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Expression of Herpes Simplex Virus Type 1 Glycoprotein D Deletion Mutants in Mammalian Cells

GARY H. COHEN,[1,2]* WESLEY C. WILCOX,[2,3] DONALD L. SODORA,[1,2,3] DEBORAH LONG,[1,2,3] JOSHUA Z. LEVIN,[4]† AND ROSELYN J. EISENBERG[2,3]

Department of Microbiology[1] and Center for Oral Health Research,[2] School of Dental Medicine; Department of Pathobiology, School of Veterinary Medicine[3]; and College of Arts and Sciences,[4] University of Pennsylvania, Philadelphia, Pennsylvania 19104-6003

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Glycoprotein D (gD) is a virion envelope component of herpes simplex virus types 1 and 2. We have previously defined seven monoclonal antibody (MAb) groups which recognize distinct epitopes on the mature gD-1 protein of 369 amino acids. MAb groups VII, II, and V recognize continuous epitopes at residues 11–19, 272–279, and 340–356, respectively. MAb groups I, III, IV, and VI recognize discontinuous epitopes. Recent studies have focused on epitopes I, III, and VI. Using truncated forms of gD generated by recombinant DNA methods and proteolysis, epitopes III, IV, and VI were located within amino acids 1–233. A portion of discontinuous epitope I was located in a region within residues 233–275. For this study, we used recombinant DNA methods to create mutations in the gD-1 gene and studied the effects of those mutations on gD as expressed in mammalian cells. Plasmid pRE4, containing the coding sequence of gD-1 and the Rous sarcoma virus long terminal repeat promoter, was transfected into mammalian cells. The expressed protein, gD-1-(pRE4), was identical in size and antigenic properties to gD-1 from infected cells. Six in-frame deletion mutations were subsequently constructed by using restriction enzymes to excise portions of the gD-1 gene. Plasmids carrying these mutated forms were transfected into cells, and the corresponding proteins were examined at 48 h posttransfection for antigenicity and glycosylation patterns. Three deletions of varying size were located downstream of residue 233. Analysis of these mutants showed that amino acids within the region 234–244 were critical for binding of DL11 (group I), but not for other MAb groups. Three other deletion mutants lost all ability to bind MAbs which recognize discontinuous epitopes. In addition, much of the gD expressed by these mutants was observed to migrate as high-molecular-weight aggregated forms in nondenaturing gels. Each of these mutations involved the loss of a cysteine residue, suggesting that disulfide linkages play an essential role in the formation of discontinuous epitopes. The extent of glycosylation of the mutant gD molecules accumulated at 48 h posttransfection suggested altered carbohydrate processing. In one case, there was evidence for increased O-linked glycosylation. Those proteins which had lost a cysteine residue as part of the deletion did not accumulate molecules processed beyond the high-mannose stage. The results suggest that carbohydrate processing during synthesis of gD is very sensitive to alterations in structure, particularly changes involving cysteine residues.

Glycoprotein D (gD) is a virion envelope component of herpes simplex virus (HSV) types 1 (HSV-1) and 2 (HSV-2) which stimulates production of high titers of virus-neutralizing antibody (8, 10, 18, 20, 21, 28, 33, 34, 37, 38, 41, 45) and is likely to play an important role in the initial stages of viral infection (24, 25, 28, 37, 38). gD has been shown to protect animals from HSV challenge (6, 33, 34, 40) and is a subunit vaccine candidate. The gene for gD of HSV-1 (gD-1) codes for a polypeptide of 394 amino acids (see Fig. 1 and 2) (52). The protein contains a signal peptide of 25 amino acids (52), which is cleaved during processing (17, 36) to yield a mature protein of 369 amino acids. In addition, gD contains three N-linked oligosaccharides (12, 52), O-linked carbohydrate (30), and a hydrophobic transmembrane anchor sequence near the carboxy terminus (36, 53).

Our goal in this and previous studies (8, 9, 15, 18, 19, 54) has been to localize more precisely the discontinuous epitopes of gD. We have examined a large number of anti-gD monoclonal antibodies (MAbs), and they have been arranged into groups according to a number of shared biological and biochemical characteristics (9, 15, 18, 19, 28, 45). It is important to note that within an antibody group, the MAbs may not recognize precisely the same epitope, but rather may react with neighboring epitopes that constitute an antigenic site (1, 55). Anti-gD polyclonal antibodies and MAbs have been used to associate biological properties of gD with one or more epitopes. For example, group I MAbs have high titers of complement-independent neutralizing activity (15, 18, 38, 41, 42, 45), inhibit fusion of infected cells (37, 38), inhibit penetration of virus into cells (25, 28), and protect animals against virus challenge (13). Group III MAbs, which exhibit neutralizing activity in the presence of complement (15, 18, 45), may be clinically important in humans, as three human anti-HSV MAbs are gD specific and are in this group (V. Isola, C. Desgranges, G. H. Cohen, and R. J. Eisenberg, manuscript in preparation). Antibodies in both groups are type common, i.e., they react with both gD-1 and gD-2 (from HSV-2). Group VI MAbs (15, 18, 45) have been shown to inhibit adsorption of virus to cells (24).

Information has been obtained about the structure and position of gD epitopes. Groups I, III, IV, and VI recognize the protein in its native but not denatured (reduced and alkylated) form (9, 19). The corresponding epitopes are referred to as discontinuous (1). Antibodies in groups VII,

* Corresponding author.
† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

II, and V are still able to react with the denatured protein (9, 19); the corresponding epitopes are defined as continuous. As diagrammed in Figure 2, the continuous epitopes have been precisely localized to residues 11–19, 272–279, and 340–356, respectively, of the mature form of gD-1 (3, 8, 9, 15, 18, 19, 36, 52). Synthetic peptides which mimic residues 1–23 induce virus-neutralizing antibodies and protect mice from a lethal or paralytic HSV-2 challenge (8, 14). In addition, passive immunization with MAbs from groups VII and II can protect mice from a lethal challenge (13, 25; G. H. Cohen, R. J. Eisenberg, and R. J. Dix, unpublished data). Blocking studies showed that MAb groups I and III react with different portions of the glycoprotein (19). To further localize discontinuous epitopes, we analyzed the antigenic activity of gD fragments generated by protease cleavage and of truncated forms of gD produced by recombinant DNA methods (8, 9, 15, 18, 19). These studies led to the conclusion that discontinuous epitopes III, IV, and VI were located entirely upstream of residue 233, but that a portion of epitope I was located between residues 233 and 275.

In the present studies, our approach was to make internal deletions in the isolated gD gene and to use a eucaryotic expression vector carrying the altered genes to test the effects of these mutations on gD expression in mammalian cells. Similar studies using procaryotic cells for expression showed that full-length gD-1 was synthesized, but did not contain any discontinuous epitopes (R. J. Eisenberg, V. Rinaldt, and G. H. Cohen, unpublished data). Three deletions of various sizes were located downstream of residue 233, between cysteines 6 and 7 (Fig. 2). Three deletions removed residues upstream of 233; two of these removed cysteine 6, and one removed cysteine 1. Antigenicity of gD was examined by testing the reactivity of the expressed protein with representative MAbs of the various groups. Effects of mutations on processing were examined by treating the expressed proteins with neuraminidase and endoglycosidases and examining their electrophoretic behavior. A deletion mutant lacking amino acids 243–286 expressed a protein which bound MAb DL11 (group I) at approximately 28% of the wild-type level; binding of DL11 was reduced to approximately 5% when amino acids 234–244 were deleted and was completely eliminated when residues 234–287 were deleted. Binding of antibodies in other groups was unaffected. Thus, amino acids within the region 234–244 are critical for binding of DL11 (group I). The extent to which each mutant protein was processed differed from that of the wild type. When the deletion involved removal of a cysteine residue (either cysteine 1 or cysteine 6), there was little or no evidence for accumulation of molecules containing complex oligosaccharides. These latter mutants shared two other properties: each lost all ability to bind MAbs which recognize discontinuous epitopes, and each formed high-molecular-weight aggregates which were dissociated under reducing conditions. The results suggest that cysteines 1 and 6 may participate in the disulfide-bonded structure of gD.

MATERIALS AND METHODS

Cell culture and virus. Conditions for the growth and maintenance of BHK cells and HSV-1 (Patton) were previously described (10, 20). Cells were infected at a multiplicity of infection of 5 PFU/cell and harvested at 18 h postinfection. Cytoplasmic extracts were prepared as previously described (10). COS-1 cells were propagated in Dulbecco minimal medium supplemented with 10% fetal bovine serum.

Construction of recombinant plasmids for expression of gD. Restriction and other DNA-modifying enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, or Boehringer Mannheim and were used as directed by the manufacturer. Standard methods (35) were used to prepare plasmid and phage vector DNAs for subcloning the gD gene and preparing the deletion mutants. Each of the mutants described below was sequenced for at least 100 bases on either side of the deletion (7, 44).

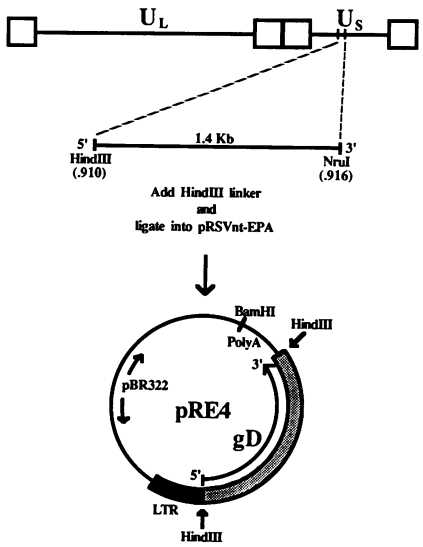

FIG. 1. Schematic representation of the HSV-1 genome (prototype arrangement) showing the position of the gD-1 gene and features of the gD expression plasmid pRE4. A 1.4-kilobase *Hin*dIII-*Nru*I fragment containing the entire coding region of gD-1, but lacking the promoter, was excised from the plasmid pRWF6 (52). A *Hin*dIII linker was added, and the fragment was cloned into the vector pRSV-nt EPA.

(i) Plasmid pRE4. Figure 1 shows the principal features of plasmid pRE4, which was designed to express full-length gD-1 when transfected into COS-1 cells. The parent vector, pRSV-nt EPA (provided by S. Carswell and J. Alwine), contains the long terminal repeat of Rous sarcoma virus as a promoter; a unique *Hin*dIII site adjacent to the long terminal repeat, used for insertion of the gD coding sequence; and the simian virus 40 early polyadenylation signal. pRSV-nt EPA is similar to pRSV0 (5), but lacks the simian virus 40 splice acceptor sequences. The gD gene was subcloned from plasmid pRWF6 (52) (supplied by Roger Watson), which contained the BamHI J fragment of HSV-1 (Patton). The 1.4-kilobase *Hin*dIII-to-*Nru*I fragment (map units 0.910 to 0.916, Fig. 1) was isolated, a *Hin*dIII linker was added to the 3′ end, and the fragment was ligated into *Hin*dIII-digested pRSV-nt EPA. The plasmid was transformed into *Escherichia coli* DH5 alpha, and plasmid DNA was isolated and purified through two cycles of CsCl-ethidium bromide centrifugation (35). The protein expressed after transfection of COS-1 cells was designated gD-1-(pRE4).

(ii) Construction of pWW52 (amino acids 234–287 deleted) and pWW49 (194–287 deleted). Plasmids pWW52 and pWW49 were obtained by digesting pRE4 DNA with *Acc*I and *Nar*I. The 5′ overhangs were filled in with the Klenow fragment, and the blunt ends were ligated. The predicted sequence contains a new serine codon at residue 234. Since no new restriction sites were created, DNAs from several clones lacking the *Acc*I and *Nar*I sites were sequenced (7, 44). Plasmid pWW52 exhibited the base sequence expected for the *Acc*I-*Nar*I deletion. However, plasmid pWW49 had

lost an additional 120 bases 5′ to the original *Acc*I site, thus creating an in-frame deletant lacking amino acids 194–287. The expressed proteins are designated gD-1-(Δ234-287) and gD-1-(Δ194-287).

(iii) Plasmid pDL24 (amino acids 243–286 deleted). pRE4 DNA was digested with *Apa*I, followed by treatment with mung bean nuclease to form blunt ends. The DNA was then digested with *Nar*I and filled in with Klenow fragment, and the blunt ends were ligated. This deletion recreates the alanine codon at 287. The expressed protein is designated gD-1-(Δ243–286).

(iv) Plasmid pWW17 (residues 234–244 deleted). pRE4 DNA was digested with *Acc*I and *Apa*I, treated with mung bean nuclease to remove 5′ and 3′ overhangs, and ligated. The resulting joint sequence created a unique *Bgl*I site and a new codon, GTC (valine), in place of the original valine codon (amino acid 233). The presence of the *Bgl*I site was used to identify clones containing the correct mutant form of the gD-1 gene. The expressed protein is designated gD-1-(Δ234-244).

(v) Plasmid pWW11 (amino acids −1 through +82 deleted). pRE4 DNA, which contains two *Sac*II sites, was digested with *Sac*II and religated. This deletion removes codons for the last amino acid of the signal sequence (glycine at −1) and the first 82 amino acids of the mature form of gD-1. The remainder of the signal sequence is connected to glycine (amino acid 83 of the normal sequence). The expressed protein is designated gD-1-(Δ minus 1-82).

(vi) Plasmid pWW13 (amino acids 196–207 deleted). The deletion in plasmid pWW13 was created by digestion with *Fsp*I and *Stu*I. Because the vector contained an *Fsp*I site outside the gD gene, we first digested pRE4 with *Hin*dIII to remove the entire gD fragment. This fragment was isolated, circularized with T4 ligase, and then digested with *Fsp*I and *Stu*I. The resulting fragment was religated, creating a codon for proline. *Hin*dIII was used to linearize the DNA, and the DNA was then reinserted into the *Hin*dIII site of pRSV-nt EPA. The expressed protein is designated gD-1-(Δ196-207).

DNA transfection. The calcium phosphate coprecipitation procedure (27) was used with two modifications to transfect COS-1 cells. First, calcium phosphate-precipitated, supercoiled plasmid DNA was incubated with the cells for 16 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 post-transfection. Cells were harvested and cytoplasmic extracts were prepared as previously described for infected cells (10).

Polyclonal antibodies and MAbs. Preparation and characterization of antibodies have been described previously, as follows: rabbit anti-gD-1 serum (21); MAb DL11 (group I) (9); MAb DL6 (group II) (9); MAb 11S (group III) (18, 45), MAb 55S (group V) (18, 45); MAb DL2 (group VI) (9); MAb 1D3 (group VII) (9, 23). 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 (8, 9, 15, 19), blocking studies (19), neutralization activity (18, 45), reactions with synthetic peptides (8, 15, 19), and their location on gD fragments (9, 18, 19).

Western blot (immunoblot) analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins under denaturing and nondenaturing ("native") conditions was carried out as previously described (9). After electrophoresis, proteins were transferred to nitrocellulose (4, 50) and incubated with antibodies and then with iodinated protein A (ICN) as described previously (8, 9, 19). The nitrocellulose blots were exposed to Kodak XAR-5 film with a Cronex Lightning-Plus screen (Du Pont) at −70°C.

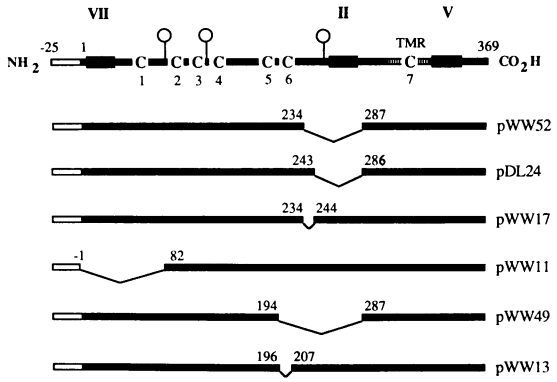

FIG. 2. Stick model of gD-1 and six deletion mutants. gD-1 contains a signal peptide (open box) of 25 amino acids (52) which is cleaved during cotranslational processing (17, 36) and a hydrophobic anchor sequence or transmembrane region (TMR) near the carboxy terminus (36, 53). In addition the protein contains seven cysteine residues (C) and three sites (balloons) for addition of N-linked oligosaccharides (12, 52). The positions of three continuous epitopes (8, 9, 15, 18, 19, 36, 52) at amino acids 11–19 (VII), 272–279 (II), and 340–356 (V) are depicted as solid boxes. The amino acids deleted from each mutant are also shown. In some cases, a new amino acid is inserted (see Materials and Methods for details).

Enzyme digestions. For each enzyme, 12 μl of cytoplasmic extract (representing approximately 1.8×10^4 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

Properties of a eucaryotic gD expression vector. The 1.4-kilobase *Hin*dIII-*Nru*I fragment containing the entire coding region of gD-1 (map units 0.910–0.916, Fig. 1), but lacking the promoter, was excised from the plasmid pRWF6 (52). A *Hin*dIII linker was added, and the fragment was cloned into the vector pRSV-nt EPA to yield the gD expression vector pRE4 (Fig. 1). A stick model of the gD-1 protein and one of each of the deletion mutants is shown in Fig. 2. The properties of each deletion mutant are summarized in Table 1.

COS-1 cells were transfected with pRE4 DNA, and a cytoplasmic extract was prepared. For comparison, a cytoplasmic extract was prepared from HSV-1 (Patton)-infected BHK cells. Proteins were separated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with polyclonal anti-gD-1 serum. gD-1 produced by pRE4-transfected cells [gD-1-(pRE4)] (Fig. 3A, lane 3) comigrated with gD-1 produced by HSV-1-infected cells (lane 2). No reaction was obtained with extracts of COS-1 cells transfected with a plasmid containing the gD gene positioned in the opposite orientation (data not

TABLE 1. Properties of HSV gD-1 deletion mutants

Plasmid designation	Protein designation	Reactivity with MAb groups:						Aggregates present
		Continuous[a]			Discontinuous[b]			
		VII	II	V	III	VI	I	
pRE4	gD-1-(pRE4)	+	+	+	+	+	+	−
pWW52	gD-1-(Δ234-287)	+	−	+	+	+	−	−
pDL24	gD-1-(Δ243-286)	+	−	+	+	+	+ (28%)[c]	−
pWW17	gD-1-(Δ234-244)	+	+	+	+	+	+ (5%)[c]	−
pWW11	gD-1-(Δ minus 1-82)	−	+	+	−	−	−	+
pWW49	gD-1-(Δ194-287)	+	−	+	−	−	−	+
pWW13	gD-1-(Δ196-207)	+	+	+	−	−	−	+

[a] MAbs to continuous epitopes were: group VII, 1D3; group II, DL6; group V, 55S.
[b] MAbs to discontinuous epitopes were: group I, DL11; group III, 11S; group VI, DL2.
[c] Expression was calculated as percent of that found for an equivalent amount of gD-1-(pRE4) (wild type). See legend to Fig. 5 for details.

shown). To further examine the antigenic properties of gD-1-(pRE4), the extract was electrophoresed on a native gel (9) and transferred to nitrocellulose (4, 50). Strips of nitrocellulose were incubated with polyclonal antibodies or MAbs (Fig. 3B). A positive reaction was detected with MAbs 1D3 (Fig. 3B, lane 1), DL6 (lane 2), and 55S (lane 3), which react with continuous epitopes VII, II, and V, respectively (Fig. 2 and Table 1). Positive reactions were also detected with MAbs to discontinuous epitopes, including DL11 (group I; Fig. 3B, lane 4), 11S (group III; lane 5), and DL2 (group VI; data not shown). We conclude that gD-1-(pRE4) synthesized in transfected cells has the same molecular size and antigenic structure as gD-1 produced in infected cells.

For each of the plasmids containing a deletion in gD-1 (Fig. 2 and Table 1), DNA was transfected into COS-1 cells, cytoplasmic extracts were prepared, and the expressed protein was analyzed in a fashion similar to gD-1-(pRE4) as described above. Each of the mutant forms of gD had a mobility consistent with its expected molecular size (Fig. 4), with two exceptions. First, gD-1-(Δ minus 1-82) (lane 7), which lacked 83 amino acids, migrated more slowly (apparent molecular weight of 53,000 [53K]) than did gD-1-(Δ243-286) (lane 6), which lacked 44 amino acids (42K). Second, gD-1-(Δ234-244) (lane 5), which lacked 11 amino acids, consisted of two polypeptides of 51K and 67K, the larger of which migrated more slowly than did gD-1-(pRE4) (60 K, lanes 3 and 10). For the latter mutant, the aberrant mobility of the 67K polypeptide appears to be due to addition of extra O-linked oligosaccharides (see Fig. 7D). However, in the case of gD-1-(Δ minus 1-82), the unexpectedly slow electrophoretic mobility appeared to be associated with the polypeptide itself, as the endo F-treated form of this protein (Fig. 7E, lane 5) migrated more slowly in a denaturing gel (45K) than did endo F-treated gD-1-(Δ243-286) (Fig. 7C, lane 5) (38K).

Analysis of continuous epitopes in the deletion mutants. Each of the mutant forms of gD reacted with MAb 55S, which recognizes residues 340–356 (Fig. 5 and 6, lanes 3), confirming that each deletion was in frame and that the expressed protein contained the correct carboxy terminus. Antibodies 1D3 and DL6 were useful in verifying deletions, since they react with residues 11–19 and 272–279, respectively. For example, gD-1-(Δ minus 1-82) lacked epitope VII (Fig. 6A, lane 1). Likewise, gD-1-(Δ234-287), gD-1-(Δ243-286), and gD-1-(Δ194-287) each lacked epitope II (Fig. 5A, 5B, and 6B, lanes 2). As expected, gD-1-(Δ234-244) and gD-1-(Δ196-207) contained each of the continuous epitopes (Fig. 5C and 6C, lanes 1 to 3).

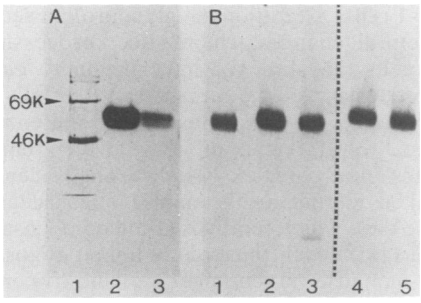

FIG. 3. Western blot analysis of gD-1-(pRE4). (A) Cytoplasmic extracts were prepared from pRE4-transfected COS-1 cells (lane 2) or from HSV-1 (Patton)-infected BHK cells (lane 3), electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gD-1 serum. Lane 1, Molecular weight markers. (B) Cytoplasmic extract of pRE4-transfected COS-1 cells was electrophoresed under nondenaturing conditions (9), transferred to nitrocellulose, and reacted with MAb 1D3 (lane 1), DL6 (lane 2), 55S (lane 3), DL11 (lane 4), or 11S (lane 5). gD-1-(pRE4) and each of the mutants were also tested with MAb DL2; however, the signal with this antibody was generally weak (9). In each case, proteins which reacted with 11S also reacted with DL2, and those which did not react with 11S did not react with DL2.

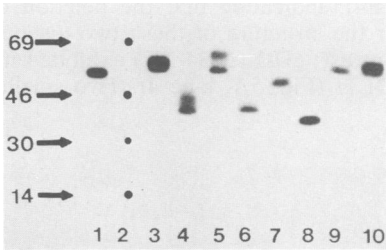

FIG. 4. Western blot analysis of gD-1-(pRE4) and deletion mutants. Cytoplasmic extracts were prepared from COS-1 cells transfected with each of the deletion mutants (Fig. 2 and Table 1), electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gD-1 serum. The apparent molecular size of each protein was calculated. Lane 1, gD-1-(pRE4), treated with endo F and run as a marker for unglycosylated gD-1, 50K; lane 2, dots indicate positions of molecular weight markers of 69K, 46K, 30K, and 14K; lane 3, gD-1-(pRE4), 60K; lane 4, gD-1-(Δ234-287), two polypeptides of 46K and 40K; lane 5, gD-1-(Δ234-244), two polypeptides of 67K and 51K; lane 6, gD-1-(Δ243-286), 42K; lane 7, gD-1-(Δ minus 1-82), 53K; lane 8, gD-1-(Δ194-287), 38K; lane 9, gD-1-(Δ196-207), 58K; lane 10, gD-1-(pRE4).

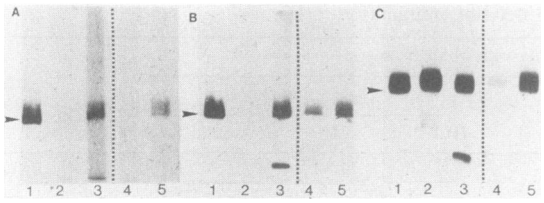

FIG. 5. Antigenic analysis of (A) gD-1-(Δ234-287), (B) gD-1-(Δ243-286), and (C) gD-1-(Δ234-244). Cytoplasmic extracts were electrophoresed in a combless 10% native gel (9), transferred to nitrocellulose, and reacted with MAb 1D3 (lane 1), DL6 (lane 2), 55S (lane 3), DL11 (lane 4), 11S (lane 5), or DL2 (not shown). To compare the level of antibody binding of each mutant with gD-1-(pRE4), the strips were all exposed to the same piece of X-ray film for the same time and quantitated by densitometry. For pRE4-1-(gD-1), the ratio of reactivity of DL11 to 55S was 1.12; for 11S to 55S it was 1.07. Similar ratios were obtained when 1D3 was the denominator. For gD-1-(Δ243-286), the ratio of DL11 to 55S was 0.31 or 28% of that of gD-1-(pRE4). For this same mutant, the 11S/55S binding ratio to gD-1-(Δ243-286) was 0.78, equal to 73% of gD-1-(pRE4). For gD-1-(Δ234-244), the ratio of DL11 to 55S was 0.056 or 5% of gD-1-(pRE4); the ratio of 11S to 55S was 0.94, or 92% of gD-1-(pRE4). The arrow in each panel indicates the position of the mutant monomeric form of gD-1.

Effect of deletions involving amino acids 234–287 on binding of MAbs to discontinuous epitopes. Our previous results (9) indicated that MAbs 11S (group III) and DL2 (group VI) react with the truncated fragment 1–233, but that DL11 (group I) does not. This antibody does react with another truncated form, 1–275, implicating amino acids between 233 and 275 in the binding of group I MAbs. Plasmids pWW17, pDL24, and pWW52 (Fig. 2 and Table 1) were constructed to further map the DL11 epitope. Reactions of the mutant forms of gD-1 with each MAb were compared with those obtained using gD-1-(pRE4) as the standard (Fig. 5 and 6). Comparison between parent and mutant forms of each discontinuous epitope were made by constructing a ratio of reactivity for antibody pairs such as DL11 (discontinuous) and 55S (continuous) (see legend to Fig. 5 for details).

Each of the deletion mutants in Fig. 5 reacted with MAb 11S (lane 5 in each panel) and MAb DL2 (data not shown). In each case, the level of reactivity was greater than 70% of that of gD-1-(pRE4), indicating that the deletion did not profoundly alter the structure of these two discontinuous epitopes. In contrast, gD-1-(Δ234-287) exhibited no detectable binding of DL11 (Fig. 5A, lane 4). Two smaller deletions, gD-1-(Δ243-286) and gD-1-(Δ234-244), reacted with DL11, but binding was reduced to 28 and 5%, respectively. These results suggest that residues within the region 234–244 are critical for DL11 binding and that downstream residues may also contribute.

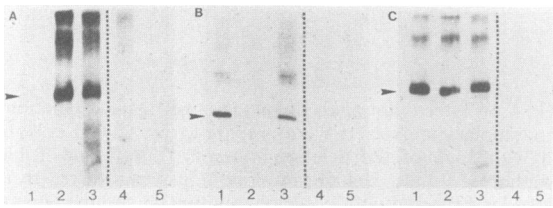

FIG. 6 Antigenic analysis of (A) gD-1-(Δ minus 1-82), (B) gD-1-(Δ194-287), (C) gD-1-(Δ196-207). Cytoplasmic extracts were electrophoresed in a combless 10% native gel (9), transferred to nitrocellulose, and reacted with MAb 1D3 (group VII; lane 1), DL6 (group II; lane 2), 55S (group V; lane 3), DL11 (group I; lane 4), 11S (group III; lane 5), or DL2 (group VI; not shown). In each case, proteins which failed to react with 11S did not react with DL2. The arrow in each panel indicates the position of the mutant monomeric form of gD-1.

Analysis of gD deletion mutants upstream of residue 233 with altered numbers of cysteine residues. gD deletion mutants were constructed to examine the contribution of upstream residues to the antigenic structure of gD. In each case, the deletion also removed a cysteine residue, and the expressed proteins shared certain properties.

(i) gD-1-(Δ minus 1-82). gD-1-(Δ minus 1-82), which lacks the first 82 amino acids of the mature form of gD, including cysteine 1 at residue 66, failed to react with MAb DL11 (Fig. 6A, lane 4), 11S (lane 5), or DL2 (data not shown). Interestingly, aggregated or multimeric forms of gD were observed in nondenaturing gels (Fig. 6A, lanes 2 through 4), which reacted with both polyclonal antibodies (not shown) and MAbs in groups II and V (see Fig. 6A, lanes 2 and 3). These forms were not seen under denaturing conditions (Fig. 4, lane 7).

(ii) gD-1-(Δ194-287). The gD-1-(Δ194-287) protein, which lacked 94 amino acids including cysteine 6, had properties similar to those of gD-1-(Δ minus 1-82), as it did not react with either DL11 (Fig. 6B, lane 4), 11S (Fig. 6B, lane 5) or DL2 (data not shown). Again, multimeric forms, which were not present under denaturing conditions (Fig. 4, lane 8), were observed in a nondenaturing gel (Fig. 6B, lanes 1 and 3).

(iii) gD-1-(Δ196-207). Protein gD-1-(Δ196-207) lacked 12 amino acids, including cysteine 6. It failed to react with DL11 (Fig. 6C, lane 4), 11S (Fig. 6C, lane 5), or DL2 (not shown) and exhibited multimeric forms under nondenaturing conditions (compare Fig. 4 lane 9 with Fig. 6, lanes 1 through 3).

Thus, mutant forms of gD-1 which lacked a cysteine formed high-molecular-weight aggregates and lacked several discontinuous epitopes (9, 19). These properties were independent of the size of the deletion. Each of these deletions could be removing residues specific to a particular epitope. However, we cannot draw any further conclusions, since the deletions apparently affected conformation in a general way.

Extent of processing of gD synthesized in transiently transfected COS-1 cells. Alterations of glycoprotein sequence can cause structural changes which affect processing of that protein in cells (26, 43). We have begun to examine the extent of processing of the mutant forms of gD-1 in transfected COS-1 cells by using sugar-modifying enzymes. The enzymes used were: (i) neuraminidase, which removes sialic acid residues from complex N-linked oligosaccharides and from O-linked (serine or threonine) oligosaccharides; (ii) *O*-glycanase (48), which removes O-linked oligosaccharides; (iii) endo H (49), which removes N-linked oligosaccharides of the high-mannose (precursor) type; and (iv) endo F (22), which removes N-linked oligosaccharides of both high-mannose and complex type. Loss of sensitivity to endo H and acquisition of sensitivity to neuraminidase or *O*-glycanase depend on transport of a glycoprotein from the endoplasmic reticulum to the Golgi apparatus, where N-linked oligosaccharides are modified and O-linked oligosaccharides are added (26, 43). Thus, changes in sensitivity to sugar-modifying enzymes also reflect alterations in transport of the glycoprotein.

Extracts of cells at 48 h posttransfection were treated with sugar-modifying enzymes and separated on a denaturing gel, transferred to nitrocellulose, and probed with polyclonal

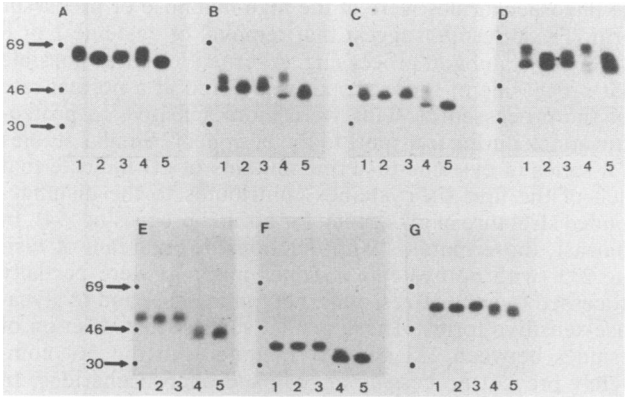

FIG. 7. Effect of sugar-modifying enzymes on gD-1 expressed in COS-1 cells transfected with pRE4 and plasmids containing gD deletions. Cytoplasmic extracts were treated with enzyme, electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gD-1 serum. (A) gD-1-(pRE4); (B) gD-1-(Δ234-287); (C) gD-1-(Δ243-286); (D) gD-1-(Δ234-244); (E) gD-1-(Δ minus 1-82); (F) gD-1-(Δ194-287); (G) gD-1-(Δ196-207). The enzymes used in each panel were: lane 1, mock-digested control; lane 2, neuraminidase; lane 3, *O*-glycanase; lane 4, endo H; lane 5, endo F. The dots in each panel indicate the positions of molecular weight markers of 69K, 46K, and 30K.

anti-gD-1 antibody (Fig. 7). gD-1-(pRE4) was sensitive to neuraminidase (Fig. 7A, lane 2), *O*-glycanase (lane 3), and endo F (lane 5); endo H (lane 4) had little effect. The decrease in electrophoretic mobility after endo F treatment indicated that the molecular weight decreased from 60K to 50K, consistent with a loss of three N-linked oligosaccharides. Approximately 70 to 90% of the N-linked oligosaccharides on gD-1-(pRE4) were of the complex or product form. The protein also contained O-linked oligosaccharides and sialic acid.

(i) Effect of deletions downstream of 233 on extent of processing of gD in transfected cells. Mutant glycoproteins with deletions between amino acids 233 and 287 exhibited alterations in processing as compared with gD-1-(pRE4). In each case the protein contained a significant amount of endo H-sensitive (high-mannose) oligosaccharide (Fig. 7B, C, and D, lanes 4). However, the protein also contained endo H-insensitive, endo F-sensitive oligosaccharides (Fig. 7B, C, and D, compare lanes 4 and 5). In each case, the decrease in electrophoretic mobility was consistent with removal of all N-linked oligosaccharides. gD-1-(Δ234-244) also contained a significant amount of O-linked oligosaccharide (Fig. 7D, lane 3), more than was seen with gD-1-(pRE4) (Fig. 7A, lane 3). Increased O-linked glycosylation probably accounts for the difference in electrophoretic mobility noted previously between gD-1-(pRE4) and the larger-molecular-weight form of gD-1-(Δ234-244) (Fig. 5, compare lanes 3 and 5). The results showed that some Golgi-associated processing of these mutant forms of gD-1 took place.

(ii) Effect of deletions upstream of 233 which remove a cysteine residue on the extent of processing of gD in transfected cells. The glycoprotein found in cells transfected by gD-1-(Δ minus 1-82), gD-1-(Δ194-287), and gD-1-(Δ196-207) contained oligosaccharides almost exclusively of the high-mannose or precursor type (Fig. 7E, F, and G). The proteins were equally sensitive to endo H and endo F digestion, as indicated by the similar electrophoretic mobility of the treated forms (lanes 4 and 5 in Fig. 7E through G). Neuraminidase had a slight effect on the mobility of gD-1-(Δ minus 1-82) (Fig. 7E, lane 2) but no effect on gD-1-(Δ194-287) (Fig. 7F, lane 2) or gD-1-(Δ196-207) (Fig. 7G, lane 2). The mobility of the proteins was unaffected by *O*-glycanase. Thus, at 48 h posttransfection, these proteins contained only high-mannose-type N-linked oligosaccharides, suggesting that processing had not proceeded past the precursor stage. Alternatively, the absence of detectable complex oligosaccharides could have been caused by proteolysis of the glycoprotein just before or after further processing.

DISCUSSION

Our goal in this and previous studies (8, 9, 15, 18, 19, 54) has been to localize more precisely the discontinuous epitopes of gD. Our current approach was to make in-frame deletion mutations in the isolated gD gene and to use a eucaryotic expression vector, pRE4, to test the effect of those mutations on antigenic activity. In addition to epitope mapping, this approach has enabled us to explore the effect of mutations on the native structure of gD, employing the presence or absence of discontinuous epitopes as a measure of that structure. A similar approach was used to probe the functional domains of the *ras* p21 protein (31). As a first step, we showed that gD-1-(pRE4) had an antigenic profile indistinguishable from that of authentic gD-1 synthesized after virus infection (8, 9, 15, 18, 19). These results confirm and extend those obtained previously by using both the gD-1 and gD-2 genes in expression vectors to transfect mammalian cells (2, 29). In analyzing the protein expressed by each deletion mutant, MAbs to the three continuous epitopes, 1D3 (group VII, residues 11–19), DL6 (group II, residues 272–279), and 55S (group V, residues 340–356), were invaluable for confirming the deletion and showing that it was in frame and for estimating the total level of gD expression.

Fine mapping of epitope I of gD. Since antibodies in group I had been associated with the major neutralizing site of gD (9, 15, 18, 19, 24) and with other activities including fusion (24, 37, 38) and penetration (25, 28), we constructed a series of gD deletion mutants which would allow us to study epitope I in more detail. Deletions which involved residues between 234 and 287 provided some important information. As expected, gD-1-(Δ234-287) had the same antigenic characteristics as the truncated form, amino acids 1–233 (9), i.e., it reacted with MAbs 11S (group III) and DL2 (group VI), but failed to react with DL11 (group I). However, gD-1-(Δ243-286) and gD-1-(Δ234-244) both reacted with DL11, but at reduced levels [28 and 5% of that of gD-1-(pRE4), respectively]. Because residues 243 and 244 are missing in both mutants, one or both amino acids may be a part of epitope I. Alternatively, the reduction in binding of DL11 to gD-1-(Δ243-286) could be due to the absence of downstream residues. Most importantly, the results suggest that residues within 234–244 are critical for binding of DL11, and we propose that one or more amino acids in this small stretch are part of epitope I.

Analysis of deletion mutants lacking a cysteine residue. Three mutants which contained deletions upstream of residue 234 were constructed to map discontinuous epitopes that might be within the 1–234 region. However, each deletion also removed a cysteine residue. The expressed proteins shared several properties. First, each contained higher-molecular-weight or aggregated forms; second, processing of the N-linked oligosaccharides to the product form was markedly reduced and no O-linked oligosaccharides were present; and third, each protein lacked discontinuous epitopes I, III, and VI. We suggest that these effects are due

more to the lack of a critical cysteine residue than to the loss of amino acids specific for any one epitope. For example, two mutant proteins, gD-1-(Δ194-287) and gD-1-(Δ196-207), each lacked cysteine 6 at residue 202. The two proteins exhibited very similar properties in spite of the large difference in the size of the deletion. Furthermore, the properties of these two mutants closely resemble those of a mutant form of gD-1, in which cysteine 6 was replaced by a serine (54). Thus, the results suggest that cysteine 6 is critical for proper folding and processing of gD-1, probably as part of a disulfide-bonded pair. Further efforts at epitope mapping in this region will have to avoid making changes that affect the ability of cysteine 6 to form the proper disulfide bond.

The third deletion mutant lacked the first 82 amino acids of mature gD-1, including cysteine 1 at residue 66, and shared the properties of the above two mutants. In addition, however, gD-1-(Δ minus 1-82) exhibited an aberrant electrophoretic mobility (Fig. 4, lane 7) in that it migrated more slowly than gD-1-(Δ243-286) (Fig. 4, lane 6), which lacked fewer amino acids. One possible explanation is that the signal peptide was not cleaved during translational processing of this mutant. Digestion of the gD gene with *Sac*II removed the codon for the last amino acid of the gD-1 signal peptide (glycine). Religation placed another glycine, normally amino acid 83, in that position, thus restoring the signal peptide sequence of gD-1. However, amino acids downstream of the signal peptide would differ in the mutant. According to the "−1, −3" rule (51), signal peptidase appears to require certain upstream signals; no specific downstream signals have been identified. Thus, it would be of interest to determine whether the signal peptide is cleaved during processing of gD-1-(Δ minus 1-82). If it is not, this would implicate downstream residues in the recognition process. In addition, the noncleaved signal peptide might contribute to the aberrant conformation of gD-1-(Δ minus 1-82). Recently (54), we constructed a mutant, gD-1-(Cys-1), in which cysteine 1 was replaced by a serine. This alteration had profound effects on both antigenic structure and extent of processing, indicating that cysteine 1 participates in an intramolecular disulfide bond which is important for proper folding of gD. However, unlike gD-1-(Δ minus 1-82), gD-1-(Cys-1) did exhibit some reactivity with MAbs to discontinuous epitopes. Studies are planned to create smaller deletions in the region 1–82 which do not remove cysteine 1, or which alter amino acids immediately downstream of the signal peptide, to continue epitope mapping in this region.

Effects of deletions on extent of processing of gD. Earlier studies (12) showed that all three potential sites for N-linked glycosylation of gD (32, 51, 52) are utilized. Some O-linked oligosaccharides are also present in gD-1 (30, 39). In infected cells, N-linked oligosaccharides are processed from the high-mannose oligosaccharide chains of the precursor, pgD-1, to the complex oligosaccharides of the product, gD-1 (10, 11, 16, 29, 46, 47). At 18 h postinfection, the two forms are present in approximately equal quantities (21, 29), although only the product form is found in the virion envelope (16, 47). In transfected cells, processing appears to be more rapid and efficient (2, 29) and gD is found primarily in the product form. We found a similar result for gD-1-(pRE4), in which approximately 70 to 90% of the protein was in the product form. For other glycoproteins, alterations of protein structure affect processing, transport, and degradation (26, 43). Here, we found that the extent of processing of each of the genetically altered gD proteins was different from that of gD-1-(pRE4) and seemed to follow two patterns. When the protein lacked either cysteine 1 or cysteine 6, virtually all of the oligosaccharides were in the high-mannose or precursor form. These results suggest that removal of cysteine 1 or 6 profoundly inhibited processing. Alternatively, it is possible that processing of these proteins occurred at a normal rate but the processed proteins were more sensitive to proteolytic attack during transport to the periphery. Similar studies of a series of cysteine-to-serine mutants of gD indicate that each of the first six cysteines contributes to the disulfide-bonded structure of gD needed for normal processing (54). In contrast, those mutants with deletions downstream of residue 233 (with no cysteine residues missing) were partially processed to endo H-resistant, neuraminidase- and *O*-glycanase-sensitive forms. These results suggest that deletion of residues between 233 and 287 inhibited but did not completely prevent processing of N-linked oligosaccharides. In one case, the deletion led to an increase in O-linked glycosylation. However, it is important to note that the studies presented here measured steady-state levels of glycosylation of 48 h posttransfection. Pulse-chase studies will be carried out to assess the effect of each mutation on the rate of processing and transport. Studies are also planned to determine whether the alterations in processing are accompanied by changes in transport of the glycoprotein to the transfected cell surface.

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