Considering that residues 7 to 21 are dispensable for gD function, it is surprising that binding of MAbs to site VII results in neutralization of wild-type virus and that immunization with synthetic peptides representing residues 1 to 23 protects mice against virus challenge (3, 18). The N terminus of the molecule may be sufficiently close to a functional region that antibody binding causes neutralization by steric hindrance.

Deletion of residues 234 to 244 results in the loss of antigenic site Ib (13, 41) and also in the addition of extra O-linked carbohydrates (13). However, other antigenic sites are retained, and the protein is transported to the cell surface, indicating that the effect of the deletion on folding is relatively minor. The predicted structure for this part of the protein is partly β-sheet and partly α-helix (Fig. 6); the deletion removes the predicted helix and destroys a downstream segment of β-sheet. The failure of this mutant to complement the growth of F-gDβ strongly suggests that residues 234 to 244 form part of a functional site on gD, although an alternative explanation, that the deletion prevents incorporation of gD into the virus envelope, has not been ruled out. We will attempt to address this possibility in future experiments.

Deletion of residues 243 to 286 results in the complete loss of some epitopes in antigenic site Ib and the partial loss of others, but, since discontinuous sites Ia and III are retained (13, 41), this large deletion does not prevent folding of the upstream portion of gD. However, the protein is not transported to the cell surface, suggesting that it has structural abnormalities that are not detected by MAbs to discontinuous epitopes. Previous work has shown that these epitopes are all present on a fragment truncated at residue 275 (12), whereas there are a number of continuous epitopes located between residues 264 and 301. Most of the latter are removed by the deletion of residues 243 to 286. According to the secondary structure prediction (Fig. 6), the deletion removes four β-turns, a region of β-sheet, and two α-helices

as well as the third N-linked glycosylation site. In addition, the β-sheet after the deletion is lost. Thus, the failure of this mutant to complement F-gDβ may be because important functional residues are contained within residues 243 to 286, but there are other equally valid interpretations. Since the protein is not transported to the cell surface, we cannot be certain that it reaches the inner nuclear membrane, in which case it would not be incorporated into the viral envelope. Perhaps the region of overlapping continuous epitopes forms an extended stalk structure, whose length cannot be greatly altered without affecting the transport and/or function of gD. Preliminary data indicate that a five-amino-acid insertion can be tolerated between residues 287 and 288 without affecting transport or function (Muggeridge et al., unpublished data).

We now intend to create a series of mutants with smaller deletions in the region of residues 234 to 286, with the expectation that these deletions will have reduced effects on protein folding and enable us to obtain more precise information about the location of the functional site. If gD does, indeed, bind to a cell receptor, this site may be involved in the interaction.

There is some evidence that HSV-1 and HSV-2 use different cell receptors (57, 58). However, studies with permanent cell lines have shown that expression of gD-1 provides some protection against HSV-2 infection (7, 35) as well as against HSV-1. These results suggested not only that gD can interact with a receptor in uninfected cells, and thus prevent subsequent interaction between the receptor and virions that attach to the cell surface, but also that gD-1 and gD-2 bind to the same receptor. In addition, attachment of UV-inactivated virions containing gD-1 blocks subsequent infection with HSV-1 or HSV-2 (34). To address the question of whether gD-2 can substitute for gD-1 to bring about penetration of HSV-1 virions, we tested its ability to complement the growth of F-gDβ, a type 1 virus, in transiently transfected Vero cells. The fact that complementation was successful does not prove that gD-1 and gD-2 bind to the same receptor, but if different entry pathways are used, they must be interchangeable at this point, as suggested previously by results with HSV-1–HSV-2 recombinants (51). Furthermore, the observation that two gD-1–gD-2 hybrids were also able to complement F-gDβ provides some support for the same receptor being involved. Alternatively, if different receptors are used, the receptor-binding site of gD must consist of residues relatively close together in the linear sequence, so that the site is not disrupted in the hybrids. To study the folding of the hybrids, Western blots of native gels were probed with anti-gD MAbs recognizing several distinct conformational epitopes (Fig. 5). Three type-common MAbs (ABD, HD1, and DL11) bound to gD-1, gD-2, and both hybrids, indicating that the central cysteine-containing section of gD-1 can be replaced en bloc by the corresponding section of gD-2 without disrupting folding of the protein. HD1 binding to gD-2 was weaker than binding to gD-1 and the hybrids, suggesting that its epitope contains residues that differ between gD-1 and gD-2. These type-specific residues would lie between 197 and 369, where both hybrids have the type 1 sequence. Moreover, since residues downstream of 233 are unnecessary for HD1 binding (41), comparison of the gD-1 and gD-2 sequences limits the possible type-specific residues in the HD1 epitope to 200, 204, 205, 206, and 233 (59, 60). The type 1-specific MAb (II-436) bound to gD-1 and the 1/2/1 hybrid but not to gD-2 or the 2/1 hybrid. The II-436 epitope therefore includes one or more type-specific residues between 1 and 66 (residues 7, 21, 35, 42, 43, 45, and 53).

Growth of F-gDβ was not complemented by gp50, the pseudorabies virus homolog of gD. Petrovskis et al. (47) showed that the yield of HSV from HeLa and Vero cell lines that constitutively express gp50 is reduced compared with that from the parent cells, suggesting that gD and gp50 have similar functions, possibly using the same receptor. Therefore, the lack of complementation may indicate that gD must interact with another virus protein as well as with its cellular receptor and that gp50 does not mimic this interaction. Alternatively, gp50 may not be incorporated into HSV virions. Presumably it is present in the inner nuclear membrane, from which both viruses acquire their envelopes (2, 56), but it may not contain the signal required for incorporation. This possibility is attractive, because HSV gC-1 expressed in PRV-infected cells is not incorporated into PRV virions (61). The nature of the signal required for incorporation is presently unknown, but clearly, as shown in this paper and by Ligas et al. (Abstr. 14th International Herpesvirus Workshop), it is not in the cytoplasmic tail of gD. It is also possible that the quantity of gp50 expressed in transfected cells was sufficient to prevent infection by F-gDβ, although this was not the case with gD-1 expression.

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