JOURNAL OF VIROLOGY, Nov. 1990, p. 5542–5552
0022-538X/90/115542-11$02.00/0

Vol. 64, No. 11

Cysteine Mutants of Herpes Simplex Virus Type 1 Glycoprotein D Exhibit Temperature-Sensitive Properties in Structure and Function

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Received 11 June 1990/Accepted 28 July 1990

We previously constructed seven mutations in the gene for glycoprotein D (gD) of herpes simplex virus type 1 in which the codon for one of the cysteine residues was replaced by a serine codon. Each of the mutant genes was cloned into a eucaryotic expression vector, and the proteins were transiently expressed in mammalian cells. We found that alteration of any of the first six cysteine residues had profound effects on protein conformation and oligosaccharide processing. In this report, we show that five of the mutant proteins exhibit temperature-sensitive differences in such properties as aggregation, antigenic conformation, oligosaccharide processing, and transport to the cell surface. Using a complementation assay, we have now assessed the ability of the mutant proteins to function in virus infection. This assay tests the ability of the mutant proteins expressed from transfected plasmids to rescue production of infectious virions of a gD-minus virus, F-gDβ, in Vero cells. Two mutant proteins, Cys-2 (Cys-106 to Ser) and Cys-4 (Cys-127 to Ser), were able to complement F-gDβ at 31.5°C but not at 37°C. The rescued viruses, designated F-gDβ(Cys-2) and F-gDβ(Cys-4), were neutralized as efficiently as wild-type virus by anti-gD monoclonal antibodies, indicating that gD was present in the virion envelope in a functional form. Both F-gDβ(Cys-2) and F-gDβ(Cys-4) functioned normally in a penetration assay. However, the infectivity of these viruses was markedly reduced compared with that of the wild type when they were preincubated at temperatures above 37°C. The results suggest that mutations involving Cys-106 or Cys-127 in gD-1 confer a temperature-sensitive phenotype on herpes simplex virus. These and other properties of the cysteine-to-serine mutants allowed us to predict a disulfide bonding pattern for gD.

Glycoprotein D (gD) of herpes simplex virus (HSV) is a structural component of the virion envelope which stimulates high titers of neutralizing antibody (8, 10, 15–18, 20, 30, 36, 40, 47) and is essential for virus entry (24, 29) in tissue culture. In addition, studies using anti-gD monoclonal antibodies (MAbs) have implicated gD in adsorption (18), penetration (19, 20), and fusion of infected cells (5, 20, 40). Although the actual function(s) of the gD molecule remains unknown, recent studies suggest that gD binds to a receptor after the virion has attached to the cells (4, 23). In addition, the basic fibroblast growth factor receptor may bind HSV and serve as a portal of entry for the virus (25). The gD-1 polypeptide contains three utilized N-linked oligosaccharide sites (12, 50, 51), two or three O-linked oligosaccharides (6), and six cysteine residues (50, 51) in its extracellular domain. A seventh cysteine residue is located in the hydrophobic membrane-anchoring domain near the carboxy terminus of the protein. The first six cysteines are strictly conserved in both number and spacing in gD-2, but the seventh cysteine is absent (28, 50). A similar pattern of cysteine spacing is also found in two gD homologs, pseudorabies virus gp50 (42) and bovine herpesvirus gIV (G. Kiel, personal communication). Conservation of cysteines implies that these residues probably play an important structural role, possibly by formation of disulfide bonds. Disulfide bonds can stabilize the native structures of proteins (26, 33, 41, 44, 46, 52) and may be important for glycosylation and cellular transport of membrane proteins (43, 56). Conformational epitopes relying on disulfide bond formation have also been described for glycoproteins of a variety of viruses (9, 35, 49, 55, 56).

We previously showed that replacement of the cysteine of gD-1 at residue 333 by serine (Cys-7) had a minimal effect on carbohydrate processing or the reactivity of MAbs that recognize discontinuous epitopes (54). In contrast, the replacement of any one of the other six cysteine residues (Cys-1 through Cys-6) resulted in either a major reduction or complete loss of antibody binding. In addition, mutations of any of these six cysteines had profound effects on oligosaccharide processing of gD. We found there was little or no accumulation of the mature form of gD, which contains complex oligosaccharides (11, 14, 34). This result implied that transport of the mutants to the Golgi apparatus was impaired. We postulated that the first six cysteine residues form three disulfide bonds which are required for the proper folding of gD and that impaired processing was due to misfolding of the protein. During these studies, we noted minor variations in the amount of antigenic activity and processing from experiment to experiment. This led us to question whether any of the mutants were temperature sensitive (*ts*). Therefore, we reexamined each of the gD-1 cysteine mutants by carrying out the transfections at either 31.5 or 39.5°C. We found that mutants Cys-1, Cys-2, Cys-4, Cys-5, and Cys-6 were *ts* for one or more of the following properties: conformation, formation of aggregates, processing, and transport to the cell surface. When we analyzed each of the mutants in a complementation assay for virus function (38), only two mutant proteins, Cys-2 and Cys-4,

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could complement the gD-minus virus, F-gDβ (24), and the complementation was *ts*. Furthermore, both F-gDβ(Cys-2) and F-gDβ(Cys-4) functioned normally in a virus penetration assay (20, 21) carried out at 37°C (the temperature used for nonpermissive infection). However, they were more susceptible to thermal inactivation than was F-gDβ(WT). On the basis of the properties of the cysteine mutants, a model for the intracellular disulfide bond pattern of gD is presented.

MATERIALS AND METHODS

Cell culture and virus. Conditions for the growth and maintenance of COS-1 cells were previously described (13). Vero cells were grown in Dulbecco modified Eagle medium containing 5% fetal bovine serum. VD60 cells (29) were grown in the same medium supplemented with 1 mM histidinol. F-gDβ virus (29) was propagated and titered in VD60 cells without histidinol.

Construction of gD cysteine mutants. The construction of plasmids containing gD-1 cysteine mutations was previously described (54). pDL100 and pDL101 were derived from pWW31 and pWW38, respectively. The latter plasmids were discovered to have a C-to-A mutation in the codon for residue 54 of gD, changing the glutamine to a proline. To correct these unwanted mutations, a 203-bp *Sac*II fragment (containing the C-to-A mutation) was excised from plasmids pWW31 and pWW38 and replaced with a *Sac*II fragment isolated from the wild-type gD expression vector pRE4 (13). Plasmid DNA was prepared and sequenced (7). Plasmid DNA used in transfections was subjected to two CsCl gradient centrifugation steps.

Expression of gD cysteine mutants in COS-1 cells. COS-1 cells were transfected as previously described (13), with modifications. The DNA was added, and the cells were incubated for 16 h at 37°C, washed, and overlaid with fresh medium. The cells were then incubated at either 31.5 or 39.5°C for an additional 32 h. Cytoplasmic extracts were prepared as described previously (13, 48).

Polyclonal antibodies and MAbs. Rabbit anti-gD serum (15) was used for Western immunoblotting and for indirect immunofluorescence experiments. MAbs DL11 (group Ib) (37), DL2 (group VI), and DL6 (group II) (17, 22; M. I. Muggeridge, S. R. Roberts, V. J. Isola, G. H. Cohen, and R. J. Eisenberg, *in* M. V. H. Van Regenmortel and A. R. Neurath, ed., *Immunochemistry of Viruses* II, in press) were isolated in our laboratory. HD1 (group Ia) (37) was kindly provided by Lenore Pereira; ABD, RIP, and VID (group III) (V. J. Isola, unpublished data) were provided by Claude Des-Granges; and LP2 (group Ia) was provided by Anthony Minson (36).

Western blot analysis and endo H digestion. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins under denaturing and nonreducing native conditions was performed in 10% acrylamide gels (9). Proteins were transferred to nitrocellulose (2) and then probed with antibody and with ^{125}I-protein A (ICN) as described previously (8). In some cases, the protein was digested with endo-β-*N*-acetylglucosaminidase H (endo H; Boehringer Mannheim) before electrophoresis (54).

Immunoblot assay. Cytoplasmic extracts were applied to nitrocellulose by using a slot blot apparatus (Hoefer) and then incubated with a gD-specific MAb. The nitrocellulose was washed with a blocking solution, incubated with ^{125}I-protein A, washed again, and exposed to X-ray film. To quantitate MAb binding, individual slots were cut out and counted in a gamma counter. The reaction of each extract with MAb DL6 was used to normalize the results obtained with the other MAbs (37, 48). Background was determined by applying equal amounts of an extract from mock-transfected cells and reacting it with the appropriate MAb.

Cell surface immunofluorescence. COS-1 cells seeded onto glass cover slips were transfected with purified plasmid DNA, incubated at 37°C for 16 h, and then shifted to 31.5 or 39.5°C for another 32 h before assay. The immunofluorescence protocol was carried out as previously described (31, 48). Briefly, cover slips were washed in phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde, and reacted with anti-gD serum, followed by a goat anti-rabbit–tetramethylrhodamine isothiocyanate conjugate. The fixed cells were permeabilized with 0.8% Nonidet P-40, incubated with anti-gD, and labeled with a goat anti-rabbit–fluorescein isothiocyanate conjugate. The cells were viewed with a Leitz epifluorescence microscope.

Complementation assay. gD-1 cysteine mutants were tested for their ability to rescue the infectivity of F-gDβ virus in Vero cells incubated at 31.5 or 37°C. The latter temperature was chosen as the nonpermissive temperature because F-gDβ and its parent virus (strain F) do not replicate at 39.5°C because of a mutation in the gene for ICP4 (1). The complementation assay was performed as described previously (38) when carried out at 37°C except that the cells were lysed by sonication rather than by Dounce homogenization. For assays carried out at 31.5°C, intracellular virus was harvested at 36 to 48 h postinfection.

Neutralization of rescued virus. Virus collected from the complementation assay was incubated for 1 h at 31.5°C with various dilutions of an anti-gD MAb, DL11 (group 1b). The virus was then titered on VD60 cells at 31.5°C. The results are expressed as the antibody dilution that gave a 50% reduction in plaques compared with the value for a control sample of virus that was incubated without antibody.

Penetration assay. The procedure is derived from that used by Highlander et al. (20). Confluent VD60 cells in 22-mm-diameter tissue culture dishes were infected with 100 to 200 PFU of virus prepared from the complementation assay. The plates were incubated at 4°C for 2 h, washed twice with PBS, overlaid with medium, and shifted to 31.5 or 37°C. At various times after the temperature shift, the infected cells were treated with acid-glycine (pH 3.0) (3) for 1 min, washed twice with PBS, and overlaid with medium. After 24 h at 37°C, plaques, representing intracellular virus, were counted, and the percentage of PFU surviving acid treatment was calculated; 100% is the number of plaques formed on cells not treated with acid-glycine. Penetration at each time point is then defined by the following formula: percent survivors = 100 × number of PFU (acid treated)/number of PFU (PBS control).

Measurement of heat sensitivity of rescued virus. Virus preparations were diluted in Dulbecco modified Eagle medium containing 5% fetal bovine serum and incubated for 1 h at various temperatures, and the residual infectivity was titered at 31.5°C on VD60 cells. To determine the rate of virus inactivation, virus samples were incubated at 37 or 45°C and samples were taken at various times, placed on ice, and titered on VD60 cells at 31.5°C.

RESULTS

The seven mutated forms of gD, designated Cys-1 through Cys-7, and the plasmids containing them are listed in Table 1. Dideoxynucleotide sequencing confirmed the cysteine-to-serine change, but upon additional sequencing we found that

TABLE 1. Properties of gD-1 cysteine mutants expressed in COS-1 cells at 37°C[a]

Plasmid	Residue mutated to Ser	Protein	Aggregates	EndoH resistance	Discontinuous epitopes
pRE4	None	Wild type	−	+	+
pWW53	Cys-66	Cys-1	+	+[b]	±[c]
pWW47	Cys-106	Cys-2	+	−	±
pDL101[d]	Cys-118	Cys-3	+	−	−
pWW48	Cys-127	Cys-4	+	+[b]	±
pDL100[d]	Cys-189	Cys-5	+	−	±
pWW54	Cys-202	Cys-6	+	−	−
pWW50	Cys-333	Cys-7	−	+	+
pRSVntEPA	Vector	None			

[a] Summarized from information in Wilcox et al. (54) as well as new information regarding pDL101 and pDL100.
[b] 5 to 10% of that seen with wild-type gD.
[c] ±, Weak or variable reaction.
[d] Altered as described in Materials and Methods to contain the cysteine-to-serine change and to remove the extraneous mutation at amino acid 54.

two mutants, pWW31 (Cys-5) and pWW38 (Cys-3), contained an additional change at amino acid 54 (glutamine to proline) of the mature protein. This mutation was corrected as described in Materials and Methods, and all of the mutants were reexamined for effects on antigenic structure and processing at 37°C (Table 1). The properties of the Cys-3 and Cys-5 proteins were essentially the same as those reported previously (54).

Properties of the mutant proteins expressed at 31.5 and 39.5°C in COS-1 cells. (i) Aggregation as detected by Western blot analysis under nonreducing conditions. Previously, when cells were transfected and incubated at 37°C, we used native gels to demonstrate that Cys-1 through Cys-6 proteins aggregate (Table 1) (54). These aggregates were not present when the proteins were electrophoresed under reducing conditions. We speculated that aggregation was due to aberrant intermolecular disulfide bonding of the mutant proteins within cysteine residues 1 to 6. A similar phenomenon has been reported for other proteins (32, 39, 45). Here we asked whether aggregation was affected by the temperature at which transfection and protein synthesis occurred. COS-1 cells were transfected at either 31.5 or 39.5°C, and cytoplasmic extracts were electrophoresed on nonreducing native gels, transferred to nitrocellulose, and reacted with polyclonal anti-gD-1 serum (Fig. 1). The Cys-7 protein (lane 8) synthesized at either temperature comigrated with wild-type (pRE4) gD-1 (lane 1; 59 RDa) and did not aggregate. This result is consistent with previous results obtained with this mutant at 37°C (Table 1) (54). When mutants Cys-1 through Cys-6 were synthesized at 39.5°C, gD migrated predominantly as a single polypeptide; in addition, higher-molecular-weight aggregates were found which were distributed in the separating gel, at the stacker-separating gel interface (arrows in Fig. 1), and at the top of the stacking gel. When the mutants were expressed at 31.5°C, there was generally less evidence of aggregation, although higher-molecular-weight bands were still seen at the top of the stacking gel for mutants Cys-1, Cys-2, Cys-3, and Cys-4. These results suggest that some of the mutants had different structural properties when expressed at the two temperatures. It should be noted that these aggregates could result from aberrant intermolecular disulfide bonds between gD molecules. Alternatively, or in addition, the aggregates could form between gD and other unidentified cell proteins.

(ii) Processing of the gD Cys mutants. Previously we

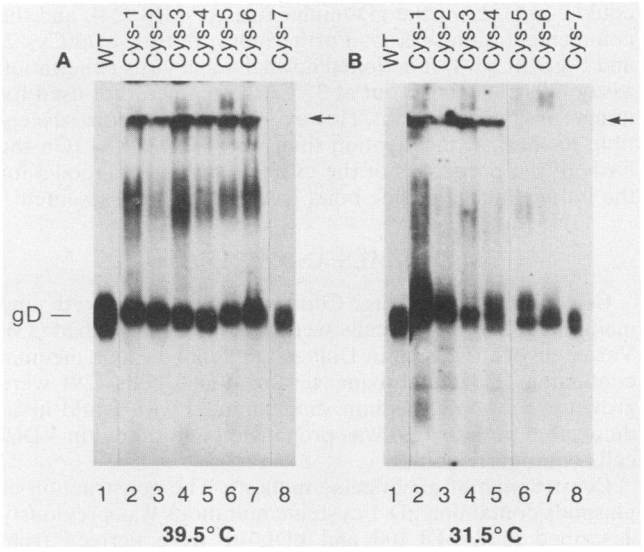

FIG. 1. Western blot analysis of wild-type gD-1 and gD-1 cysteine mutants expressed at 39.5 or 31.5°C, electrophoresed under nonreducing native conditions. Cytoplasmic extracts prepared from cells transfected at 39.5°C (A) or 31.5°C (B) were electrophoresed on 10% native gels, transferred to nitrocellulose, and reacted with polyclonal anti-gD-1 serum and ^{125}I-protein A. WT, Wild-type gD-1(pRE4). The separation between the stacking and separating gel is indicated by the arrow. Panel B is a composite of two gels.

showed that four of the mutant proteins (Cys-2, Cys-3, Cys-5, and Cys-6) synthesized at 37°C contained only high-mannose oligosaccharides (Table 1). In contrast, Cys-1 and Cys-4 were partially processed (5 to 10% compared with wild-type gD) beyond the precursor stage. Here we examined processing of each of the mutants synthesized at either 39.5°C (Fig. 2A) or 31.5°C (Fig. 2B). Cytoplasmic extracts were treated with endo H, electrophoresed under reducing conditions, transferred to nitrocellulose, and reacted with anti-gD-1 serum. Mutants Cys-1 through Cys-6 synthesized at 39.5°C were completely sensitive to endo H, indicating that these proteins contained only high-mannose oligosaccharides (precursor forms). Cys-7 and wild-type gD were both almost completely endo H resistant, indicating that these proteins contained predominantly complex oligosaccharides (product forms).

When the mutants were expressed at 31.5°C, several patterns of endo H digestion were observed (Fig. 2B). First, the Cys-7 protein showed the same mobility shift as did wild-type gD. In both cases, only a small proportion of the protein was endo H sensitive. Second, Cys-3 was entirely endo H sensitive, showing that the carbohydrate moieties present on this protein are all of the high-mannose type. The third pattern was exhibited by the remaining five mutants, Cys-1, Cys-2, Cys-4, Cys-5, and Cys-6. In each case, a significant portion of the N-linked oligosaccharides was endo H resistant when the proteins were synthesized at 31.5°C, even though there was no evidence of processing of these mutants to complex forms at 39.5°C. At 37°C (54), there was partial processing of Cys-1 and Cys-4. Taken together, the results suggest that Golgi-associated oligosaccharide processing of Cys-1, Cys-2, Cys-4, Cys-5, and Cys-6 mutants is *ts*.

(iii) Transport of the mutant gD proteins to the cell surface. The *ts* difference in processing of some of the mutant proteins suggested that their transport within the cell and to

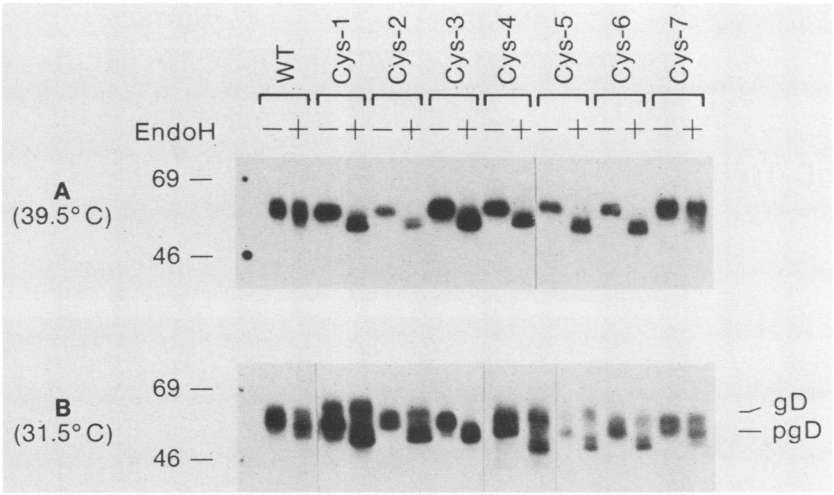

FIG. 2. Endo H analysis of gD-1 cysteine mutants synthesized at 39.5 or 31.5°C. Cytoplasmic extracts prepared from cells transfected at 39.5°C (A) and 31.5°C (B) were mock treated (−) or treated (+) with endo H, electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with anti-gD-1 serum and ^{125}I-protein A. Molecular weight markers of 46,000 and 69,000 are indicated. WT, wild type.

the cell surface might also be *ts*. Cells were transfected with plasmids containing the wild-type or mutant forms of gD, incubated at either 31.5 or 39.5°C, then examined by a double-label indirect immunofluorescence assay that detects cell surface and internal expression of gD in the same cell (Fig. 3 and 4). A mutant form of gD-1, truncated after residue 182 [gDt(182)] and therefore lacking the transmembrane region, was used as a negative control for cell surface staining (Fig. 3D and 4D) (48). All of the proteins were detected internally at both temperatures (Fig. 3 and 4, panels A, C, E, and G). At 31.5°C, all of the cells transfected with either wild-type gD (Fig. 3B) or Cys-7 (not shown) that exhibited internal fluorescence also had gD on the cell surface. This was also the case for Cys-2 (Fig. 3H) as well as Cys-1, Cys-4, Cys-5, and Cys-6 (not shown). In contrast, Cys-3 exhibited no cell surface fluorescence on any cells at this temperature (Fig. 3F). These results suggested that all of the mutants except Cys-3 were transported to the cell surface at 31.5°C. At 39.5°C (Fig. 4), the same basic pattern was observed, with one important difference. For Cys-1, Cys-2, Cys-4, Cys-5, and Cys-6, only 15 to 35% of the cells exhibiting internal fluorescence were also stained on the cell surface (shown for Cys-2 in Fig. 4G and H). These results suggest that transport of Cys-1, Cys-2, Cys-4, Cys-5, and Cys-6 was *ts*. Transport of Cys-7 to the cell surface occurred at both temperatures (not shown), and transport of Cys-3 did not occur at either temperature (Fig. 3F and 4F).

(iv) Antigenic analysis. Thus far, we have shown that five of the cysteine mutants (Cys-1, -2, -4, -5, and -6) exhibit *ts* differences in aggregation, processing, and transport. We asked whether there were also *ts* differences in protein conformation as detected by alterations in antigenic structure. To address this question, we used a panel of MAbs in a slot blot assay to examine the antigenic properties of each mutant protein expressed at 31.5 or 39.5°C. Results for three MAbs are shown in Fig. 5. DL6, a group II MAb that binds to a continuous epitope (residues 272 to 279), was used to normalize the total amount of gD loaded in each slot (Fig. 5A). MAbs HD1 (group Ia) (37) and ABD (group III) recognize distinct discontinuous epitopes (Fig. 5B and C) and were used to detect differences in the conformation of the mutant forms of gD. Three patterns of reactivity were observed. First, the Cys-7 mutant expressed at either temperature showed good binding of ABD but somewhat reduced binding of HD1. The binding of other MAbs in group I resembled that of HD1, and the binding of other MAbs in group III resembled that of ABD. These results suggest that the absence of cysteine 7 had some effect, albeit limited, on protein structure. Second, Cys-3 and Cys-6 expressed at either 31.5 or 39.5°C were unreactive with either HD1 or ABD as well as with five other MAbs representing other antigenic sites (not shown). This finding indicated that cysteines 3 and 6 are indispensable for the correct antigenic conformation of gD. Third, mutants Cys-1, Cys-2, Cys-4, and Cys-5 exhibited major reductions in binding to both HD1 and ABD. However, in each case there was at least two- to threefold more reactivity with these MAbs when the proteins were expressed at 31.5°C than when they were expressed at 39.5°C. A similar step-up in reactivity of Cys-1, Cys-2, Cys-4, and Cys-5 synthesized at 31.5°C was found with each of the five other MAbs to discontinuous epitopes that were tested (results not shown). These results suggest that folding of Cys-1, Cys-2, Cys-4, and Cys-5 is *ts*.

Functional analysis of gD cysteine mutants. (i) Rescue of F-gDβ virus by gD cysteine mutants expressed in *trans*. A complementation assay was used to examine whether the gD-1 Cys mutants were functional in vivo (3, 38). The assay uses F-gDβ, a mutant virus that was derived from HSV-1(F) by replacement of the gD-coding sequence and part of the gI gene with the β-galactosidase gene of *Escherichia coli* (29). It can replicate in VD60 cells, which contain integrated copies of the gD gene, but not in Vero cells, from which the VD60 cell line was derived (29). Vero cells infected with F-gDβ produce noninfectious virions lacking gD. In the complementation assay, gD transiently expressed from transfected plasmids is tested for its ability to rescue production of infectious F-gDβ virions in Vero cells. In this case, Vero cells first were transfected with plasmids containing wild-type gD-1 or one of the Cys mutants and then were infected with F-gDβ and incubated at 37°C as the nonpermissive temperature. Because F-gDβ was derived from strain F, it contains a *ts* mutation in the immediate-early ICP4 gene and does not grow at 39.5° (1; D. Long, unpublished data). Intracellular virus was collected after 24 to 48 h,

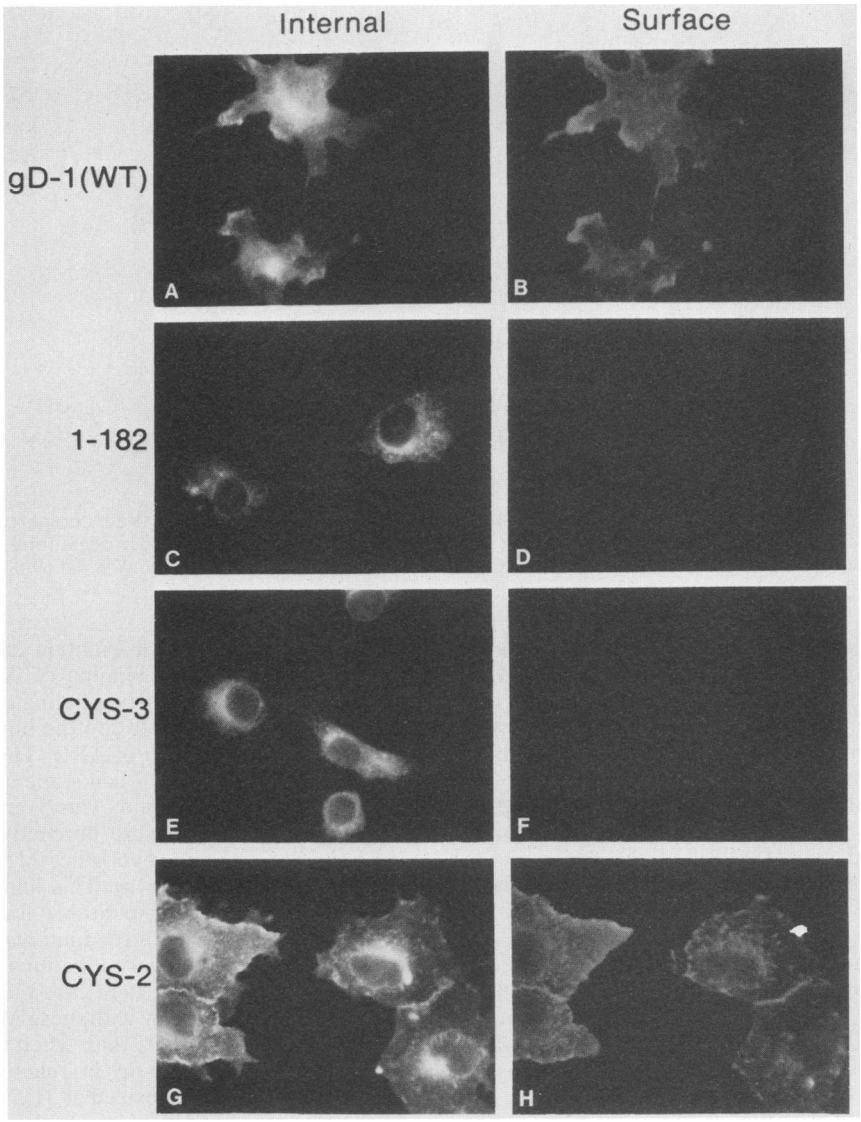

FIG. 3. Immunofluorescence analysis of cells expressing wild-type and mutant forms of gD-1 at 31.5°C. Double-label immunofluorescence was used to detect gD-1 internally and on the cell surface. Cells were fixed with paraformaldehyde and reacted with anti-gD serum and then with goat anti-rabbit–tetramethylrhodamine isothiocyanate to label gD-1 on the cell surface (B, D, F, and H). Cells were then permeabilized with Nonidet P-40 and reacted with anti-gD-1 serum and anti-rabbit–fluorescein isothiocyanate to detect gD-1 inside the cell (A, C, E, and G). Proteins analyzed were wild-type (WT) gD-1 (A and B), a gD-1 truncation mutant containing residues 1 to 182 (C and D), Cys-3 (E and F), and Cys-2 (G and H).

and virus titers were determined on VD60 monolayers (Fig. 6). When cells were transfected with the wild type or Cys-7, the titers were at least 50-fold higher than those obtained when cells were transfected with vector alone. Thus, cysteine 7 at residue 333 may not be critical for the function of gD. Although it remains functional at 37°C, it appears that Cys-7 is actually better than wild-type gD-1 at rescuing F-gDβ. Thus, its ability to rescue at 31.5°C may actually be somewhat impaired. In contrast to the results for Cys-7, virus titers obtained from cells transfected with any one of the other six cysteine mutants were similar to background levels when the experiment was carried out at 37°C. Thus, alteration of any one of these six cysteines to serine renders gD nonfunctional. However, when the assay was carried out at 31.5°C, two of the mutant proteins, Cys-2 and Cys-4, exhibited complementation activity. Titers for F-gDβ(Cys-2) were 16-fold higher than background levels, and the titers for F-gDβ(Cys-4) were 13-fold higher. Thus, these proteins were functional when synthesized at the lower temperature, though the level of complementation was never as high as with wild-type gD. The viruses produced in the complementation assay and used in the following experiments are designated as F-gDβ(WT), F-gDβ(Cys-2), F-gDβ(Cys-4), and F-gDβ(Cys-7).

(ii) Neutralization of rescued virus. To confirm that these virions actually contained gD in their envelope, we determined whether a gD MAb, DL11 (group Ib), could neutralize the infectivity of virus obtained in the complementation assay (Table 2). DL11 neutralized both F-gDβ(Cys-2) and F-gDβ(Cys-4) as efficiently as it neutralized F-gDβ(WT). We conclude that Cys-2 and Cys-4 mutant proteins are incorporated into the virus envelope of F-gDβ at the permissive

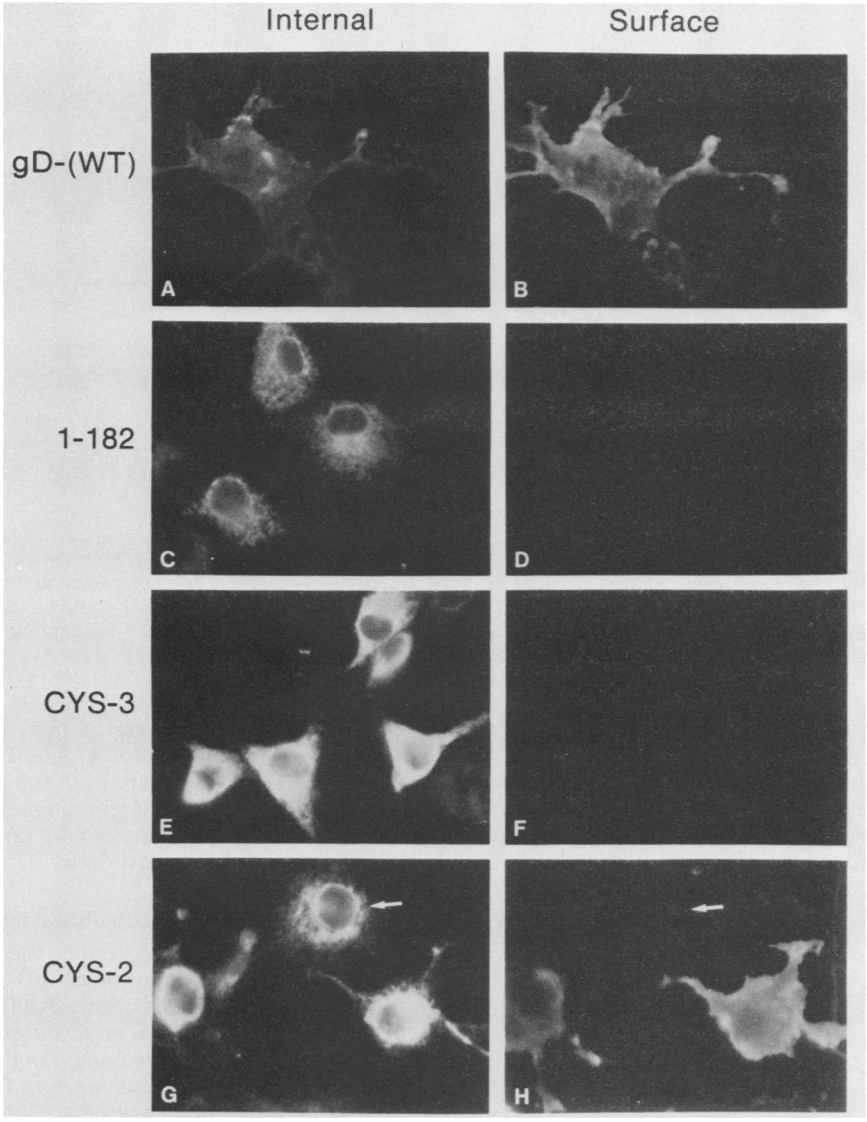

FIG. 4. Immunofluorescence analysis of cells expressing wild-type (WT) and mutant forms of gD-1 at 39.5°C. Double-label immunofluorescence was used to detect gD-1 internally (A, C, E, and G) and on the cell surface (B, D, F and H) as described in the legend to Fig. 3. The arrows in panels G and H point to a cell that exhibits internal but not cell surface fluorescence.

temperature. Moreover, the structure of the mutant protein incorporated into the virus envelope at 31.5°C appears, at least by the criterion of the neutralization assay used here, to be indistinguishable from that of wild-type gD-1.

(iii) Penetration of F-gDβ(Cys-2) and F-gDβ(Cys-4) virions into VD60 cells. Since gD is essential for HSV-1 penetration (24, 29), we decided to compare the ability of F-gDβ(Cys-2) and F-gDβ(Cys-4) to penetrate cells at 31.5 or 37°C. VD60 cells were incubated with virus produced in the complementation assay for 2 h at 4°C to allow for attachment and were then shifted either to 31.5 or 37°C; after various amounts of time, extracellular virus was inactivated with low-pH glycine buffer (20, 21), and the remaining (intracellular) virus was allowed to develop into visible plaques. We found no difference in the rate of penetration of any of the viruses tested at either temperature (Fig. 7). In each case, penetration proceeded more rapidly at 37°C (Fig. 7B) than at 31.5°C (Fig. 7A). Previous studies have shown that penetration of HSV is temperature dependent (21). We found that 50% of the total input virus became resistant to acid inactivation within 22 min of incubation at 37°C (Fig. 7B), whereas at 31.5°C (Fig. 7A), 50% of the input virus became acid resistant within 60 min. The results suggest that Cys-2 and Cys-4, once synthesized at 31.5°C and incorporated into virions, were able to function normally so that penetration was unaffected at 37°C. Alternatively, penetration occurred before thermal inactivation of F-gDβ(Cys-2) or F-gDβ(Cys-4) at 37°C.

(iv) Thermal susceptibility studies. We examined the thermal stability of each of the viruses obtained in the complementation assay by incubating them at various temperatures for 1 h and then testing their infectivity (Fig. 8). We found that the infectivity of F-gDβ(Cys-7) and F-gDβ(WT) was not significantly affected at temperatures below 43°C. In contrast, the infectivity of F-gDβ(Cys-2) and F-gDβ(Cys-4) decreased approximately 50% at 37°C, and at 45°C there was a 4-log drop in virus titer.

The rate of heat inactivation was determined by incubating virus preparations at 37 or 45°C for increasing amounts of

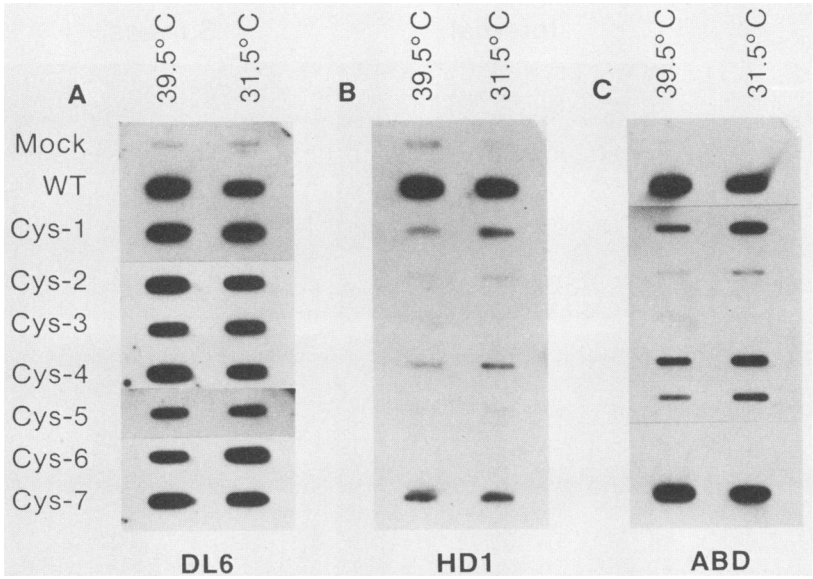

FIG. 5. Slot immunoblot analysis, using MAbs, of wild-type gD-1 and gD-1 cysteine mutants expressed at 39.5 or 31.5°C. Cytoplasmic extracts of cells transfected with wild-type (WT) or mutant gD-1 genes were applied to nitrocellulose by using a slot blot apparatus (Hoefer). Membranes were incubated with the following MAbs: DL-6 (continuous epitope, group II) (A), HD-1 (discontinuous epitope, group Ia) (B), and ABD (discontinuous epitope, group III) (C).

time and then titering the residual virus. Compared with the titer of F-gDβ(WT), the titers of F-gDβ(Cys-2) and F-gDβ (Cys-4) were not significantly affected by preincubation at 37°C for less than 30 min (Fig. 9A). It should be noted that by 30 min at this temperature approximately 60% of the virus would have penetrated the cell (Fig. 7B). Longer incubation times decreased infectivity by approximately 10-fold. At 45°C (Fig. 9B), the infectivity of both F-gDβ(WT) and F-gDβ (Cys-7) slowly decreased; approximately 50% of these viruses were inactivated after 20 min, and there was a fourfold decrease in virus titer after 32 min. In contrast, F-gDβ(Cys-2) and F-gDβ(Cys-4) were both significantly more sensitive to inactivation at 45°C; approximately 50% of these viruses were inactivated within 4 min, and there was almost a complete loss of infectivity after 32 min.

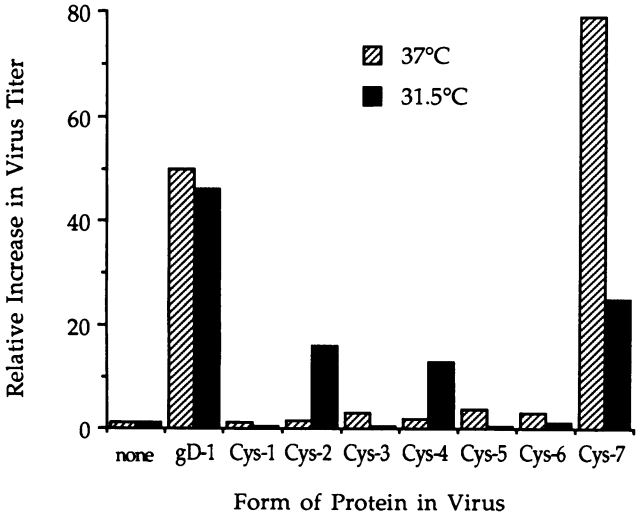

FIG. 6. Rescue of F-gDβ by gD-1 cysteine mutants expressed in *trans* at 37 or 31.5°C. Vero cells were transfected with a test plasmid, infected 16 to 18 h later with F-gDβ, and incubated at 37 or 31.5°C for 24 to 48 h. Intracellular virus was collected and titered on VD60 cells. Data are expressed as the titer obtained with the test plasmid divided by the titer obtained when cells were transfected with the control plasmid, pRSVntEPA. Titers for pRSVntEPA were approximately 10^4 at 37°C and 6×10^2 at 31.5°C. The results represent the averages of three separate experiments.

DISCUSSION

We have previously described the construction and characterization of seven cysteine gD-1 mutants (54). These results indicated that six of the seven cysteines are important in maintaining the native structure of gD, probably through the formation of disulfide bonds. In this study, we found that five of the seven mutants were *ts* for one or more of the following properties: aggregate formation, conformation, processing from a high-mannose precursor to a product form containing complex oligosaccharides, and transport to the cell surface. Furthermore, two mutant proteins, Cys-2 and Cys-4, rescued the infectivity of a gD null mutant, F-gDβ, in a *ts* fashion.

The substitution of serine for any of the first six cysteines of gD-1 resulted in the appearance of high-molecular-weight forms, presumably due to aggregation of the protein as a

TABLE 2. Neutralization of virus produced in the complementation assay

Rescued virus[a]	Antibody dilution (10^5) for 50% plaque reduction[b]
F-gDβ(WT)	1.28
F-gDβ(Cys-2)	0.96
F-gDβ(Cys-4)	1.28

[a] Names refer to the phenotype of the virus obtained in the complementation assay.

[b] Equal amounts of rescued virus were incubated with various dilutions of DL11 at 31.5°C for 1 h, and the mixture was added to VD60 cells. After 3 to 4 days at 31.5°C, plaques were counted; the averages of triplicate samples are shown.

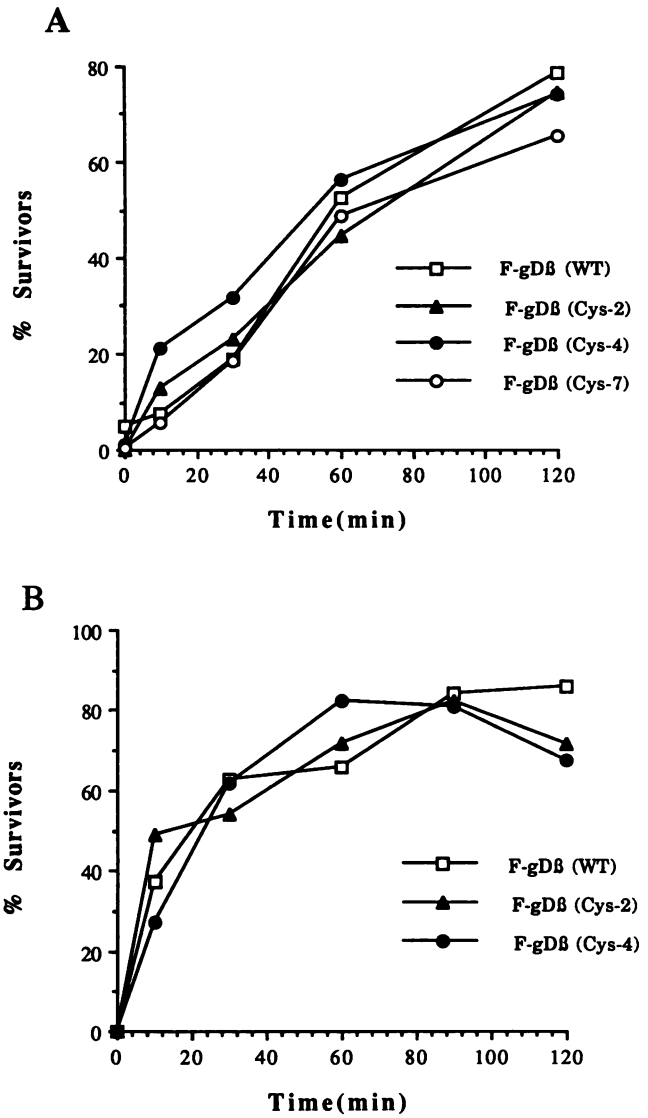

FIG. 7. Rates of penetration of viruses obtained from the complementation assay carried out at 31.5 or 37°C. Virus samples (100 to 200 PFU) prepared from the complementation assay (Fig. 6) were added to VD60 cell monolayers and allowed to adsorb to VD60 cells for 2 h at 4°C. The cultures were then incubated at 31.5°C (A) or 37°C (B). At the times indicated, cells were washed with acid-glycine buffer (pH 3.0). Virus plaques were counted after 24 h at 37°C. The results are reported as percent survivors when compared with controls in which PBS was substituted for acid-glycine buffer.

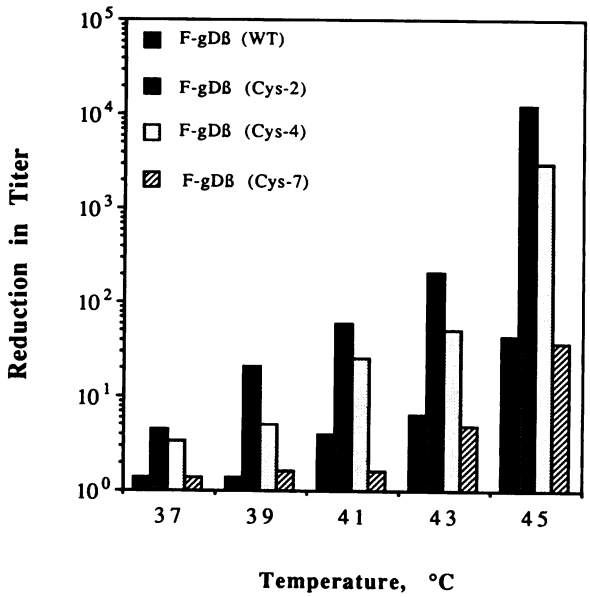

FIG. 8. Effect of preincubation temperature on infectivity. Virus samples were incubated for 1 h at the indicated temperatures and the titers were determined on VD60 cell monolayers at 31.5°C.

result of incorrect disulfide bonding. Several of the mutants appeared to be less aggregated when synthesized at 31.5°C than at 39.5°C. One explanation is that folding occurs more slowly, decreasing the inappropriate hydrophobic interactions, resulting in a more accurately folded polypeptide at the lower temperature (27). In addition, the processing which occurs at 31.5°C may also be important for achieving or maintaining the native conformation (32, 39).

All seven mutant proteins accumulated intracellularly, and all but Cys-3 were observed to be present on the cell surface when expressed at 31.5°C. In contrast, when the transfections were carried out at 39.5°C only a small fraction of cells expressing Cys-1, -2, -4, -5, and -6 internally were also found to express the protein on the cell surface. In addition, the oligosaccharides added to these proteins remained endo H sensitive, suggesting that at this high temperature much of the protein was not reaching the Golgi compartment, where processing to endo H resistance occurs. In contrast, when the proteins were expressed at 31.5°C, all of the mutants except Cys-3 were transported to the cell surface and a significant amount of the accumulated protein was endo H resistant. In these studies, we examined cell surface expression only in a qualitative way. Whitt et al. (53) have shown that the ability of some of the mutated G proteins of vesicular stomatitis virus to rescue a *ts* mutant, *ts*O45, was proportional to the amount of each mutant protein on the cell surface. It would be of interest to do similar studies with Cys-2 and Cys-4. However, it should be noted that vesicular stomatitis virus matures at the plasma membrane, whereas HSV matures intracellularly.

Our studies with MAbs that recognize discontinuous epitopes suggest that there are temperature-sensitive structural differences in four of the Cys mutant proteins. However, the MAb reactivities of the mutant proteins synthesized at 31.5°C never reached wild-type levels. Our conclusion is that although all of the disulfide bonds are critical for correct folding and maintenance of antigenic structure, the absence of any one of these bonds is compensated to some degree by lowering the temperature.

To determine whether any of the cysteine-to-serine changes affected the function of gD, we used a complementation assay in which gD transiently expressed by a plasmid can rescue the infectivity of F-gDβ, a virus strain of HSV-1 that lacks a gD gene. In this assay, Cys-7 was found to function normally, which is consistent with our prediction that this cysteine residue is not involved in formation of disulfide bonds. None of the other six mutants were able to complement at 37°C. However, when the assay was performed at 31.5°C, two mutant proteins, Cys-2 and Cys-4, were able to complement, albeit at a lower level than wild-type gD-1. Neutralization of the infectivity of virions containing Cys-2 or Cys-4 with the gD-specific MAb DL11 is evidence that these forms of gD become incorporated into

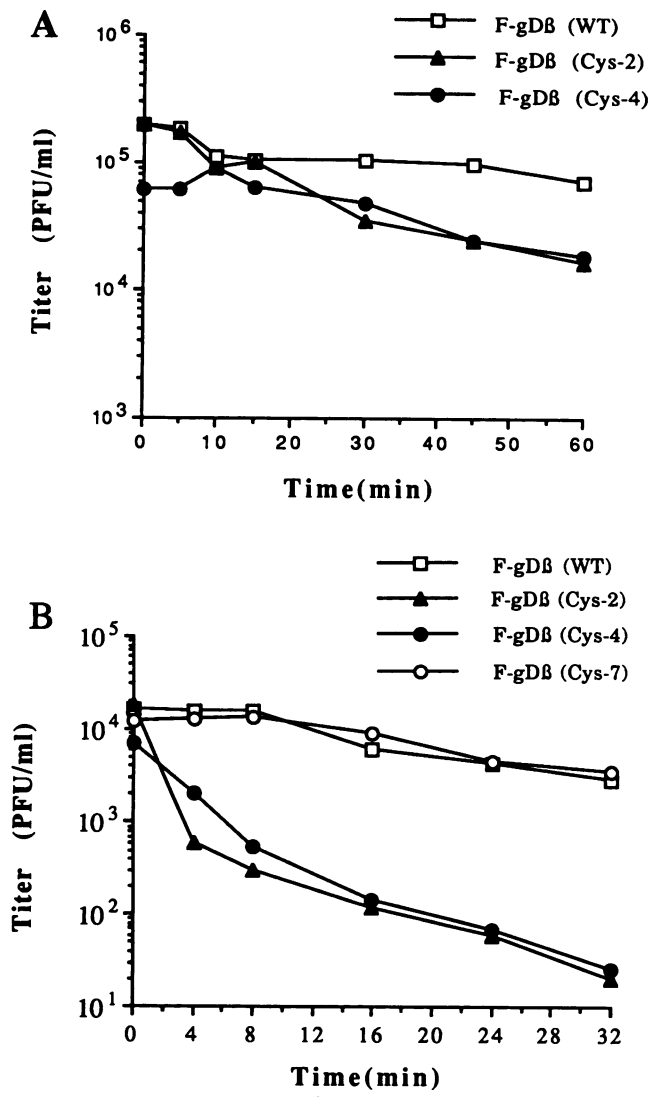

FIG. 9. Rate of virus inactivation at 37 and 45°C. Virus samples were incubated at 37°C (A) or 45°C (B) for the times indicated and then chilled at 4°C. Residual virus titers were determined on VD60 cell monolayers at 31.5°C.

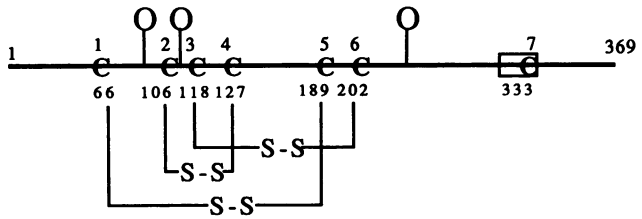

FIG. 10. Model of predicted intramolecular disulfide bonding of gD. The essential features of gD-1 are depicted as a stick figure. The seven cysteine residues are designated by a C, and their positions in the gD-1 sequence are indicated beneath. The box surrounding Cys-7 represents the transmembrane region. The protein contains three sites for addition of N-linked oligosaccharides, depicted as ballons. The disulfide bond pattern (-S-S-) predicted from the properties of the gD-1 cysteine mutants is shown.

the virion envelope at 31.5°C. In addition, it is possible that when Cys-2 and Cys-4 are synthesized at 37°C, they are not incorporated into the envelope. Perhaps the other Cys mutants do not complement because they are not incorporated into the virion at either temperature. Future experiments will attempt to address these questions by purifying noninfectious virions produced in the complementation assay and examining them for the presence of gD.

It is interesting that when incorporated into the envelope at 31.5°C, both Cys-2 and Cys-4 displayed normal entry kinetics at both 31.5 and 37°C. Viruses containing either of these proteins were fairly stable when preincubated at 37°C but more thermolabile than viruses containing wild-type gD-1 when preincubated at 45°C. One possibility is that the mutant proteins are more sensitive to thermal inactivation during synthesis at 37°C, whereas once the protein is incorporated into the envelope it is not as easily denatured. Further experiments will be necessary to clarify this issue. The complementation results predict that both the Cys-2 and Cys-4 mutations will produce virions with an interesting *ts* phenotype. Recombination experiments using these mutant forms of gD are now in progress. It is of interest to note that Highlander et al. (20) reported a slower rate of entry of HSV-1(KOS-321) into Vero cells at 37°C than we observed in our study. This discrepancy could be due to strain differences in one or more of the envelope proteins. This issue will best be resolved by comparing the entry kinetics of the recombinant viruses with that of KOS-321 in the same experiment.

Our experiments suggest that Cys-1 through Cys-6 form three disulfide bonds (54). In addition, preliminary experiments using [^{14}C]iodoacetamide are consistent with this notion (Long, unpublished data). To address the question of which cysteine residue pairs might be involved in these three intramolecular disulfide linkages, we compared the phenotypes of the various gD-1 Cys mutants. This strategy was used to predict disulfide bonds of the Tac receptor protein and was confirmed by a preliminary peptide map of cystine-linked enzymatic fragments (43). As another example, the differing properties of two cysteine mutants of the envelope protein of human immunodeficiency virus type 1 suggested that these two cysteines might not be bonded to each other (49). The properties of the cysteine mutants predict the pattern of disulfide bonding for gD-1 shown in Fig. 10. Cys-2 (residue 106) and Cys-4 (residue 127) are paired because they were similar in all properties and were the only mutants to function in the complementation assay. Cys-1 (residue 66) and Cys-5 (residue 189) are paired because they both exhibited similar *ts* properties, including ability to bind to MAbs that recognize discontinuous epitopes, processing to endo H resistance, and transport to the cell surface. In addition, and unlike Cys-2 and Cys-4, neither of these mutants was able to function in the complementation assay. In contrast, Cys-3 (residue 118) and Cys-6 (residue 202) both showed a lack of reactivity to MAbs to discontinuous epitopes; however, Cys-6 did display some *ts* processing that was not seen for Cys-3. Thus, these two cysteines are disulfide bonded in our model partly by default. Confirmation of this disulfide bond pattern by biochemical analysis is now in progress.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants DE-08239 from the National Institute of Dental Research and AI-18289 from the National Institute of Allergy and Infectious Diseases and by a grant to G.H.C. and R.J.E. from the American Cyanamid Co. D.L. is a predoctoral trainee supported by Public Health Service grant NS-07180 from the National Institute of Neurological Diseases and Stroke.

We thank D. C. Johnson for the VD60 cells and F-gDβ virus; W. Wilcox for the mutants constructed by site-directed mutagenesis; D. L. Sodora for the discovery of the mutation at amino acid 54 and for help in preparation of the manuscript; L. Pereira, C. Desgranges, and A. Minson for antibodies; and M. Cohen for excellent technical assistance.

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