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# The Contribution of Cysteine Residues to Antigenicity and Extent of Processing of Herpes Simplex Virus Type 1 Glycoprotein D

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**Glycoprotein D (gD) is an envelope component of herpes simplex virus types 1 (gD-1) and 2 (gD-2). The gD-1 polypeptide contains seven cysteine residues among its 369 amino acids; six are located on the N-terminal or luminal portion of the glycoprotein, and a seventh is located in the transmembrane region. Previous studies used a panel of monoclonal antibodies (MAbs) to define gD epitopes as continuous or discontinuous. Purified gD, denatured by reduction and alkylation, loses discontinuous epitopes, whereas continuous epitopes are retained. The contribution of disulfide bonds to maintenance of discontinuous epitopes is, therefore, significant. In the present study, our objective was to determine the contribution of individual cysteine residues to folding of gD-1 into its native conformation. Site-directed oligonucleotide mutagenesis was used to create seven mutants, each with a serine residue replacing a cysteine. The mutated genes were cloned into a eucaryotic expression vector and transfected into COS-1 cells, and the proteins were separated by nondenaturing polyacrylamide gel electrophoresis, followed by immunoblotting. Replacement of cysteine 7 (residue 333) had only a minimal effect on the antigenic properties of gD-1. In contrast, replacement of any one of the other six cysteine residues resulted in either a major reduction or a complete loss of binding of those MAbs that recognize discontinuous epitopes, with no effect on the binding of MAbs which recognize continuous epitopes. These mutations also had profound effects on the extent of oligosaccharide processing of gD-1. This was determined by digestion of the expressed proteins with various endoglycosidases, followed by electrophoresis and Western blotting (immunoblotting) to observe any mobility changes. Three mutant gD proteins which did not express discontinuous epitopes contained only high-mannose-type oligosaccharides, suggesting that processing had not proceeded beyond the precursor stage. Two mutant forms of gD exhibited reduced binding of MAbs to discontinuous epitopes. A small proportion of the molecules which accumulated at 48 h posttransfection contained complex oligosaccharides. One mutant exhibited reduced binding of MAbs to discontinuous epitopes, but was present at 48 h posttransfection only in the precursor form. The cysteine 7 mutant was processed to the same extent as wild-type gD. We conclude that the first six cysteine residues are critical to the correct folding, antigenic structure, and processing of gD-1, and we speculate that they form three disulfide-bonded pairs.**

Glycoprotein D (gD) of herpes simplex virus (HSV) is a structural component of the virion envelope which stimulates high titers of neutralizing antibody (6, 8, 16, 18, 19, 22, 28, 34, 37, 38, 44, 45) and is likely to play an important role in the initial stages of viral infection. Studies using anti-gD monoclonal antibodies (MAbs) have implicated gD-1 in virus attachment (22) and penetration (23, 28) and in fusion of infected cells (37, 38). Furthermore, animals immunized with various forms of gD are protected from a lethal HSV challenge (4, 12, 32, 34, 39). Genes for gD of both HSV type 1 (HSV-1) (gD-1) and HSV-2 (gD-2) have been localized (33, 50) and sequenced (31, 49, 50). The two proteins are highly conserved, having 85% homology over their predicted amino acid sequences of 394 amino acids for gD-1 and 393 for gD-2. In each case, the first 25 amino acids of the predicted amino acid sequence are cleaved from the protein during posttranslational processing (15). Each protein contains three predicted sites for N-linked glycosylation (31, 49, 50), all of which are utilized (10); O-linked oligosaccharides (reviewed in reference 45); and a hydrophobic membrane-anchoring domain near the carboxy-terminal end (36, 51). gD-1 contains seven cysteine residues, six of which are clustered between amino acids 66 and 202 of the mature glycoprotein (Fig. 1). These six cysteines are strictly conserved both in number and in relative position in gD-2 (31, 49, 50). A similar pattern of spacing was also found in the pseudorabies virus gp50, which appears to be a gD homolog (40). Three continuous antigenic epitopes have been mapped to specific amino acids at widely separated regions of gD (6, 13, 16, 17, 36). Also present on the native glycoprotein are discontinuous epitopes which are characterized by their loss of MAb reactivity after denaturation of the glycoprotein (7, 13, 16, 28, 37). Recently (11), we used deletion mutants of gD-1 to map a portion of the group I epitope to residues 234–244. Three other discontinuous epitopes are located within amino acids 1–233.

The loss of discontinuous epitopes after denaturation drew our attention to the possible importance of cysteine residues in the antigenic structure of gD, especially since denaturing conditions employing reduction and alkylation disrupt disulfide bonds. Tertiary configurations related to the presence of disulfide bonds have been shown to be critical for various biological activities (1, 26, 35, 48). We found that a truncated form of gD-1, residues 1–275, which lacked cysteine 7 (residue 333), retained all of the discontinuous epitopes (7). This suggests that intramolecular disulfide bonding might be

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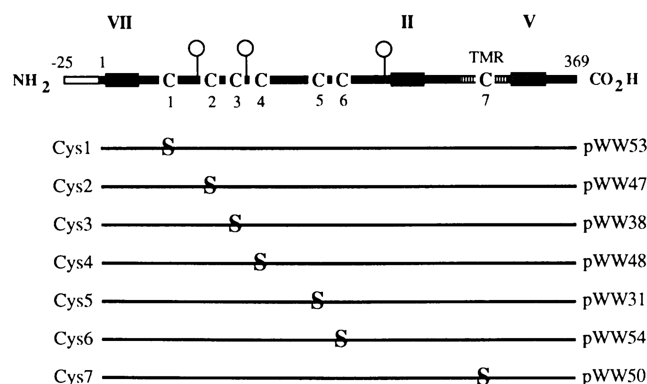


FIG. 1. Schematic representation of gD-1 and the seven cysteine mutants. The essential features of gD-1 protein are depicted as a stick figure. The protein (49) contains a signal peptide of 25 amino acids, which is cleaved during cotranslational processing (15, 36), and a hydrophobic anchor sequence (TMR) near the carboxy terminus (36). The seven cysteine residues are located at amino acids 66, 106, 118, 127, 189, 202, and 333. The protein contains three sites for addition of N-linked oligosaccharides (10, 50), depicted as balloons. The positions of three continuous epitopes (6, 7, 13, 16, 17, 36) at amino acids 11–19 (VII), 272–279 (II), and 340–356 (V) are shown. Stick models of each of the cysteine (C)-to-serine (S) mutants are also shown. The plasmid designations for each mutant are indicated on the right. The name given to each mutant is indicated on the left. Mutant proteins are referred to as gD-1-(Cys-1) through gD-1-(Cys-7), and the wild-type or parental form of gD expressed from plasmid pRE4 is referred to as gD-1-(pRE4).

limited to the first six cysteines. Recently (11), we observed that internal deletion mutants of gD-1 which lacked either cysteine 1 (residue 66) or cysteine 6 (residue 189) were unable to bind MAbs to discontinuous epitopes. In addition, these mutant forms of gD-1 accumulated only high-mannose-type oligosaccharides (8, 9, 14, 36), implying that conformations involving disulfide bonds are necessary for correct processing. For these reasons we undertook a systematic analysis of the contribution of each cysteine residue to the antigenic structure and extent of processing of gD-1.

Oligonucleotide-directed mutagenesis (29, 30, 53) was employed to create seven gD-1 mutants, each with a serine rather than a cysteine codon. Each of the seven mutant gD genes was transfected into mammalian cells, and the expressed proteins were examined with MAbs specific for continuous and discontinuous epitopes. Replacement of cysteine 7 had only a minimal effect on the antigenic properties or processing of gD-1. In contrast, replacement of any one of the first six cysteine residues had profound effects on both antigenicity and extent of processing. The six altered forms of gD-1 shared several common features. First, they suffered either a major reduction or a complete loss of binding of those MAbs that recognize discontinuous epitopes, with no effect on binding of those MAbs that recognize continuous epitopes. Second, the glycoproteins which accumulated at 48 h posttransfection contained only high-mannose oligosaccharides, suggestive of an abnormal pattern of oligosaccharide processing in transfected cells. Third, they formed high-molecular-weight aggregates which were dissociated by denaturation by heat and reduction. These properties were similar to those recently noted for several deletion mutants of gD which lacked either cysteine 1 or cysteine 6 (11).

## MATERIALS AND METHODS

**Cell culture and virus.** Conditions for the growth and maintenance of BHK cells and HSV-1 (Patton) were previously described (8, 18). An input multiplicity of 5 PFU was used for infection. COS-1 cells (a gift from J. Alwine) were propagated in Dulbecco minimal medium supplemented with 10% fetal bovine serum.

**Construction of gD mutants by oligonucleotide-directed mutagenesis.** A 1,420-base-pair *Hin*dIII fragment containing the entire gD-1 (Patton) coding sequence (50) was excised from the gD expression vector pRE4 (11) and subcloned into the *Hin*dIII site of the replicative form of M13mp18. Mutagenesis was carried out by the method of Zoller and Smith (53) as modified by Kunkel et al. (29, 30) to allow for phenotypic selection of phage containing the desired mutation. Seven synthetic oligonucleotide primers ranging in size from 17- to 21-mer were used, each of which had a single-base mismatch designed to change a cysteine anticodon to one for serine (AGC or ACA to TGC or TCA). The sequences of the oligonucleotides were: Cys-1 (residue 66), CACGCTGCGGCTGGCGCGCT; Cys-2 (residue 106), GAT AGCACTGTTGCCTCC; Cys-3 (residue 118), TTGTAGG AGCTTTCGGTGTAC; Cys-4 (residue 127), TTCGGATGG GACTGGCCCCC; Cys-5 (residue 189), GCGTACTTACTG GAGCCCTT; Cys-6 (residue 202), GGAGAGGCTGGCT GAC; Cys-7 (residue 333), CAATTCCGCTAATGACC. The anticodon is underlined here, and the T in each case was the mismatch. Mutants were identified, and their DNA sequences were verified by the dideoxynucleotide method (42). In our hands, 40 to 80% of the plaques contained virus with the desired cysteine-to-serine alteration. The mutated gD gene was removed from the replicative-form DNA of each mutant clone with *Hin*dIII and inserted into the eucaryotic expression vector pRSV-nt EPA (3, 11), which has a unique *Hin*dIII site adjacent to the Rous sarcoma virus long terminal repeat promoter (gift of S. Carswell and J. Alwine). This vector was also the parent vector for pRE4 (11). Before transfection of mammalian cells, the correctness of the mutation was again confirmed by employing the supercoil sequencing technique (5).

**Transfection procedure.** The calcium phosphate coprecipitation procedure (27) was used with two modifications. First, calcium phosphate-precipitated supercoiled plasmid DNA was incubated with the cells for 16 h, rather than for 3 h. Second, the cells were not subjected to glycerol shock. For each plasmid, the DNA concentration and incubation time leading to maximum expression of gD were determined. In most cases, 10 μg of DNA per 60-mm plate was used and the cells were harvested at 48 h posttransfection. Cells were harvested and cytoplasmic extracts were prepared as previously described for infected cells (8).

**Polyclonal antibodies and MAbs.** The antibodies used in this study were rabbit anti-gD-1 serum (19), MAb DL11 (group I) (7), MAb DL6 (group II) (7), MAb 11S (group III) (16, 44), MAb 55S (group V) (16, 44), MAb DL2 (group VI) (7), and MAb 1D3 (group VII) (7, 21). MAbs 11S and 55S were supplied by Martin Zweig. Antibodies were grouped by their ability to react with native or denatured gD-1 and gD-2 (6, 7, 13, 17), blocking studies (17), neutralization activity (16, 44), reactions with synthetic peptides (6, 7, 13), and their location on fragments of gD (7, 16, 17). Each MAb was chosen to represent a group; however, it is possible that different MAbs within a group react with distinct epitopes within an antigenic site (52).

**Western blot analysis.** Sodium dodecyl sulfate-polyacryl-

amide gel electrophoresis of proteins under denaturing and nondenaturing ("native") conditions was carried out as previously described (7). After electrophoresis, proteins were transferred to nitrocellulose (2) and incubated with antibodies and then with iodinated protein A (ICN) as described previously (6, 7, 17). The nitrocellulose blots were exposed to Kodak XAR-5 film with a Cronex Lightening-Plus screen (Du Pont) at −70°C.

**Enzyme digestions.** For each enzyme, 12 μl of cytoplasmic extract (representing approximately $1.8 \times 10^4$ transfected COS-1 cells) in lysing buffer (20 mM Tris hydrochloride [pH 7.5] containing 50 mM NaCl, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) was digested for 6 h at 37°C. The enzymes and amounts used per 12 μl of extract were as follows: type VIII neuraminidase from *Clostridium perfringens* (Sigma Chemical Co.), 150 mU; endo-α-*N*-acetylgalactosaminidase (*O*-glycanase) (Genzyme), 2 mU; endo-β-*N*-acetylglucosaminidase F (endo F) (Boehringer Mannheim), 80 mU; endo-β-*N*-acetylglucosaminidase H (endo H) (Boehringer Mannheim), 2 mU. After digestion, the extracts were stored at −70°C until analyzed.

## RESULTS

The seven mutated forms of the gD gene, designated Cys-1 through Cys-7, and the plasmids containing them are diagrammed in Fig. 1. The proteins produced by these mutations are designated gD-1-(Cys-1) through gD-1-(Cys-7), respectively. Wild-type gD-1 produced by pRE4 will be referred to as gD-1-(pRE4). The stick figure of gD-1 also depicts the positions of continuous epitopes VII (residues 11–19), II (residues 272–279), and V (residues 340–356) (6, 7, 13, 17, 36, 50). The three discontinuous epitopes chosen for study (I, III, and VI) are located within residues 1–275 (1, 13, 14). Detailed studies indicate that these epitopes occupy separate regions of the native molecule (7, 11, 17).

**Analysis of the mutants with polyclonal anti-gD-1 serum.** COS-1 cells were transfected and cytoplasmic extracts were separated by denaturing or nondenaturing (native) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7), transferred to nitrocellulose (2), and reacted with polyclonal anti-gD-1 serum (Fig. 2). In denaturing gels, all of the proteins had mobilities similar to that of gD-1-(pRE4) (Fig. 2A, compare lanes 2 through 8 with lane 1). Slight differences [e.g., gD-1-(Cys-2), lane 3] can be accounted for by differences in oligosaccharide processing, as described later (see Fig. 4). However, in a nondenaturing gel (Fig. 2B), three of the mutant proteins, gD-1-(Cys-1) (lane 2), gD-1-(Cys-3) (lane 4), and gD-1-(Cys-5) (lane 6), migrated more slowly than the wild type, gD-1-(pRE4) (lane 1). These differences may be related to structural changes associated with alterations in disulfide bonding (43).

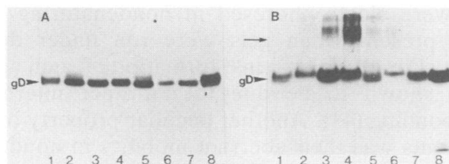


FIG. 2. Western blot analysis of gD-1-(pRE4) and the proteins produced by mutants Cys-1 through Cys-7 (see Fig. 1). Cytoplasmic extracts were prepared from COS-1 cells transfected with pRE4 (lane 1), or with mutants Cys-1 through Cys-7 (lanes 2 through 8), electrophoresed under denaturing (A) or nondenaturing (B) conditions (7), transferred to nitrocellulose (2), and reacted with polyclonal anti-gD-1 serum and then with iodinated protein A. The arrow in panel B indicates the position of the aggregates of gD-1.

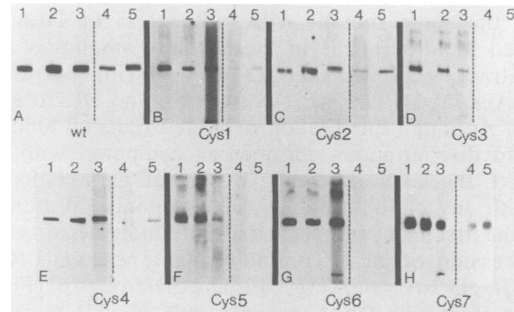


FIG. 3. Antigenic analysis of gD-1-(pRE4) and the seven cysteine mutants of gD-1. Cytoplasmic extracts were prepared from COS-1 cells transfected with pRE4 (A) or with mutants Cys-1 through Cys-7 (B through H) and were electrophoresed under nondenaturing conditions (7), transferred to nitrocellulose (2), and reacted with MAb 1D3 (group VII; lane 1), DL6 (group II; lane 2), 55S (group V; lane 3), DL11 (group I; lane 4), or 11S (group III; lane 5) and then with iodinated protein A. The nitrocellulose blots in panels A and H were exposed to film for 24 h. Lanes 1 to 3 in panels B through G were exposed to film for 24 h. To detect the reduced signal, lanes 4 and 5 of panels B through G were exposed for 48 h. (A) WT, Wild type.

Recently (11), we reported that several deletion mutants of gD expressed aggregated forms of the glycoprotein which were observed in nondenaturing gels. The higher-molecular-weight forms were not present under denaturing conditions. In each of these cases, a cysteine residue was deleted. Here, we found that for mutants Cys-1 through Cys-6, aggregates were present in nondenaturing gels (Fig. 2B, lanes 2 through 7, and Fig. 3B through G, lanes 1 to 3). In contrast, gD-1-(Cys-7) behaved like gD-1-(pRE4) in that no aggregates were seen in nondenaturing gels (Fig. 2B, compare lanes 1 and 8, and Fig. 3, compare panels A and H). Thus, it appears that aggregation is related to the absence of any one of the first six cysteines.

**Effect of cysteine-to-serine substitutions upon continuous and discontinuous epitopes of gD.** To examine the antigenic profile of each mutant, extracts were electrophoresed on native gels (7), prepared without a comb, and transferred to nitrocellulose (2). Strips of nitrocellulose were incubated with MAb. For gD-1-(pRE4) (Fig. 3A), a positive reaction was detected with MAbs 1D3 (lane 1), DL6 (lane 2), and 55S (lane 3), which react with continuous epitopes VII, II, and V, respectively. Positive reactions were also detected with MAbs to discontinuous epitopes, i.e., DL11 (group I; lane 4), 11S (group III; lane 5), and DL2 (group VI; data not shown). These results were the same as in previous studies (11) and serve as a basis for comparison of the reactivity of the cysteine-to-serine mutants. Each mutant protein reacted with MAbs 1D3, DL6, and 55S (Fig. 3, lanes 1, 2, and 3 in each panel). In two cases, gD-1-(Cys-3) (Fig. 3D, lane 3) and gD-1-(Cys-5) (Fig. 3F, lane 5), the reaction with 55S seemed reduced relative to that for 1D3 (Fig. 3D and F, lanes 1) or DL6 (Fig. 3D and F, lanes 2). These differences were not seen when the protein was Western blotted from a denaturing gel. Thus, it is possible that the 55S epitope was partially masked in the native gel by differences in conformation of these mutants. However, for each of the other mutants, the reactivities of 1D3, DL6, and 55S were equivalent. To evaluate the reactivity of each mutant form with antibodies DL11 and 11S, we compared the intensity of the signal with

that of the same mutant with either 1D3 or DL6. This corrected for differences in the absolute amount of gD in each extract. We found that gD-1-(Cys-7) (Fig. 3H) reacted with MAbs DL11 (lane 4), 11S (lane 5), and DL2 (data not shown). Although this mutant exhibited reduced binding of MAbs to discontinuous epitopes as compared with pRE4 (Fig. 3A), the level of binding was much greater than that seen with any of the other cysteine mutants. Mutations in any of the first six cysteines had a profoundly negative effect on expression of all discontinuous epitopes. gD-1-(Cys-3) (Fig. 3D), gD-1-(Cys-5) (Fig. 3F), and gD-1-(Cys-6) (Fig. 3G) failed to react with DL11 (group I; lanes 4), 11S (group III; lanes 5) or DL2 (group VI; data not shown). gD-1-(Cys-1) (Fig. 3B) and gD-1-(Cys-2) (Fig. 3C) reacted weakly with DL11 (lanes 4), 11S (lanes 5), and DL2 (data not shown). It should be noted that the film in lanes 4 and 5 of Fig. 3B through G was exposed for twice as long as that for lanes 1 to 3. In the case of gD-1-(Cys-4), the very weak reaction with DL11 (Fig. 3E, lane 4) could only be detected with much longer film exposure times (not shown). However, this mutant reacted somewhat better with 11S (Fig. 3E, lane 5). The results indicate that each mutation had some effect on the discontinuous epitopes of gD; however, the effect was greatest with changes in cysteines 1 through 6 and least with cysteine 7.

**Effect of cysteine-to-serine substitutions on extent of processing of gD-1 in transiently transfected COS-1 cells.** Recently (11), we showed that cells transfected with the wild-type gD-1 gene accumulated gD molecules which contained N-linked oligosaccharides primarily of the complex type and also contained O-linked oligosaccharides. This indicated that much of the accumulated protein had been processed to the product form at 48 h posttransfection. The glycoproteins produced by a series of deletion mutants exhibited alterations in the extent of processing, with the most pronounced effects occurring when the deletion involved removal of a cysteine residue; in those cases, the N-linked oligosaccharides on the mutant proteins were primarily or entirely of the high-mannose or precursor form. A similar experiment was carried out on the cysteine mutants. Extracts were treated with sugar-modifying enzymes, electrophoresed under denaturing conditions, transferred to nitrocellulose, and probed with polyclonal anti-gD-1 antibody (Fig. 4). An increase in the electrophoretic mobility of a treated sample indicates that the protein contained moieties sensitive to digestion by that enzyme. Thus, neuraminidase and *O*-glycanase (46) treatment increased the mobility of gD-1-(pRE4) (Fig. 4A, lanes 2 and 3), indicating the presence of sialic acid and O-linked oligosaccharides, respectively. The mobility shift with *O*-glycanase was small, but reproducible. Endo H, which digests N-linked high-mannose oligosaccharides (47) (Fig. 4A, lane 4), caused a slight increase in mobility; endo F treatment (lanes 5), which removes both high-mannose and complex oligosaccharides (20), significantly increased the mobility of the glycoprotein. These results indicate that the N-linked oligosaccharides of gD-1-(pRE4) were primarily of the complex or product form and that O-linked oligosaccharides and sialic acid were also present (11, 45).

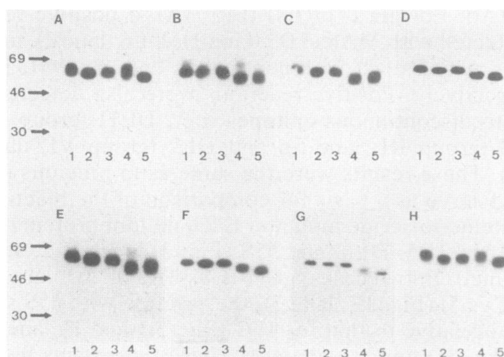


FIG. 4. Effect of sugar-modifying enzymes on gD-1 expressed in COS-1 cells transfected with pRE4 (A; WT, wild-type gD-1) or with plasmids containing each of the mutants Cys-1 through Cys-7 (B through H). Cytoplasmic extracts were treated with enzyme, electrophoresed under denaturing conditions, transferred to nitrocellulose (2), and reacted with anti-gD-1 serum. The enzymes used in each panel were: lane 1, mock-digested, no enzyme; lane 2, neuraminidase; lane 3, *O*-glycanase; lane 4, endo H; lane 5, endo F.

gD-1-(Cys-7) (Fig. 4H, lanes 1 through 5) showed the same mobility shifts as gD-1-(pRE4) in response to the different sugar-modifying enzymes, indicating that this mutation had no obvious effect on oligosaccharide processing. Two other mutant proteins, gD-1-(Cys-1) (Fig. 4B) and gD-1-(Cys-4) (Fig. 4E), contained oligosaccharides which were partially processed beyond the precursor stage, as evidenced by the sensitivity of one of these proteins to neuraminidase (compare lanes 1 and 2 in Fig. 4E) and their partial resistance to endo H (Fig. 4B and E, lanes 4). We estimate that approximately 5 to 10% of the total gD-1 present at 48 h posttransfection was endo H resistant in these two cases. Four mutant proteins, gD-1-(Cys-2) (Fig. 4C), gD-1-(Cys-3) (Fig. 4D), gD-1-(Cys-5) (Fig. 4F), and gD-1-(Cys-6) (Fig. 4G), contained only high-mannose oligosaccharides. In each case, the protein was not sensitive to neuraminidase (Fig. 4B through G, lanes 2) or *O*-glycanase (lanes 3), but was equally sensitive to endo H (lanes 4) and endo F (lanes 5). These results suggest that alteration of any of the first six cysteine residues in gD-1 caused a marked change in the extent of oligosaccharide processing. Alternatively, the absence of further processed forms of the glycoproteins could be due to enhanced degradation.

## DISCUSSION

Our findings show that substitution of a serine for any one of the first six cysteine codons in the gD-1 gene resulted in major changes in the properties of the expressed protein. In contrast, alteration of cysteine 7 had less apparent effect on these properties. First, gD-1-(Cys-1) through gD-1-(Cys-6) exhibited a dramatic decrease in reactivity with MAbs specific for discontinuous epitopes; the magnitude of this effect varied from a major reduction in antibody binding to a complete loss. Second, each of these proteins formed higher-molecular-weight aggregates which were observed when proteins were electrophoresed in nondenaturing gels, but were not present when gels were run under denaturing conditions. For other proteins, formation of such aggregates has been shown to be due to improper intermolecular disulfide bonding (43). Another peculiar property of three of these mutants was their aberrant mobility in nondenaturing gels. It has been demonstrated that serine protease zymogens show marked differences in mobility shifts in gels containing 0.1% sodium dodecyl sulfate, depending on the extent of formation or disruption of disulfide bonds. "In the presence of intact disulfide bonds, these proteins are more compact and migrate further into the gel than their reduced and carboxyamidomethylated counterparts" (43). We speculate that the absence of cysteines 1, 3, and 5 leads to

incomplete intramolecular disulfide bonding among the remaining cysteines. Third, gD-1-(Cys-1) through gD-1-(Cys-6) were underglycosylated as compared with gD-1-(pRE4) or with gD-1-(Cys-7). In recent studies (11), we found a similar set of properties in three gD-1 deletion mutants which lacked either cysteine 1 or cysteine 6. We suggested that the general loss of conformation was due either to deletion of specific amino acids in several epitopes or to absence of a critical cysteine residue. Here, we showed directly that the absence of cysteine 6 alone led to the loss of three discontinuous epitopes. We conclude that a disulfide bond involving cysteine 6 is critical for the proper tertiary structure of gD-1. Furthermore, each of the first six cysteines contributes to protein conformations necessary for recognition of MAbs against discontinuous epitopes and for proper processing of gD in the cell.

These observations led to several conclusions and some speculation. The finding that gD-1-(Cys-7) was similar to gD-1-(pRE4) suggests that cysteine 7 may not be disulfide bonded to any of the upstream cysteine residues. Two other observations support this idea. First, two truncated forms of gD-1 (residues 1–287 and 1–275) which lack cysteine 7 were fully reactive with MAbs to discontinuous epitopes (7). Second, gD-2, which lacks cysteine 7, reacts with MAb groups I and III. Although cysteine 7 appears not to be involved in intramolecular disulfide bonding, it may be important in the formation of the gD-1 dimer observed at the plasma membrane of HSV-1-infected cells (25). A similar form of gD-1 is routinely detected after immunosorbent purification of gD-1 isolated from infected (19) or transfected cells (D. Long, G. H. Cohen, and R. J. Eisenberg, unpublished results). gD-2 has never been observed to form a dimer under the above conditions.

Cysteines 1 through 6 appear to be necessary for full activity of the discontinuous epitopes and for proper oligosaccharide processing of gD. On the basis of the results presented here, we propose that they form three disulfide bridges, and that cysteine 7 is normally unpaired. A somewhat analogous situation was observed for interleukin 2 (48), which contains three cysteine residues. Substitution of serine for cysteine residues at positions 58 and 105 substantially reduced biological activity. Alteration of the third cysteine had no effect. The authors (48) argued that cysteines 58 and 105 might be disulfide bonded to each other and that the third cysteine was unpaired. Although our data suggest that cysteine 7 is unpaired, we cannot draw conclusions concerning disulfide bond patterns within cysteines 1 through 6. One might expect that if a particular disulfide bond were essential for expression of a particular epitope, loss of either cysteine in that pair would lead to the total loss of reactivity for that epitope. However, we found that gD-1 proteins expressed by three mutants (Cys-3, Cys-5, and Cys-6) were in that category, whereas the glycoprotein expressed by three other mutants (Cys-1, Cys-2, and Cys-4) retained some affinity for MAbs DL11, 11S, and DL2. These results make it impossible to pair cysteines on the basis of antigenic activity and emphasize the need for chemical studies to determine the disulfide bonding pattern of gD.

Our results indicate that alteration of cysteines 1 through 6 alters the extent of processing of oligosaccharides on gD-1 from the high-mannose to the complex form. The importance of conformation for correct processing and transport has been implicated in studies of other viral glycoproteins (24, 41). For six of the seven cysteine mutants of gD-1, there did appear to be a correlation between correct conformation, as measured by reactivity with MAbs DL11 (group I), 11S (group III), and DL2 (group VI), and extent of processing, as measured by sensitivity to neuraminidase and *O*-glycanase and insensitivity to endo H. More specifically, gD-1-(Cys-7) closely resembled wild-type gD [gD-1-(pRE4)] in conformation and processing. Three mutant proteins, gD-1-(Cys-3), gD-1-(Cys-5), and gD-1-(Cys-6), did not contain discontinuous epitopes and did not contain complex oligosaccharides. Two mutants, gD-1-(Cys-1) and gD-1-(Cys-4), reacted weakly with MAbs DL11, 11S, or DL2, and approximately 5 to 10% of the expressed glycoprotein was present as the product form at 48 h posttransfection. In contrast, gD-1-(Cys-2) contained no complex oligosaccharides, yet retained discontinuous epitopes. It is possible that processing is more sensitive to conformational differences in this mutant than is antibody binding. Alternatively, this mutant could be more sensitive to degradation than other mutants.

Further studies are needed to determine the effect of cysteine-to-serine changes on synthesis, processing, and transport of gD in transfected cells. It is possible that conformational changes inhibit the transport of these gD mutants into the medial Golgi (24, 41) and thereby prevent further processing. Alternatively, transport may occur, but incorrect folding of gD may prevent interaction of oligosaccharides with processing enzymes in the medial Golgi. Third, it is possible that incorrect folding directs gD to another cell compartment in which oligosaccharide processing does not occur. Fourth, the protein might be degraded before or as soon as it is further processed. Pulse-chase studies and fluorescence microscopic analyses will enable us to address some of these questions.

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