Antigenic and Functional Analysis of a Neutralization Site of HSV-1 Glycoprotein D

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Herpes simplex virus glycoprotein D is a component of the virion envelope and appears to be involved in attachment, penetration, and cell fusion. Monoclonal antibodies (MAbs) against this protein can be arranged in groups, on the basis of a number of biological and biochemical properties. Group I antibodies are type-common, have high complement-independent neutralization titers, recognize discontinuous (conformational) epitopes, and block each other in a binding assay. The sum of their epitopes constitutes antigenic site I of gD. Using a panel of neutralization-resistant mutants, we previously found that group I MAbs can be divided into two subgroups, Ia and Ib, such that mutations selected with Ia antibodies have little or no effect on binding and neutralization by Ib antibodies, and vice versa. Antigenic site I therefore consists of two parts, Ia and Ib. We have now identified the point mutations which prevent neutralization. Two Ib MAbs (DL11 and 4S) selected a Ser to Asn change at residue 140; this alteration creates a new N-linked glycosylation site, which is used. A third Ib MAb (D2) selected a Gln to Leu change at 132. The mutation selected by the Ia MAb HD1 (Ser to Asn at residue 216) is identical to that selected by MAb LP2, another Ia antibody. By using oligonucleotide-directed mutagenesis, we have produced gD genes with combinations of the above mutations. Attempts to recombine these genes into the virus genome were unsuccessful, suggesting that the combinations are lethal. This was confirmed by a complementation assay which measures the ability of gD transiently expressed in transfected Vero cells to rescue the production of infectious virus by the gD-minus mutant F-gDβ. © 1990 Academic Press, Inc.

INTRODUCTION

Glycoprotein D (gD) of herpes simplex virus (HSV) is involved in virus infection and pathogenesis, induces humoral and cellular immune responses (Dix et al., 1981; Eisenberg et al., 1982; Heber–Katz et al., 1985; Martin et al., 1987; Rouse and Lopez, 1984; Zarling et al., 1986), and protects animals against a lethal virus challenge (Berman et al., 1985; Chan, 1983; Cohen et al., 1985; Cremer et al., 1985; Dix and Mills, 1985; Eisenberg et al., 1985; Lasky et al., 1984; Long et al., 1984; Paoletti et al., 1984). It is present on the surface of purified virions (Stannard et al., 1987), and appears to be essential for virus replication in tissue culture, in that mutants lacking gD can attach to, but not penetrate, target cells (Johnson and Ligas, 1988; Ligas and Johnson, 1988). In addition, studies with monoclonal antibodies (MAbs) specific for gD have indicated possible roles in adsorption (Fuller and Spear, 1985), penetration (Fuller and Spear, 1987; Highlander et al., 1987), and cell fusion (Highlander et al., 1987; Noble et al., 1983). We have previously arranged anti-gD MAbs in several groups, on the basis of various charac-

teristic properties (Eisenberg and Cohen, 1986; Eisenberg et al., 1985; Isola et al., 1989; Muggeridge et al., 1989). MAbs in groups II, V, VII, and XI recognize denatured gD and define continuous epitopes. Antibodies in groups I, III, IV, and VI recognize discontinuous epitopes, and therefore bind only to native gD. Group I MAbs are type-common, have high complement-independent neutralization titers (Eisenberg et al., 1982; Fuller and Spear, 1985; Kümel et al., 1985; Minson et al., 1986; Pereira et al., 1980; Showalter et al., 1981), and inhibit penetration (Fuller and Spear, 1987; Highlander et al., 1987); some are able to inhibit cell fusion by syncytial strains of HSV-1 (Highlander et al., 1987; Noble et al., 1983).

We recently reported a detailed study of the relationships between seven group I MAbs (Muggeridge et al., 1988). Six of the antibodies blocked each other in a competitive binding assay with purified gD as the target antigen, so their epitopes on gD are clustered to form an antigenic site (site I). Analysis of a panel of monoclonal antibody-resistant (i.e., neutralization-resistant) mar mutants showed, however, that the antibodies fall into two subgroups (Ia and Ib). Mutants selected with Ia MAbs were still neutralized by Ib MAbs, and vice versa. The two subgroups can also be distin-

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guished by their reactivity with gD deletion mutants (Muggeridge *et al.*, 1988).

We have now used DNA sequencing to identify the changes responsible for resistance to neutralization, and the locations of these changes reveal a previously unrecognized part of antigenic site I, confirming its discontinuous nature. In addition, one of the mutations creates a new consensus site for N-linked glycosylation. We used oligonucleotide-directed mutagenesis to combine *mar* mutations within antigenic site I, and then studied the ability of these multiple mutants to function, using recombination and complementation assays.

MATERIALS AND METHODS

Cells and virus strains

CV1 and BHK cells were grown in Eagle's MEM, supplemented with 5% fetal bovine serum (FBS). Vero and COS-1 cells were grown in Dulbecco's modified MEM (DME) containing 5% FBS. VD60 cells were grown in DME containing 5% FBS and 1 mM histidinol (Ligas and Johnson, 1988). Isolation of the *mar* mutant viruses has been described previously (Highlander *et al.*, 1987; Minson *et al.*, 1986; Muggeridge *et al.*, 1988); MarD.4S was derived from the parent strain KOS, MarDL11 was derived from Patton, and MarD2.1 and MarD.HD1 were derived from KOS321. Isolation of the gD-minus virus, F-gDβ, has been described previously (Ligas and Johnson, 1988); F-gDβ was propagated in VD60 cells.

Antibodies

The antibodies used in this study are listed in Table 1. Of the group I MAbs, DL11 and D2 were isolated in the authors' laboratories; HD1 was kindly provided by Lenore Pereira, LP2 by Anthony Minson, and 4S by Martin Zweig. The other two group I MAbs, III-114-4 and III-174-1 (Para *et al.*, 1985), were not used in this study. DL6 is a group II MAb that recognizes a continuous epitope of gD-1 (residues 272 to 279 of the mature protein) (Eisenberg *et al.*, 1985; Isola *et al.*, 1989). 1D3 (Friedman *et al.*, 1984) and LP14 (Minson *et al.*, 1986) are group VII MAbs that recognize overlapping epitopes within residues 1 to 23 (Bosch *et al.*, 1987; Dietzschold *et al.*, 1984). DL2 is a group VI MAb that recognizes a discontinuous epitope (Cohen *et al.*, 1986). RIP is a group III MAb that recognizes a discontinuous epitope (Seigneurin *et al.*, 1983; Isola *et al.*, manuscript in preparation).

Isolation of viral DNA and cloning of *Bam*HI J fragments

Total intracellular viral DNA was isolated from BHK cells infected with virus at 0.01 PFU per cell, essentially

as described by Walboomers and Ter Schegget (1976). Centrifugation was for 16 hr at 50,000 rpm/20° in a Beckman 50Ti vertical rotor. The resulting band of virion DNA was removed with a syringe, extracted with butanol to remove ethidium bromide, and dialyzed against 10 mM Tris–HCl, pH 8.0, 1 mM EDTA. The DNA was digested with *Bam*HI and electrophoresed on a 0.8% agarose gel. Bands between 5 and 8 kb were electroeluted onto NA45 nitrocellulose, from which DNA was recovered by incubation in 20 mM Tris–HCl, pH 7.5, 1.0 M NaCl, followed by phenol/chloroform extraction and ethanol precipitation. These fragments were then cloned into the *Bam*HI site of pGEM1, using standard techniques (Maniatis *et al.*, 1982). Clones containing the *Bam*HI J fragment were identified by hybridization of a gD riboprobe to Southern blots of plasmid DNA.

DNA sequencing and oligonucleotide-directed mutagenesis

Double-stranded plasmid DNA was sequenced by the procedure of Chen and Seeburg (1985), using a set of oligonucleotide primers spaced at intervals along the gD gene. Mutagenesis of the gD gene cloned into the *Hind*III site of M13mp18 was performed by the method of Zoller and Smith (1982), as modified by Kunkel (1987). The sequences of the oligonucleotides were Gln 132 to Leu, CAGCGGGGCAGCGTTCGGA; Ser 140 to Asn, CGCTGAAGTTGTCATAGT; Ser 216 to Asn, ATCCCGATGTTGTCCACC; Ser 216 to Phe, GCATCCCGATGAAGTCCACCGTC. To create double and triple mutants, equimolar mixtures of the appropriate oligonucleotides were used. *Hind*III fragments containing mutated gD genes were excised from replicative-form phage DNA and inserted into pRSVnt EPA, a eukaryotic expression vector (Carswell *et al.*, 1986). Construction of the analogous plasmid pRE4, which expresses wild-type gD (Patton strain), has been described previously (Cohen *et al.*, 1988).

Antigenic analysis of mutants

Infection of CV1 cells and transfection of COS-1 cells were performed by previously described methods (Gorman *et al.*, 1982; Muggeridge *et al.*, 1988). Cytoplasmic extracts were prepared from these cells (Muggeridge *et al.*, 1988) and analyzed either by the native gel/Western blot procedure (Cohen *et al.*, 1986) or on nitrocellulose dot blots (Cohen *et al.*, 1984).

Native gel electrophoresis and Western blotting

The native polyacrylamide gel system and Western blotting procedure have been described previously (Cohen *et al.*, 1986). All gels used in this study con-

tained 10% acrylamide/0.1% bis-acrylamide (separating gel) and 5% acrylamide/0.3% bis-acrylamide (stacking gel).

Endoglycosidase F (endo F) digestion and nitrocellulose dot blots

Cytoplasmic extracts were incubated with endo F (Boehringer-Mannheim) for 15 min at 37° (0.05 units of enzyme per 15 μl of extract). Two-microliter samples were spotted onto nitrocellulose, then probed with MAbs followed by ^{125}I-Protein A (Cohen et al., 1984).

Construction of a cassette vector for recombination of gD mutants into the virus genome

Construction of the cassette vector pMM77 and its derivatives is outlined in Fig. 1. Plasmid pJB3 consists of a 3.6-kb Smal subclone of the HSV-1 BamHI J fragment (Betz et al., 1983), cloned into pACYC177 (Chang and Cohen, 1978); it includes the gD coding sequences, together with 0.7 and 1.6 kb of the upstream and downstream flanking sequences, respectively. pUC18ΔHN was derived from pUC18 by digestion with HindIII and NarI, followed by religation, which destroyed both sites. The Smal fragment from pJB3 was recloned into the Smal site of pUC18ΔHN to form pMM77. Since pMM77 has HindIII and NarI sites only within the viral insert, partial digestion with these enzymes produced a 5.5-kb fragment and a 1-kb fragment, the latter including the codons for amino acids 1 to 287 of the mature gD protein. The 5.5-kb fragment was isolated and ligated to 1-kb HindIII–NarI fragments from mutated gD genes. The resulting plasmids were named pMM82 (140 and 216 mutations) and pMM89 (132, 140, and 216 mutations).

Transfection and isolation of viral recombinants

Freshly trypsinized Vero cells were transfected by the procedure of DeLuca et al. (1985), with the exception that the glycerol shock was omitted. One microgram of virion DNA was used, together with 1 μg of Smal-digested plasmid DNA (either pMM82 or pMM89), and 10 μg of salmon sperm DNA. Virus was harvested after 4 to 5 days, when CPE was widespread, and replated on CV1 monolayers for screening. Recombinants were identified by their reaction with MAbs in an immunoperoxidase (black plaque) assay (Holland et al., 1983; Kousoulas et al., 1984).

Immunoperoxidase assay

This procedure is modified from that described by Holland et al. (1983) and Kousoulas et al. (1984). Cell monolayers were washed once with DME containing
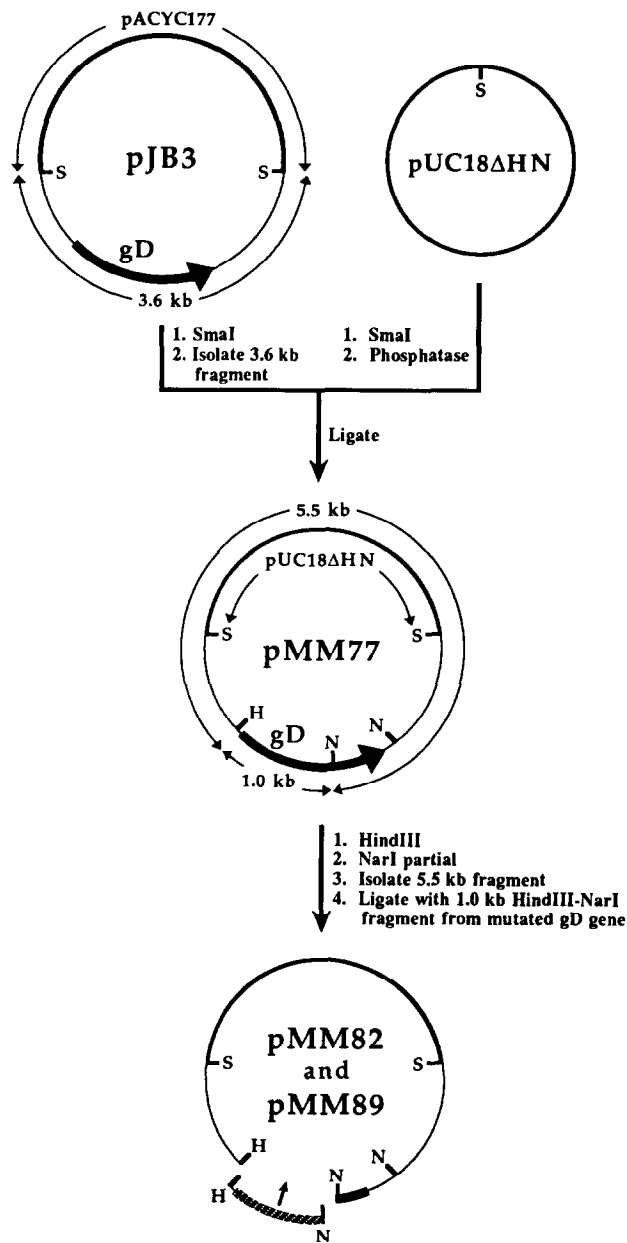

Fig. 1. Construction of cassette vector pMM77 and its derivatives. The 3.6-kb Smal insert containing the gD gene was moved from pJB3 to pUCΔHN so that a HindIII/NarI partial digest could be used to remove most of the gD coding sequence. The resulting gap was filled with the corresponding 1.0-kb HindIII/NarI fragment from mutant gD plasmids; pMM82 contains the fragment from pMM76, and pMM89 contains the fragment from pMM88 (see Results). S, Smal; H, HindIII; N, NarI.

5% FBS, then incubated in DME containing 5% FBS and an appropriate MAb (1 to 1000 dilution) for 1 hr at room temperature (RT). After two washes with DME, the cells were incubated in DME containing 1% FBS and 2 μl/ml protein A-peroxidase (Boehringer-Mannheim) for 1 hr at RT. The cells were then washed once with DME and once with PBS, before incubation with

peroxidase substrate for 5 min at RT. The substrate was prepared by adding 5 µl of 30% H_2O_2 and 5 mg of 4-chloro-1-naphthol in 0.5 ml of ethanol to 50 ml of PBS.

Complementation assay

The procedure is derived from that used by Cai et al. (1988) for the analysis of HSV glycoprotein B mutants. Sixty-millimeter petri dishes were seeded with 8×10^5 Vero cells, and transfected with DNA/calcium phosphate precipitates the next day. Ten to 30 µg of plasmid DNA in 219 µl of dH_2O was mixed with 31 µl of 2 M $CaCl_2$, then added to 250 µl of 2× HBS (0.27 M NaCl, 10 mM KCl, 1.4 mM NaH_2PO_4, 42 mM HEPES, pH 7.08). After 15 min, the suspension was added to a subconfluent monolayer of Vero cells, which had been fed with fresh medium 1 hr previously. The cells were incubated at 37° for 5 hr, treated with 15% glycerol in HBS for 2 min at 37°, then washed once and incubated in DME/5% FBS for 16 hr at 37°. Each dish of cells was subsequently infected at RT with 10^6 PFU of F-gDβ virus in 0.25 ml of tricine-buffered saline (TBS) (Cai et al., 1988), followed by the addition of 5 ml of DME/5% FBS and incubation for 1 hr at 37°. The medium was then removed, and extracellular virus was inactivated by incubating the monolayer for 1 min in glycine–saline, pH 3.0 (Cai et al., 1988), followed by two washes with TBS. After 24 hr in DME/5% FBS at 37°, the cells were lysed by Dounce homogenization; nuclei were pelleted by low-speed centrifugation, and the supernatant was stored at −70° for subsequent determination of virus titers on VD60 cells. One hundred percent complementation is defined as the titer obtained after transfection with plasmid pRE4, which expresses wild-type gD. Complementation with a mutant is then defined by the formula

% complementation =

$$100 \times \left(\frac{\text{titer with mutant plasmid} - \text{titer with no DNA}}{\text{titer with pRE4} - \text{titer with no DNA}} \right).$$

RESULTS

Cloning and sequencing of mutant gD genes

The gD gene is contained within the 6.5-kb BamHI J fragment of HSV-1 (Lee et al., 1982). This fragment was isolated from mutant virion DNAs and cloned into pGEM1, as described under Materials and Methods. The plasmids were designated pMM72 (MarD2.1), pTW83 (MarDL11), and pTW84 (MarD.HD1). pMar62, a plasmid containing the MarD.4S BamHI J fragment cloned into pBR325, was provided by Randal Byrn.

The entire ectodomain of the MarD.4S gD gene was sequenced. As the sequence of gD from its parent strain (KOS) has not been reported, we compared MarD.4S to the wild-type Patton strain (Watson et al., 1982). The only difference was at residue 140, where MarD.4S has an Asn and Patton has a Ser (Fig. 2). This difference is responsible for the resistance of MarD.4S to neutralization by MAb 4S, as confirmed by site-directed mutagenesis (see below). Since KOS, like Patton, is neutralized by 4S, it does not have an Asn at 140. The other three mutants were selectively sequenced (Fig. 2). MarDL11, which behaves identically to MarD.4S in neutralization and binding assays, has the same mutation. MarD2.1, the third mutant selected with a Ib MAb, has a Gln to Leu substitution at amino acid 132. The Ia mutant, MarD.HD1, has a Ser 216 to Asn change, the same as that previously reported for a mutant selected with LP2, another group Ia MAb (Minson et al., 1986).

Confirmation of mutant phenotypes by site-directed mutagenesis

Each of the three mutations identified by sequencing was reproduced individually in a Patton gD gene cloned into M13mp18. This was accomplished by oligonucleotide-directed mutagenesis, using the procedure of Kunkel (1987). HindIII fragments containing the mutated gD genes were then excised from double-stranded phage DNA and cloned into the expression vector pRSVnt EPA (Carswell et al., 1986). Plasmids pMM80, pMM85, and pMM87 have mutations in the codons for amino acids 140, 132, and 216, respectively. The corresponding mutant proteins were expressed in transfected COS-1 cells, and their antigenicity tested with the appropriate MAbs (Table 1). Cell extracts were electrophoresed on native (nondenaturing) gels, transferred to nitrocellulose, then probed with a MAb followed by ^{125}I-Protein A. The results (Fig. 3) confirm that the mutations at residues 132, 140, and 216 are responsible for the altered antigenicity of the respective mar mutants. The reactions of each MAb with wild-type gD expressed by plasmid pRE4 are shown in lanes 1, 3, 5, 7, and 9. The 216 mutation abolished binding of LP2 (Fig. 3, lane 4) but not HD1 (Fig. 3, lane 2), as previously shown for the interaction of these MAbs with gD from the MarD.LP2 and MarD.HD1 viruses (Muggeridge et al., 1988). The 140 mutation abolished binding of DL11 (Fig. 3, lane 6) and 4S (Fig. 3, lane 8), and the 132 mutation abolished binding of D2 (Fig. 3, lane 10).

One explanation for the failure of HD1 to neutralize MarD.HD1 and MarD.LP2 even though it can still bind is that the orientation of bound antibody is changed in

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                          CAG->CTG AGC->AAC
                           \      / \      /
                            \    /   \    /
101                         132'    140'          150
   RMGGNCAIPITVMEYTECSYNKSLGACPIRTQPRWNYYDSFSAVSEDNLG  Patton
   ...............................L...................  MarD2.1
   ..................................N................  MarDL11
   ..................................N................  MarD.4S
   ...................................................  MarD.HD1


151                                                200
   FLMHAPAFETAGTYLRLVKINDWTEITQFILEHRAKGSCKYALPLRIPPS  Patton
   .................................................  MarD2.1
   .................................................  MarDL11
   .................................................  MarD.4S
   .................................................  MarD.HD1


                 AGC->AAC
                  \      /
                   \    /
201               216'                              250
   ACLSPQAYQQGVTVDSIGMLPRFIPENQRTVAVYSLKIAGWHGPKAPYTS  Patton
   .................................................  MarD2.1
   .................................................  MarDL11
   .................................................  MarD.4S
   ..............N..................................  MarD.HD1
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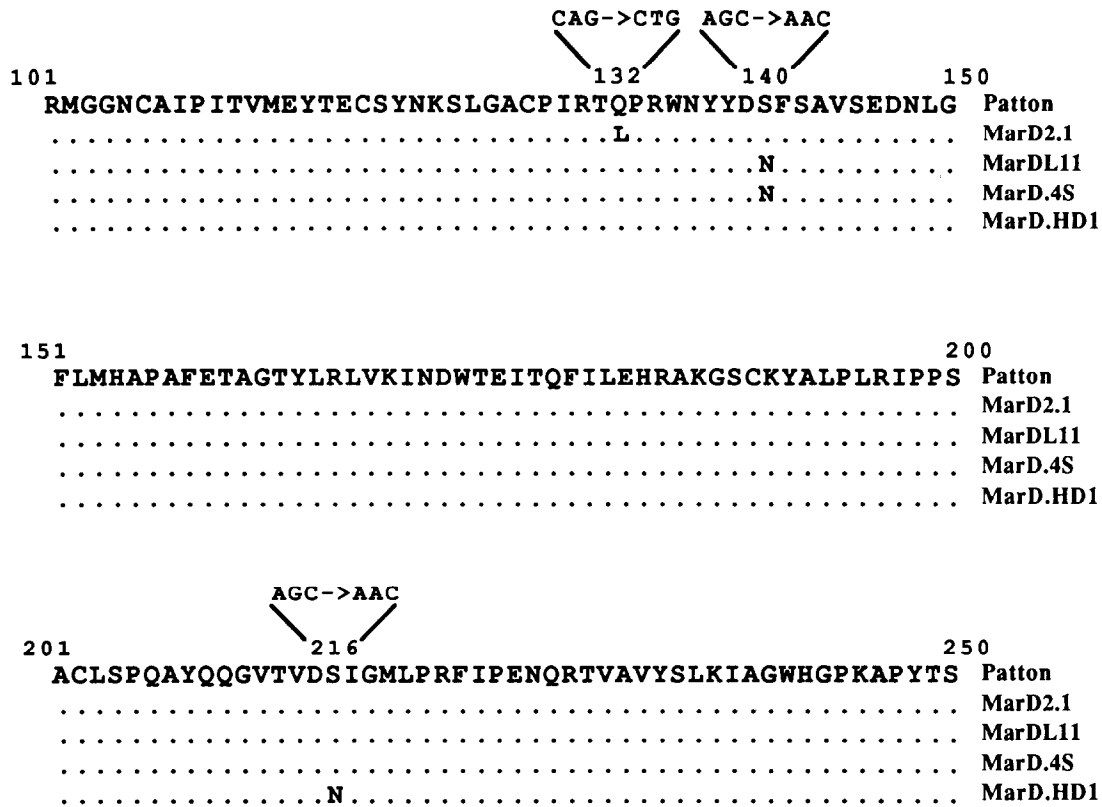
FIG. 2. Amino acid changes in gD-1 *mar* mutants. The sequence shown is that of the Patton strain, from residues 101 to 250 of the mature protein. Below it are the single amino acid substitutions identified in the four *mar* mutants, with the corresponding codons shown above.

such a way that it no longer blocks the participation of gD in penetration. Alternatively, amino acid 216 might itself be outside of the HD1 epitope but be able to influence whether neutralization occurs as a consequence of antibody binding. If the former possibility was correct, then changing 216 to a residue other than Asn might abolish binding of HD1. We therefore used oligonucleotide-directed mutagenesis to change 216 from Ser to Phe. The resulting protein was expressed in transfected COS-1 cells and analyzed by the native gel/Western blot procedure (Fig. 4). As expected, the

TABLE 1

ANTI-gD MONOCLONAL ANTIBODIES USED IN THIS STUDY[a]

MAb group	MAb	Epitope
II	DL6	Continuous: 272–279
VII	1D3, LP14	Continuous: 1–23
Ia	HD1, LP2	Discontinuous: 1–233
Ib	DL11, 4S, D2	Discontinuous: 1–275
III	RIP	Discontinuous: 1–233
VI	DL2	Discontinuous: 1–233

[a] See Muggeridge *et al.* (1989) for review.

mutation had no effect on binding of Ib MAbs (Fig. 4, lanes 6, 8, and 10) or nongroup I MAbs (Fig. 4, lanes 12, 14, and 16). However, binding of both the Ia MAbs was abolished (Fig. 4, lanes 2 and 4), indicating that residue 216 lies within the epitopes of HD1 and LP2.

The Ser140 to Asn mutation creates a new N-linked glycosylation site

The consensus sequence for attachment of N-linked carbohydrate chains is Asn–X–Ser/Thr, where X can be any amino acid except Pro (Mononen and Karjalainen, 1984; Roitsch and Lehle, 1989). gD has three of these sequences, with the Asn residues at positions 94, 121, and 262; all three are used (Matthews *et al.*, 1983). Residues 141 and 142 of gD are Phe–Ser, so the conversion of residue 140 to Asn in MarD.4S and MarDL11 creates a new consensus sequence. To test for glycosylation at this site, we compared the electrophoretic mobilities of the wild-type and mutant proteins, before and after enzymatic removal of N-linked carbohydrates (Fig. 5). Cytoplasmic extracts of infected CV1 cells were incubated for 15 min at 37° with endo F; mock digests were performed with no enzyme. The samples were then electrophoresed on a native
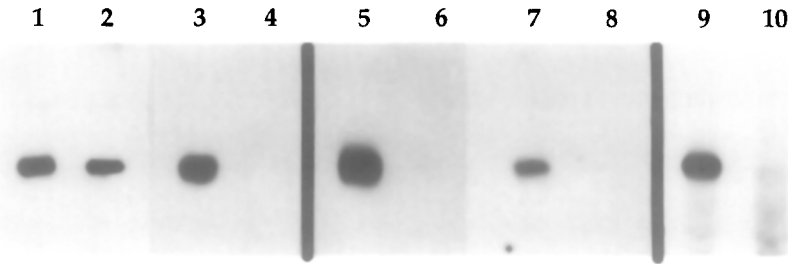
FIG. 3. Reaction of MAbs with gD containing site-directed mutations corresponding to those identified by sequencing. Cytoplasmic extracts of COS-1 cells transfected with plasmids expressing gD mutants were electrophoresed on native polyacrylamide gels, transferred to nitrocellulose, and probed with one of the group I MAbs followed by ^{125}I-Protein A. Lanes 1, 3, 5, 7, and 9: plasmid pRE4, which expresses wild-type (Patton) gD. Lanes 2 and 4: plasmid pMM87, which has a mutation at 216. Lanes 6 and 8: plasmid pMM80, which has a mutation at 140. Lane 10: plasmid pMM85, which has a mutation at 132. Lanes 1 and 2 were probed with HD1; lanes 3 and 4 were probed with LP2; lanes 5 and 6 were probed with DL11; lanes 7 and 8 were probed with 4S; lanes 9 and 10 were probed with D2.

gel, Western blotted, and probed with DL6 (an anti-gD MAb that recognizes a continuous epitope; see Table 1) followed by ^{125}I-Protein A. Mock-digested gD from the two mutants (Fig. 5, lanes 2 and 3) had a lower mobility than wild-type KOS gD (Fig. 5, lane 1). After deglycosylation, the mutant (Fig. 5, lanes 5 and 6) and wild-type (Fig. 5, lane 4) proteins had identical mobilities. The extra glycosylation site created by the mutation at amino acid 140 is therefore used in both mutants.

Antigenic site Ib is partially covered by a carbohydrate chain in the MarD.4S and MarDL11 mutants

These two mutants are resistant to neutralization by the Ib MAbs 4S, DL11, and D2. It seemed likely that the loss of antibody recognition was caused by covering of the Ib site by the new carbohydrate chain. To test this possibility, we compared antibody binding before and after deglycosylation. Initially, cytoplasmic extracts of

infected cells were digested with endo F, or mock-digested, for 15 min at 37°, then electrophoresed on native gels, Western blotted, and probed with MAbs followed by ^{125}I-Protein A. However, we found significant loss of conformation of deglycosylated wild-type KOS gD, such that binding of MAbs recognizing discontinuous epitopes was reduced (Fig. 6). Any specific effect of deglycosylation on site Ib could therefore not be evaluated. To determine whether loss of conformation was occurring during the endo F incubation or during subsequent electrophoresis and blotting, the effect of longer incubation times was investigated. If denaturation was occurring during the endo F incubation, then binding of MAbs with discontinuous epitopes, such as HD1, should eventually be reduced to zero. Samples were taken from endo F and mock digests at 15 min, 30 min, 1 hr, and 2 hr, and two identical native gels were run (Fig. 7). One was probed with DL6, which reacts with denatured and native gD, the other with HD1. DL6 reacted similarly with all mock-digested (Fig. 7A,
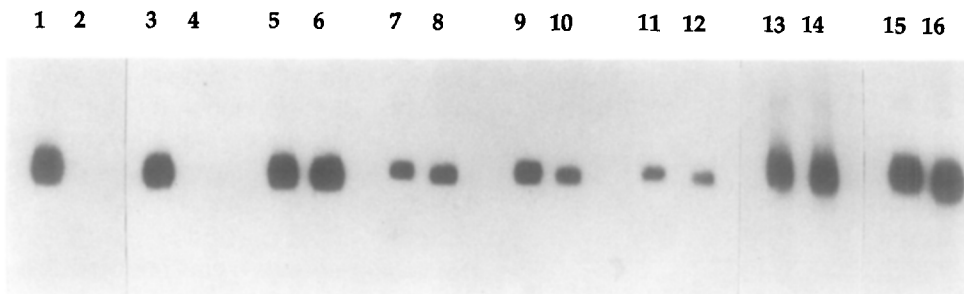

FIG. 4. Reaction of MAbs with gD containing a site-directed mutation of Ser 216 to Phe. Cytoplasmic extracts of transfected COS-1 cells were electrophoresed on native polyacrylamide gels with no comb and Western blotted. The nitrocellulose blots were cut into strips, which were probed with a MAb followed by ^{125}I-Protein A, then arranged in pairs for autoradiography. Lanes 1, 3, 5, 7, 9, 11, 13, and 15: plasmid pRE4, which expresses wild-type (Patton) gD. Lanes 2, 4, 6, 8, 10, 12, 14, and 16: plasmid pD1S216F, which expresses gD with a Ser 216 to Phe mutation. Lanes 1 and 2 were probed with HD1; lanes 3 and 4 were probed with LP2; lanes 5 and 6 were probed with DL11; lanes 7 and 8 were probed with 4S; lanes 9 and 10 were probed with D2; lanes 11 and 12 were probed with the group VI MAb DL2; lanes 13 and 14 were probed with the group III MAb RIP; lanes 15 and 16 were probed with the group II MAb DL6.
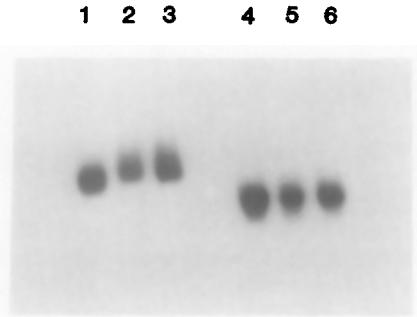
1 2 3   4 5 6

FIG. 5. Endo F digestion of gD. CV1 cells were infected with the wild-type KOS strain, or with the MarD.4S or MarDL11 mutants. Cytoplasmic extracts were incubated at 37° for 15 min, with or without the addition of endo F, then electrophoresed on native polyacrylamide gels, transferred to nitrocellulose, and probed with MAb DL6, followed by ^{125}I-Protein A. Lanes 1 and 4: KOS. Lanes 2 and 5: MarD.4S. Lanes 3 and 6: MarDL11. Lanes 1, 2, and 3: mock digests. Lanes 4, 5, and 6: endo F digests.

A **B**

1 2 3 4   5 6 7 8   1 2 3 4   5 6 7 8

FIG. 7. Effect of endo F on stability of gD. A cytoplasmic extract of CV1 cells infected with KOS virus was incubated at 37°, with or without the addition of endo F. Samples were removed at various times for electrophoresis on native polyacrylamide gels, transfer to nitrocellulose, and analysis with MAbs followed by ^{125}I-Protein A. (A) Probed with DL6. (B) Probed with HD1. Lanes 1 to 4 of A and B are mock digests; lanes 5 to 8 of A and B are endo F digests. The digestion times were as follows: lanes 1 and 5, 15 min; lanes 2 and 6, 30 min; lanes 3 and 7, 1 hr; lanes 4 and 8, 2 hr.

lanes 1 to 4) and endo F-digested (Fig. 7A, lanes 5 to 8) samples. The reaction of HD1 with endo F-digested samples (Fig. 7B, lanes 5 to 8) was weaker than with mock-digested samples (Fig. 7B, lanes 1 to 4), as expected. However, there was no progressive loss of binding with increasing digestion time. The loss of conformation caused by deglycosylation of gD is therefore manifest during native gel electrophoresis and Western blotting, rather than during the 37° incubation with endo F.

As an alternative to native gels, we turned to nitrocellulose dot blots. As before, cytoplasmic extracts of cells infected with MarD.4S or KOS virus were digested with endo F or mock-digested. To confirm that the carbohydrate chains had indeed been removed by endo F, aliquots were run on gels, Western blotted, and probed with DL6 (data not shown). For the dot blots, 2-μl aliquots of the digested extracts were spotted on nitrocellulose, then probed with a MAb followed by ^{125}I-Pro-

tein A (Fig. 8). Four identical sets of spots were made; as a control to show that equal amounts of gD were present in each extract, one set was probed with DL6, which reacts equally with wild-type and mutant gD and is not affected by carbohydrate removal. The other three sets were probed with 4S, DL11, and D2. Binding of DL11 and D2 was partially recovered after endo F digestion, indicating that their epitopes are indeed blocked by carbohydrate. However, 4S failed to bind to MarD.4S gD after digestion. After carbohydrate removal, the only difference between the mutant and the

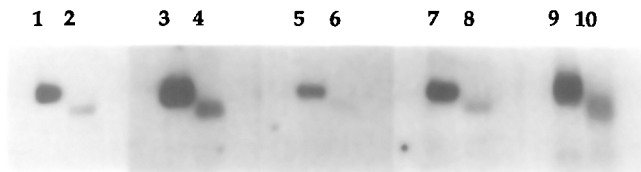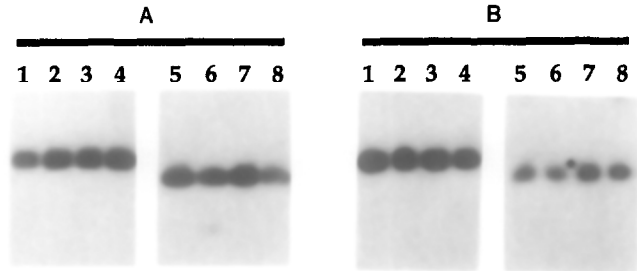**1 2   3 4   5 6   7 8   9 10**

FIG. 6. Effect of N-linked carbohydrate removal on discontinuous epitopes of gD. A cytoplasmic extract of CV1 cells infected with KOS virus was incubated at 37° for 15 min, with or without the addition of endo F, then electrophoresed on native polyacrylamide gels, transferred to nitrocellulose, and probed with MAbs followed by ^{125}I-Protein A. Lanes 1, 3, 5, 7, and 9: mock digests. Lanes 2, 4, 6, 8, and 10: endo F digests. Lanes 1 and 2 were probed with HD1; lanes 3 and 4 were probed with DL11; lanes 5 and 6 were probed with 4S; lanes 7 and 8 were probed with D2; lanes 9 and 10 were probed with DL2 (group VI).

ENDO F   **ENDO F**

$-$ $+$ $-$ $+$

KOS   KOS

MarD.4S   MarD.4S

DL6   **DL11**

KOS   KOS

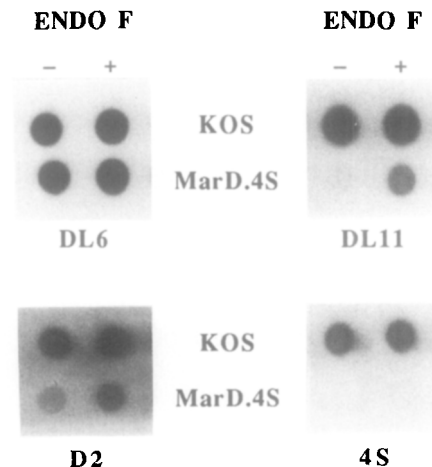MarD.4S   MarD.4S

D2   **4S**

FIG. 8. Effect of N-linked carbohydrate removal on binding of Ib MAbs. CV1 cells were infected either with the wild-type KOS strain or with the MarD.4S mutant. Cytoplasmic extracts were incubated at 37° for 15 min, with or without the addition of endo F, and 2-μl aliquots were then spotted on nitrocellulose and probed with a MAb followed by ^{125}I-Protein A. One set of samples was probed with the group II MAb DL6; the other three sets were probed with the Ib MAbs DL11, D2, and 4S.
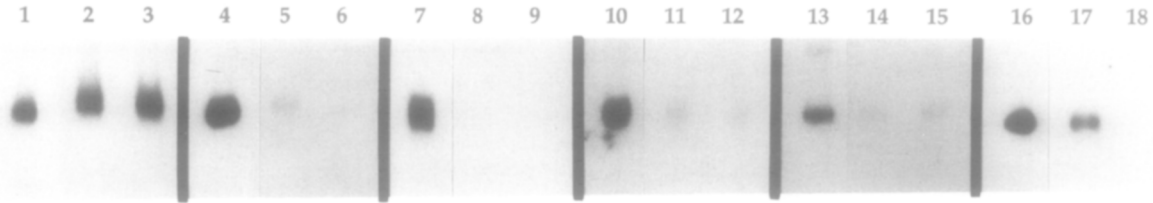
FIG. 9. Reaction of MAbs with gD containing multiple site-directed mutations within antigenic site I. Cytoplasmic extracts of COS-1 cells transfected with plasmids expressing gD mutants were electrophoresed on native polyacrylamide gels, transferred to nitrocellulose, and probed with a MAb followed by ^{125}I-Protein A. Lanes 1, 4, 7, 10, 13, and 16: plasmid pRE4, which expresses wild-type (Patton) gD. Lanes 2, 5, 8, 11, 14, and 17: plasmid pMM76, which has mutations at 140 and 216. Lanes 3, 6, 9, 12, 15, and 18: plasmid pMM88, which has mutations at 132, 140, and 216. Lanes 1, 2, and 3 were probed with DL6; lanes 4, 5, and 6 were probed with HD1; lanes 7, 8, and 9 were probed with LP2; lanes 10, 11, and 12 were probed with DL11; lanes 13, 14, and 15 were probed with 4S; lanes 16, 17, and 18 were probed with D2.

wild-type is the amino acid at 140. Therefore, Asn140 must be part of the antigenic site and not just an attachment point for carbohydrate.

Construction of multiple *mar* mutants

Since group I MAbs inhibit virus penetration and cell–cell fusion (Fuller and Spear, 1987; Highlander *et al.*, 1987; Noble *et al.*, 1983), it is likely that antigenic site I overlaps a functionally important region of gD. In that case, it may be possible to alter the structure of site I in such a way that the protein can no longer function. Since the individual *mar* mutations are found in viable viruses, the structural changes they cause are able to affect antibody binding but not gD function. However, a combination of these mutations may be sufficient to do both. To test this possibility, we used mixtures of mismatched oligonucleotides to create gD genes carrying two (140 and 216) or three (132, 140 and 216) mutations. The mutated genes were cloned into pRSVnt EPA (Carswell *et al.*, 1986) to form plasmids pMM76 (double mutant) and pMM88 (triple mutant). The corresponding proteins were expressed in transfected COS-1 cells, and their antigenicity analyzed by the native gel/Western blot procedure (Fig. 9). The reactions of each MAb with wild-type gD expressed by plasmid pRE4 are shown in lanes 1, 4, 7, 10, 13, and 16. Both mutants reacted with DL6 (Fig. 9, lanes 2 and 3). As expected, both mutants showed reduced or negligible binding with the Ib MAbs DL11 (Fig. 9, lanes 11 and 12), 4S (Fig. 9, lanes 14 and 15), and D2 (Fig. 9, lanes 17 and 18). Residual binding of D2 to the double mutant (Fig. 9, lane 17) was probably caused by incomplete glycosylation at amino acid 140. Neither mutant reacted significantly with the Ia MAbs (Fig. 9, lanes 5, 6, 8, and 9), even though HD1 reacts well with the three single mutants (Muggeridge *et al.*, 1988). Combining mutations at different residues within antigenic site I therefore has unpredictable effects on antigenicity.

Recombination of multiple *mar* mutants into the virus genome

If gD with two or three mutations within antigenic site I is functional, it should be possible to isolate viable viruses after recombining the genes into the virus genome. We attempted to do this by cotransfection of Vero cells with viral and plasmid DNA. To increase the frequency of recombination, we first recloned the mutant gD genes from pMM76 and pMM88 into a cassette vector containing viral sequences normally found on either side of the gD gene, as described under Materials and Methods. The resulting plasmids were named pMM82 (140 and 216 mutations) and pMM89 (132, 140 and 216 mutations). To facilitate the screening process, virion DNA was from MarD.LP14, a *mar* mutant that does not bind group VII MAbs, such as 1D3, owing to an Arg to His change at residue 16 (Minson *et al.*, 1986). The gD gene in pMM82 and pMM89 is wild-type at residue 16, and so rescues binding of 1D3 to plaques formed by recombinants. Virus harvested from the transfected Vero cells was plated on CV1 cells, and the plaques were screened for binding of 1D3 in a black plaque assay (Holland *et al.*, 1983; Kousoulas *et al.*, 1984). Recombinant plaques were cloned twice, then tested for resistance to neutralization by group I MAbs (Muggeridge *et al.*, 1988). Of two independent recombinants obtained with pMM82, both were resistant to LP2 but not DL11. Of nine independent recombinants obtained with pMM89, all were resistant to LP2, but not to DL11 or D2. These results suggested that the 216 mutation had been transferred, and that the 132 and 140 mutations had not. This was confirmed by cloning the *Bam*HI J fragments from one pMM82 recombinant and one pMM89 recombinant, followed by sequencing of the appropriate sections of their gD genes. Both had the Ser216 to Asn mutation, but were wild-type at positions 132 and 140. Loss of these mutations suggested that the combination of Ia and Ib *mar* mutations is lethal to the virus, presumably

TABLE 2

COMPLEMENTATION OF F-gDβ VIRUS BY gD MUTANT PLASMIDS

Plasmid	Mutation	% Complementation
pMM80	Ser140 to Asn	30
pMM85	Gln132 to Leu	137
pMM87	Ser216 to Asn	106
pMM76	140 + 216	4
pMM88	132 + 140 + 216	8
pCD64 (gC-2)	None	0

because it prevents gD from carrying out its essential function.

Complementation of a gD-minus virus by plasmid-encoded gD mutants

F-gDβ is a mutant virus that was derived from HSV-1 (strain F) by replacement of the gD coding sequence, and part of the gI gene, with DNA coding for *Escherichia coli* β-galactosidase (Ligas and Johnson, 1988). It can replicate in VD60 cells, which contain an integrated copy of the gD gene, but not in Vero cells, from which the VD60 line was derived. Vero cells infected with F-gDβ produce noninfectious virions lacking gD. Following a procedure used to analyze gB linker insertion mutants (Cai et al., 1988), we have developed an assay in which gD transiently expressed from transfected plasmids is tested for its ability to rescue the production of infectious F-gDβ in Vero cells. Vero cells are first transfected with an expression vector containing a wild-type or mutant gD gene, then, on the next day, infected with F-gDβ. On the third day, virus is harvested and titered on VD60 monolayers. Titers obtained from Vero cells transfected with plasmid pRE4, which expresses wild-type gD-1, were 50- to 100-fold higher than those from cells transfected with calcium phosphate alone or with plasmid pCD64, which expresses gC-2 (Seidel–Dugan et al., submitted for publication).

Approximately 5% of Vero cells transfected with 10 μg of pRE4 DNA showed surface expression of gD (detected by immunoperoxidase staining). Quantities of mutant plasmid DNA were adjusted to produce a similar percentage of expressing cells. Complementation results obtained with the gD mutants are shown in Table 2, each value representing the average of three independent experiments. Complementation with pMM85 and pMM87, which have single mutations at 132 and 216, respectively, was similar to that with pRE4. Complementation with pMM80, which has a mutation at 140, was reduced to 33%, which may be

close to the lower limit for viability of a virus carrying this mutation. However, complementation by the mutants with two changes (pMM76) or three changes (pMM88) was much lower (between 4 and 8%), confirming that these combinations of mutations within antigenic site I of gD are detrimental to its function.

DISCUSSION

MAbs which bind to antigenic site I of gD prevent virus penetration into the cell (Fuller and Spear, 1987; Highlander et al., 1987), block cell fusion (Campadelli–Fiume et al., 1988b; Highlander et al., 1987; Noble et al., 1983), and have high complement-independent neutralization titers (Eisenberg et al., 1982; Fuller and Spear, 1985; Kümel et al., 1985; Minson et al., 1986; Pereira et al., 1980; Showalter et al., 1981). Because this site, or an adjacent region, is vital for the proper functioning of gD during infection, we have begun to map it in detail.

Previously, we have precisely mapped several continuous antigenic sites of gD, using synthetic peptides (Cohen et al., 1984; Dietzschold et al., 1984; Eisenberg et al., 1985). Site I, being discontinuous, cannot be mapped in this way. Instead, we have used a combination of two approaches. First, group I MAbs were used to select neutralization-resistant mutant viruses for sequence analysis (Highlander et al., 1987; Minson et al., 1986; Muggeridge et al., 1988). Prior to sequencing, we tested the panel of *mar* mutants for reactivity with all seven group I MAbs, and were able to divide the site into parts Ia and Ib, recognized by two MAbs and five MAbs, respectively. Second, a series of gD truncation and deletion mutants was created (Cohen et al., 1988), and the corresponding proteins were expressed in transfected cells. Their analysis indicated that site Ia is contained within residues 1 to 233, whereas site Ib requires residues between 234 and 275 (Muggeridge et al., 1988).

We have now used DNA sequencing and oligonucleotide-directed mutagenesis to identify the altered amino acids in the *mar* mutants. The Ia mutant (MarD.HD1) has a change at 216, which is consistent with the truncation mutant data. The mutation is identical to that in MarD.LP2, a mutant selected with another Ia MAb (Minson et al., 1986). We anticipated that the Ib mutants would have changes between 234 and 275, but that was not the case. Instead, the mutations were at 132 (MarD2.1) and 140 (MarDL11 and MarD.4S), emphasizing the discontinuous nature of the antigenic site. The change at 140 creates a new consensus sequence for attachment of an N-linked oligosaccharide; this is not uncommon in *mar* mutants of other viruses (Caust et al., 1987; Skehel et al., 1984; Vrati et al.,

1988). Removal of oligosaccharides by endo F digestion restored binding of two Ib MAbs, but not a third. Some Ib MAbs are therefore sensitive to the amino acid at 140, which is Ser in wild-type gD but Asp in the endo F-treated mutants. In the course of the endo F analysis, we also discovered that removal of N-linked oligosaccharides from gD leaves the protein more susceptible to denaturation during electrophoresis in low-SDS native acrylamide gels and/or Western blotting.

A computer analysis of the gD sequence (Eisenberg et al., 1985) predicted that amino acids 132 to 140 lie within a hydrophilic sequence which is a candidate for an antigenic site (Hopp and Woods, 1981). However, peptides corresponding to amino acids 124 to 136 and 130 to 139 were not recognized by rabbit antisera prepared against native gD (Isola et al., 1989), so this region probably does not form a continuous antigenic site. It therefore seems likely that folding of the protein brings amino acids 132 to 140 close to 234 to 275 in the native protein to form discontinuous site Ib.

The repeated isolation of a limited number of mutants is striking. Thus, a Ser to Asn change at 140 was selected by two Ib MAbs, and a Ser to Asn change at 216 was selected by two Ia MAbs (Minson et al., 1986). In addition, the 216 change was selected by MAb LP2 in two strains of HSV-1 and one of HSV-2 (Minson et al., 1986). This may reflect a limited potential for non-deleterious amino acid substitutions within antigenic site I of gD, or indeed for the whole protein, given the near identity of known gD-1 sequences and 85% homology between gD-1 and gD-2 (McGeoch et al., 1985; Minson et al., 1986; Watson, 1983; Watson et al., 1982). There is a marked contrast to the influenza HA and NA proteins, for example, which yield a variety of mutants when grown in the presence of a neutralizing MAb (Daniels et al., 1987; Webster et al., 1987).

Each of the three mutations within site I occurs individually in a viable mutant virus. To determine whether antigenically intact site I is critical for virus infectivity, we have used oligonucleotide-directed mutagenesis to combine Ia and Ib mutations in a cloned gD gene. A combination of two mar mutations in gB was previously shown to produce a temperature-sensitive phenotype in the virus (Marlin et al., 1986). A similar study with gD (Highlander et al., 1987) did not produce temperature-sensitive or lethal combinations; however, the mar mutations were from different antigenic sites. In this case, we have combined mutations within an antigenic site. The effect on antigenicity was greater than predicted by the properties of the individual mutations: the 132/140/216 triple mutant (pMM88) showed negligible binding with all group I MAbs, including HD1, which binds to all three single mutants. Binding of MAbs that recognize discontinuous antigenic site III was not di-

minished, and the mutant proteins were transported to the cell surface (data not shown), so their folding was not grossly altered. To determine if the loss of site Ia and Ib antigenicity is correlated with loss of gD function, we attempted to recombine the mutant genes into the virus genome. The recipient genome was from MarD.LP14, an HSV-1 mutant that fails to bind group VII MAbs, such as LP14 and 1D3, because of an Arg to His change at residue 16 of gD (Minson et al., 1986). This facilitated the screening assay, because only recombinants could bind 1D3 in a black plaque assay. The recombinants were resistant to neutralization by LP2, and DNA sequencing confirmed that the mutation at residue 216 had been transferred to the virus genome, but they remained sensitive to DL11 and D2. Sequencing showed that the recombinants still had the wild-type residues at 132 and 140. Therefore, of the four amino acid substitutions, only the two outermost (16 and 216) were transferred. One explanation is that the combination of mutations within sites Ia and Ib is lethal, and that a complex crossover event must occur during recombination to remove the 132 and 140 mutations and generate a functional protein.

To test the function of gD mutants, we used a complementation assay, in which gD transiently expressed by a plasmid can rescue the production of infectious virions by F-gDβ, a mutant lacking a gD gene. A stock of F-gDβ virus was grown in VD60 cells, which synthesize gD from an integrated copy of the gene, passaged through Vero cells, which do not produce gD, and then titered on VD60s. Vero cells previously transfected with plasmid pRE4, which expresses wild-type gD, produced 50- to 100-fold higher titers than cells transfected with calcium phosphate alone. It has been demonstrated recently that cells expressing large amounts of gD are resistant to infection by HSV-1 (Campadelli-Fiume et al., 1988a; Johnson and Spear, 1989); evidently our expression vector does not produce sufficient gD for this to be a problem. As expected, none of the three mar mutations individually prevented complementation, although the mutation at 140 reduced complementation approximately 3-fold. However, the combination of two (140 and 216) or three (132, 140, and 216) mutations within site I reduced the complementation to low levels. These combinations of mutations, which cause no apparent structural changes beyond site I, therefore prevent gD from performing its essential function in HSV replication. This assay is applicable to any gD mutant which can be expressed in transfected cells, and can be used to construct a functional map of the gD protein.

ACKNOWLEDGMENTS

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