

The Major Neutralizing Antigenic Site on Herpes Simplex Virus Glycoprotein D Overlaps a Receptor-Binding Domain

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Herpes simplex virus (HSV) entry is dependent on the interaction of virion glycoprotein D (gD) with one of several cellular receptors. We previously showed that gD binds specifically to two structurally dissimilar receptors, HveA and HveC. We have continued our studies by using (i) a panel of baculovirus-produced gD molecules with various C-terminal truncations and (ii) a series of gD mutants with nonoverlapping 3-amino-acid deletions between residues 222 and 254. Binding of the potent neutralizing monoclonal antibody (MAb) DL11 (group Ib) was unaffected in forms of gD containing residues 1 to 250 but was greatly diminished in molecules truncated at residue 240 or 234. Both receptor binding and blocking of HSV infection were also affected by these C-terminal truncations. gD-1(234t) bound weakly to both HveA and HveC as determined by enzyme-linked immunosorbent assay (ELISA) and failed to block infection. Interestingly, gD-1(240t) bound well to both receptors but blocked infection poorly, indicating that receptor binding as measured by ELISA is not the only gD function required for blocking. Optical biosensor studies showed that while gD-1(240t) bound HveC with an affinity similar to that of gD-1(306t), the rates of complex formation and dissociation were significantly faster than for gD-1(306t). Complementation analysis showed that any 3-amino-acid deletion between residues 222 and 251 of gD resulted in a nonfunctional protein. Among this set of proteins, three had lost DL11 reactivity (those with deletions between residues 222 and 230). One of these proteins (deletion 222–224) was expressed as a soluble form in the baculovirus system. This protein did not react with DL11, bound to both HveA and HveC poorly as shown by ELISA, and failed to block HSV infection. Since this protein was bound by several other MAbs that recognize discontinuous epitopes, we conclude that residues 222 to 224 are critical for gD function. We propose that the potent virus-neutralizing activity of DL11 (and other group Ib MAbs) likely reflects an overlap between its epitope and a receptor-binding domain of gD.

The herpes simplex virus (HSV) genome codes for at least 11 glycoproteins, most of which are detectable in the virion envelope (50). Infection of susceptible cells is initiated by the attachment of virions, via glycoprotein C (gC) and/or gB, to cell surface heparan sulfate proteoglycans (21, 22, 59). This is followed by the interaction of gD with a cellular receptor. Then, pH independent fusion occurs between the virus envelope and the host cell plasma membrane (58); gB, gD, and the gH-gL complex have all been implicated in this step (50, 52).

Recently, expression cloning was used to identify several human genes whose products convert the normally nonpermissive Chinese hamster ovary cells into cells that are permissive for HSV type 1 (HSV-1) and HSV-2 entry (9, 19, 40, 53). These mediators of HSV entry are known as HveA, HveB, and HveC. HveA is a member of the tumor necrosis factor receptor superfamily of proteins (40) and interacts with both lymphotoxin α and LIGHT (38). HveB (also called PRR2) and HveC (also called PRR1) are closely related members of the immunoglobulin superfamily of proteins (36.1% amino acid sequence identity within the predicted extracellular domains) which share

53.2 and 33.9% amino acid sequence identities, respectively, with the poliovirus receptor extracellular domain (14, 19, 37, 53). The normal cellular functions of these proteins remain unknown, although recent data suggest that the murine homolog of HveB may be a cell-cell adhesion molecule (1). A splice variant of HveC, called HIgR, can also mediate HSV infection of nonpermissive cells (9). Soluble forms of gD have been shown to bind directly to soluble forms of HveA, HveC, and HIgR but not to HveB (8, 9, 31, 54, 55). In addition, antibodies to the receptors have been shown to block infection by HSV (9, 40, 53). Thus, it is clear that HSV can utilize several different and structurally unrelated cell surface proteins as receptors and that two of these receptors bind directly to HSV gD.

Two approaches were used in previous studies to try to define the relationship between gD structure and function: (i) examination of the properties of a panel of monoclonal antibodies (MAbs) to gD (11, 12, 23, 41, 43) and (ii) examination of the properties of a panel of gD mutants (7, 17, 42). First, the antigenic site I of gD was defined by seven MAbs, all of which possess potent virus-neutralizing activity in the absence of complement (41). Although all group I MAbs block the binding of other group I antibodies to gD, further subdivision of these MAbs into groups Ia and Ib was done on the basis of studies with truncated and other mutant forms of gD. Two group Ia MAbs, HD1 and LP2 (11), bind to gD truncated at amino acid residue 233, whereas DL11 and four other group Ib antibodies do not (11, 43). More recently, we showed that, whereas DL11 blocks the binding of soluble HveA or HveC to

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*Hind*III site) which was excised from plasmid pRE4 (12) by *Hind*III digestion. The specific mutagenic primers used were as follows: $\Delta 222-224$, 5'-TGGTCTCGGGGGCAGCATC-3'; $\Delta 225-227$, 5'-ACGGTGGCGCTGGATGAAGCGGGCC-3'; $\Delta 228-230$, 5'-GTATACGGCGACGTTCTCGGGGAT-3'; $\Delta 231-233$, 5'-CTTCAAGCTGTAGGTGCGTGGTT-3'; $\Delta 234-236$, 5'-CCCAGCGATCTTACGGCGACGGT-3'; $\Delta 237-239$, 5'-CCCGTGCACCCCAAGCTGTATAC-3'; $\Delta 240-242$, 5'-GGCCTTGGGCCCGCGCATCTCAAGC-3'; $\Delta 243-245$, 5'-CGTGTATGGGGCGTCCACCCGGC-3'; $\Delta 246-248$, 5'-CAGGGTGCTGTCTTGGGCCCGTGC-3'; $\Delta 249-251$, 5'-TCCGGGGCAGCAGGTATGGGCTT-3'; $\Delta 252-254$, 5'-GGACAGCTCCGGGGTGTCTCGTGTAT-3'.

After mutagenesis, the gD ORFs were excised (*Hind*III) from M13 replicative-form DNA and transferred into the mammalian expression plasmid, pRSVnt-EPA (5). Plasmids containing the gD ORF in the desired orientation were sequenced by using the method of Chen and Seeberg (6) to confirm the presence of the anticipated mutations (nine nucleotide deletions) within the gD ORF.

Antigenic analysis of mutants. Transfection of COS cells and the subsequent preparation of cytoplasmic extracts were performed as previously described (12, 41).

Immunoperoxidase staining. This procedure, which was performed as previously described (43), is a modification of that of Holland et al. (24) and Koussoulas et al. (30). Surface staining of transfected cells was studied with unfixed cells; for detection of intracellular antigens, the cells were fixed with 5% methanol in PBS before incubation with the MAbs.

Complementation assay. The assay was performed essentially as previously described (43), except that COS cells were used instead of Vero cells. Briefly, cells were transfected with DNA-calcium phosphate precipitates for 16 h at 37°C and then washed and incubated in DMEM-5% FBS for 8 h at 37°C. Each dish of cells was subsequently infected at room temperature with 10^6 PFU of F-gD β virus, followed by the addition of 5 ml of DMEM-5% FBS and incubation for 1 h at 37°C. The medium was then removed, and extracellular virus was inactivated by incubating the monolayer for 1 min in glycine-saline (pH 3.0) (4, 25). After 18 h in DMEM-5% FBS at 37°C, the medium was removed and stored at -70°C for subsequent determination of the virus titers. The cells were lysed by freeze-thawing and sonication with a Microson cell disruptor. Nuclei were then pelleted by low-speed centrifugation, and the supernatant was stored at -70°C for subsequent determination of the virus titers. Both intracellular and extracellular virus titers were determined on VD60 cells. Transfection with salmon sperm DNA was used as the negative control. One hundred percent complementation is defined as the titer obtained after transfection with plasmid pRE4, which expresses wild-type gD (12). Complementation with a mutant is then defined by the following formula: % complementation = $100 \times (\text{titer with mutant plasmid} - \text{titer with carrier DNA}) / (\text{titer with pRE4} - \text{titer with carrier DNA})$.

Optical biosensor experiments. Biosensor experiments were carried out on a Biacore X optical biosensor (Biacore AB) at 25°C as previously described (32, 47). Biosensor data were analyzed by using a global fitting routine with BIA-evaluation software, version 3.0 (2). Model curve fitting was carried out by using a 1:1 Langmuir interaction with drifting baseline. This models the simple interaction between ligand (L) and receptor (R) as follows: $L + R \rightleftharpoons LR$. The rate of association (k_{on}) was measured from the forward reaction, and k_{off} was measured from the reverse reaction. For gD-1(234t), a maximum k_{off} was estimated as previously described (47) by using the equation $\ln(R_0/R_n) = k_{off}(t_n - t_0)$, where R_0 is the response at time zero (t_0) of dissociation and R_n is the response at time n (t_n) (29). Scatchard analyses of the gD-1(234t)-receptor complexes were performed as previously described (47).

RESULTS

C-terminal truncations. Krummenacher et al. (31) and Rux et al. (47) showed that, compared to gD truncated at residue 306 [gD-1(306t)], molecules truncated at residues 285 [gD-1(285t)] and 275 [gD-1(275t)] exhibited enhanced receptor binding. In contrast, a form consisting of residues 1 to 234 [gD-1(234t)] exhibited greatly diminished receptor binding. gD-1(234t) was shown to retain much of the native structure of the full-length molecule in that most MAbs recognizing discontinuous epitopes of gD reacted with gD-1(234t) (47). One exception was that the group Ib MAb, DL11, bound poorly to gD-1(234t). Since gD-1(234t) lacks a significant portion of functional region III (7) (residues 225 to 246), we reasoned that the diminished receptor binding of gD-1(234t) was consistent with the idea that functional region III is directly involved in receptor binding. To define more precisely the C-terminal gD residues required for receptor binding as well as for the binding of MAb DL11, we expressed three additional forms of gD in the baculovirus system. These gD molecules were truncated after residues 260 [gD-1(260t)], 250 [gD-1(250t)], and 240 [gD-1(240t)]. Stick diagrams of these, as well

as other recombinant baculovirus products, are shown in Fig. 1A. Each truncated form of gD was constructed such that six histidine residues were present at the C terminus to allow for purification by nickel chromatography. The gD truncation mutants were purified by immunoaffinity chromatography [gD-1(306t) and gD-1(285t)] or by nickel chromatography (all other forms of gD). To assess the purity of the recombinant proteins, similar amounts were loaded onto an SDS-10% polyacrylamide gel, electrophoresed, and stained with silver nitrate. All of the proteins were purified to near homogeneity and were of the expected sizes (Fig. 1B). Western blot analysis with the group VII MAb 1D3 (Fig. 1C) confirmed that all of these purified proteins retained the correct N terminus of gD (Fig. 1A).

Antigenic analysis of C-terminal gD truncations. To assess the antigenic structure of the recombinant gD molecules, the proteins were separated on nonreducing ("native") SDS-polyacrylamide gels, and blots were probed with various MAbs. The blot shown in Fig. 2A was reacted with the group II MAb DL6, which recognizes a linear epitope (residues 272 to 279) (26) (Fig. 1A). The expected pattern of reactivity with DL6 was observed in that proteins smaller than gD-1(285t) were not reactive. The blots shown in Fig. 2B to D were reacted with MAbs DL2, ABD, and HD1, each of which recognizes a separate discontinuous epitope on gD. All of the truncated proteins reacted similarly with these MAbs, indicating that the native structure of gD was not grossly altered by the truncations. The blots shown in Fig. 2E and F were reacted with two group Ib MAbs, DL11 and D2. Although DL11 bound strongly to gD-1(306t), gD-1(285t), gD-1(260t), and gD-1(250t), it bound weakly to gD-1(240t) and gD-1(234t). In previous studies, we showed that DL11 competed with soluble HveA and HveC for binding to gD in HSV virions, suggesting that it binds within or near a region of gD involved in receptor interaction (31, 44). According to the data presented in Fig. 2E, gD residues immediately upstream of 250 contribute to the DL11 epitope. MAb D2, like DL11, bound weakly to gD-1(240t) and gD-1(234t) but also exhibited reduced reactivity with gD-1(250t) when compared with molecules truncated after residues 260, 285, and 306. These results confirm and extend previous work mapping residues critical to the formation of antigenic site Ib (11, 12, 41-43).

ELISA. Previous studies showed that, compared to gD-1(306t), molecules truncated after residues 285 and 275 bound to HveA and HveC with increased affinity, while a molecule truncated after residue 234 bound with reduced affinity (31, 47). To assess the effect of the C-terminal truncations on receptor interaction, we analyzed their binding to truncated forms of HveA [HVEM(200t)] and [HveC (HveC(346t))] by ELISA (Fig. 3). Figure 3A shows the binding of truncated forms of gD to HveA, while Fig. 3B shows their binding to HveC. As previously reported (31, 47), gD-1(285t) bound to both HveA and HveC, as seen by ELISA, ca. 100-fold better than did gD-1(306t). gD-1(260t) and gD-1(250t) bound to both receptors as well as gD-1(285t). However, gD-1(240t) bound to both receptors about as well as gD-1(306t), whereas the binding of gD-1(234t) was nearly undetectable. We conclude from these observations that gD residues between positions 234 and 240 are critical for receptor binding and that residues between positions 240 and 250 may also be involved (since the receptor-binding activity of gD-1(240t), though not eliminated, is reduced relative to larger forms of gD). It is of interest to note that gD-1(240t), which showed diminished reactivity with the MAb DL11, still bound to both receptors.

Biosensor analysis of gDt binding to HveA and HveC. Previously, we used optical biosensor technology to show that the increased affinity of gD-1(285t) for both HveA and HveC rel-

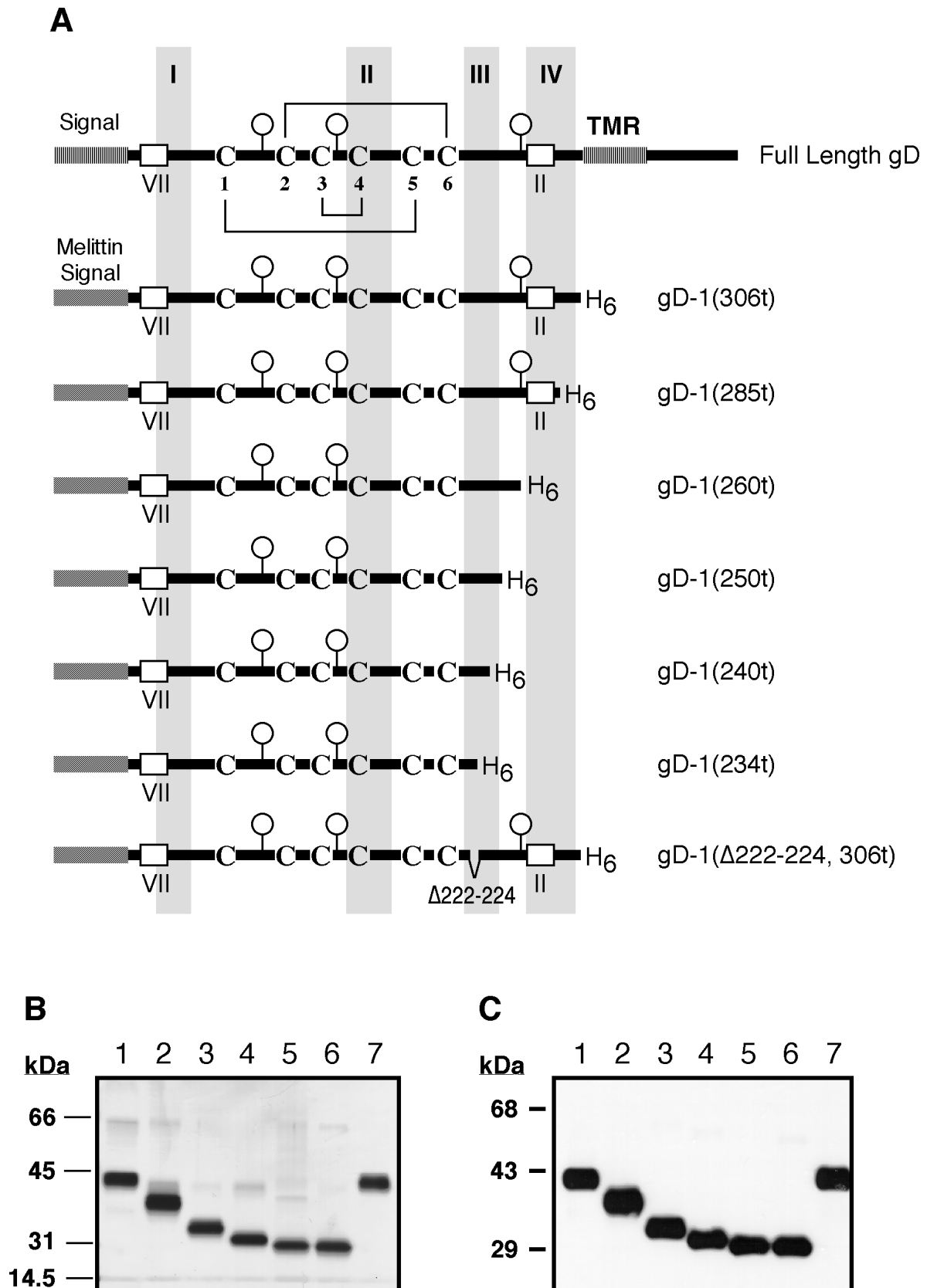


FIG. 1. Recombinant baculovirus-produced proteins. (A) Stick diagrams of full-length HSV gD and the truncated forms used in this study (produced in recombinant baculovirus-infected cells). Functional regions I to IV as defined by Chiang et al. (7) are shown as shaded regions. The positions of linear epitopes for group II and group VII are indicated. The positions of consensus sites for *N*-glycosylation are marked by balloons. The disulfide bond pattern for the six cysteine residues located in the extracellular domain of gD (36) is shown on the full-length gD stick diagram. (B) Silver-stained SDS-polyacrylamide gel (10%) showing the purified recombinant baculovirus-produced proteins used in this study. Lane 1, gD-1(306t); lane 2, gD-1(285t); lane 3, gD-1(260t); lane 4, gD-1(250t); lane 5, gD-1(240t); lane 6, gD-1(234t); lane 7, gD-1(Δ 222-224, 306t). (C) Western blot of the purified proteins shown in panel B probed with MAb 1D3 (group VII).

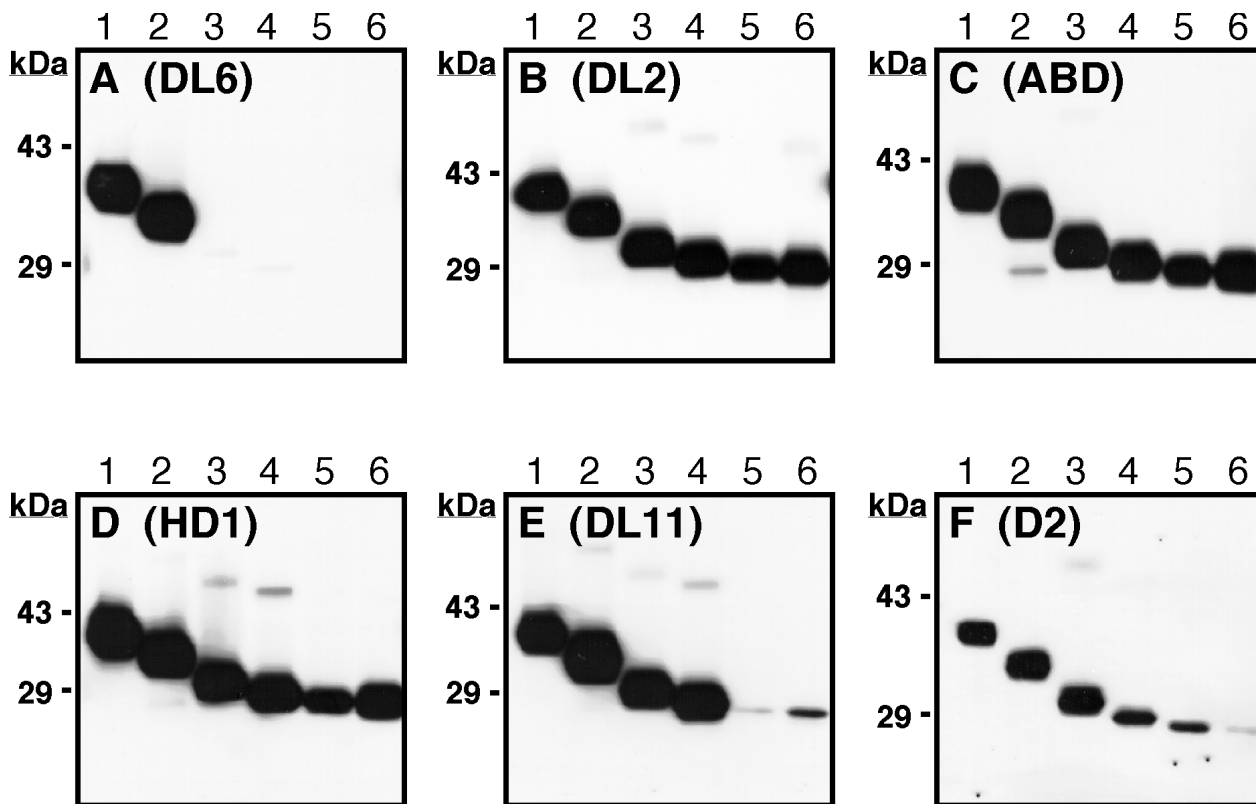


FIG. 2. Antigenic analysis of baculovirus-produced gD truncation mutants. Purified proteins were separated by "native" SDS-PAGE, transferred to nitrocellulose, and probed with gD-specific MAbs. Lane 1, gD-1(306t); lane 2, gD-1(285t); lane 3, gD-1(260t); lane 4, gD-1(250t); lane 5, gD-1(240t); lane 6, gD-1(234t). (A) Blot probed with DL6 (group II MAb). (B) Blot probed with DL2 (group VI MAb). (C) Blot probed with ABD (group III MAb). (D) Blot probed with HD1 (group Ia MAb). (E) Blot probed with DL11 (group Ib MAb). (F) Blot probed with D2 (group Ib MAb).

ative to gD-1(306t) resulted almost exclusively from a faster rate of complex formation (47). In contrast, gD-1(234t) exhibited a faster rate of complex dissociation (k_{off}) with HveA compared to gD-1(306t), suggesting that some gD residues involved in HveA binding had been removed. Here we found

that the binding kinetics and affinities of gD-1(285t), gD-1(260t), and gD-1(250t) for both receptors were quite similar (Table 1). In each case, the higher affinity was due primarily to a faster rate of complex formation (k_{on}). gD-1(240t) exhibited binding kinetics and an affinity similar to gD-1(306t) in its

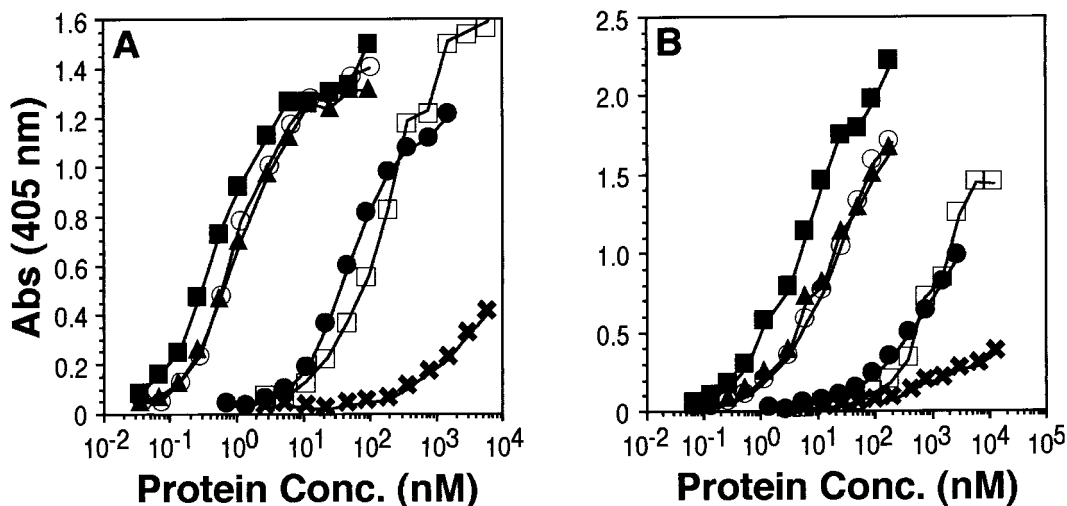


FIG. 3. Analysis of gD truncation mutants for receptor binding by ELISA. The wells of an ELISA plate were coated with an excess of HveA(200t) or HveC(346t) and incubated with increasing concentrations (shown on the x axis) of gD truncation mutants. Bound gD was detected by incubating sample wells with a rabbit antiserum raised against gD (R7), followed by peroxidase-conjugated goat anti-rabbit antibody and then peroxidase substrate. (A) Binding to HveA(200t). (B) Binding to HveC(346t). Symbols: □, gD-1(306t); ■, gD-1(285t); ▲, gD-1(260t); ○, gD-1(250t); ●, gD-1(240t); ×, gD-1(234t).

TABLE 1. Kinetic and affinity values for gD-receptor complex formation

Immobilized receptor	gD	k_{on} ($10^3 \text{ s}^{-1} \text{ M}^{-1}$)	k_{off} (10^{-2} s^{-1})	K_D (10^{-6} M) (k_{off}/k_{on})
HveAt	306t ^a	6.1 ± 0.6^b	2.0 ± 0.2^b	3.2 ± 0.6
	285t ^a	300	1.1	0.037
	260t	130	0.75	0.058
	250t	520	0.94	0.018
	240t	18	1.6	0.89
	234t	ND ^c	20 ^d	20 ^f
HveCt	306t ^a	2.6 ± 0.7^b	0.71 ± 0.09^b	2.7 ± 0.2
	285t ^a	190	0.73	0.038
	260t	280	0.71	0.025
	250t	440	0.66	0.015
	240t	25	2.9	1.2
	234t	ND ^c	10 ^d	2 ^f

^a Values for gD-1(306t) (32, 57), gD-1(285t) (32, 47), and the HveA-gD-1(234t) complex (47) were reported previously and are shown here for comparison.

^b Average \pm the standard deviation from at least three separate experiments.

^c ND, not determined.

^d Estimated maximum k_{off} . As a control, this method was also used to calculate a k_{off} for the dissociation of gD-1(306t) from HveA and HveC. The values obtained were $1.5 \times 10^{-2} \text{ s}^{-1}$ and $0.66 \times 10^{-2} \text{ s}^{-1}$, respectively.

^e χ^2 values for the global fits ranged from 0.7 to 4.5.

^f From Scatchard analysis.

interaction with HveA, consistent with its similar binding properties as determined by ELISA. Although gD-1(240t) exhibited an overall affinity for HveC similar to that of gD-1(306t), the k_{on} was 10-fold faster and the k_{off} was 4-fold faster than gD-1(306t). Thus, in contrast to the ELISA results, the optical biosensor enabled us to distinguish the binding of gD-1(306t) versus gD-1(240t) to HveC. As observed previously (47), gD-1(234t) bound to HveAt, but the data failed to fit a 1:1 Langmuir binding model. A similar result was obtained when gD-1(234t) binding to HveC was examined. Because of this, the binding kinetics for gD-1(234t) could not be analyzed by using the global fitting routine of the instrument software. Instead, maximum k_{off} values were estimated by plotting $\ln(R_0/R_n)$ ver-

sus time for the initial part of the dissociation phase. For both HveA and HveC, the maximum k_{off} for gD-1(234t) was approximately 10-fold faster than for gD-1(306t). Finally, equilibrium dissociation constants (K_D) for gD-1(234t) binding to HveA and HveC were calculated from data collected under conditions of binding equilibrium by using Scatchard analysis. For gD-1(234t) binding to HveA this calculation yielded a K_D approximately sixfold lower than gD-1(306t), while for binding to HveC the K_D was nearly identical to that of gD-1(306t).

Blocking of virus entry by soluble forms of gD. Soluble gD blocks HSV entry into susceptible cells, presumably by binding to and occupying cell surface receptors (28, 45). We tested the abilities of truncated gD to block infection of HeLa and Vero cells (Fig. 4A and B). gD-1(285t), gD-1(260t), and gD-1(250t) blocked HSV infection at similar concentrations (50% inhibition occurred between 1 and 10 nM) and were more potent than gD-1(306t) (50% inhibition occurred between 100 and 200 nM). These results were consistent with the ELISA and biosensor data showing that gD-1(285t), gD-1(260t), and gD-1(250t) bound to both HveA and HveC with greatly increased affinities relative to gD-1(306t). Similarly, the weak ability of gD-1(234t) to block HSV infection is consistent with its diminished capacity to bind HveA or HveC relative to gD-1(306t) (at least by ELISA). Surprisingly, gD-1(240t) blocked HSV infection much less effectively than gD-1(306t) (50% inhibition occurred at approximately 5 μM). This result was unexpected because both the ELISA and biosensor data indicated that this protein bound to both receptor molecules with an affinity similar to gD-1(306t). We have repeated these experiments several times with similar results. These data suggest that gD-1(240t) may lack a portion of a gD functional domain.

Antigenic structure of 3-amino-acid deletion mutants. In order to characterize further the region of gD encompassing functional region III, as well as the residues involved in group Ib MAb binding, we used site-directed mutagenesis to generate a series of plasmids encoding full-length gD with nonoverlapping 3-amino-acid deletions spanning amino acid residues 222 through 254 (Fig. 5). COS cells were transfected separately with plasmids expressing each of the gD mutants. With the

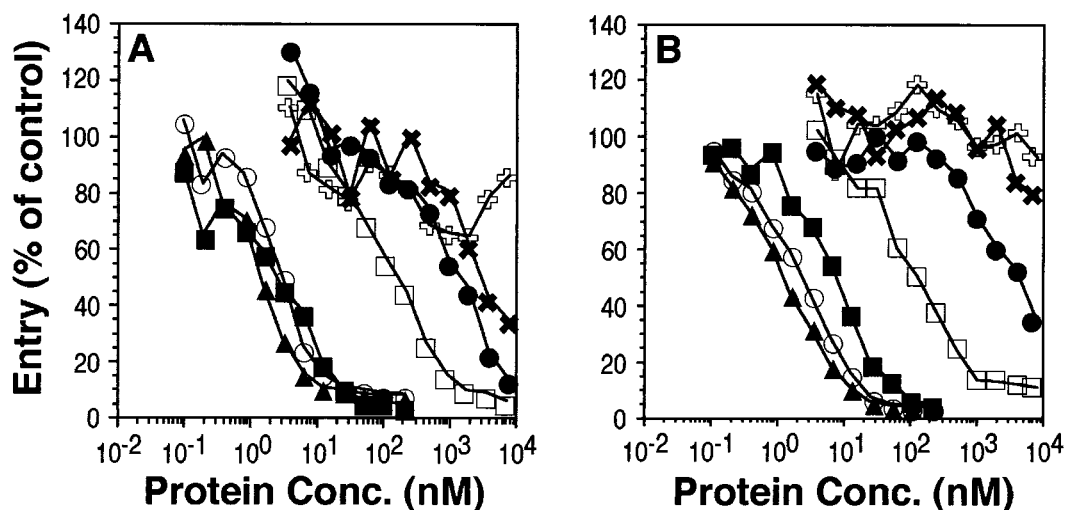


FIG. 4. Analysis of gD truncation mutants for blocking of HSV entry. Cells in 96-well tissue culture plates were incubated with increasing concentrations (shown on the x axis) of various forms of gD prior to infection with HSV-1(KOS) carrying a β -galactosidase reporter gene (*hrrR3*). At 5 h postinfection, cells were lysed and assayed for β -galactosidase activity. (A) HeLa cells. (B) Vero cells. Symbols: \square , gD-1(306t); \blacksquare , gD-1(285t); \blacktriangle , gD-1(260t); \circ , gD-1(250t); \bullet , gD-1(240t); \times , gD-1(234t); \oplus , BSA.

gD-1 Amino Acid Sequence	Deletion	Complementation
220 LPRFIPENQRTVAVYSLKIAGWHGPKAPYTSLLLP	255 NONE	100%
LP---PENQRTVAVYSLKIAGWHGPKAPYTSLLLP	222-224	2
LPRFT---QRTVAVYSLKIAGWHGPKAPYTSLLLP	225-227	0
LPRFIPEN---VAVYSLKIAGWHGPKAPYTSLLLP	228-230	1
LPRFIPENQRT---YSLKIAGWHGPKAPYTSLLLP	231-233	1
LPRFIPENQRTVAV---KIAGWHGPKAPYTSLLLP	234-236	0
LPRFIPENQRTVAVYSL---GWHGPKAPYTSLLLP	237-239	2
LPRFIPENQRTVAVYSLKIA---GPKAPYTSLLLP	240-242	N/D
LPRFIPENQRTVAVYSLKIAGWH---APYTSLLLP	243-245	0
LPRFIPENQRTVAVYSLKIAGWHGPK---TSTLLLP	246-248	0
LPRFIPENQRTVAVYSLKIAGWHGPKAPY---LLLP	249-251	1
LPRFIPENQRTVAVYSLKIAGWHGPKAPYTS---P	252-254	15

FIG. 5. Complementation analysis of 3-amino-acid deletion mutants of HSV-1 gD. The predicted amino acid sequence for residues 220 to 255 of HSV-1 (KOS) gD is shown at the top left (numbering is based on the assignment of residue number 1 to the lysine at the N terminus of the signal-peptidase-processed molecule, which is the 26th residue of the primary translation product). HSV-2 (strain 333) has two amino acid changes relative to gD from the KOS strain in this region of the protein, V→L at residue 233 and A→P at residue 246. Below the wild-type sequence, the corresponding sequences of the 3-amino-acid deletion mutants are shown. The level of complementation of a gD-null virus is shown for each construct in the column at the right and is expressed as a percentage of that achieved with the wild-type construct (the mutant lacking residues 240 to 242 was never detected in transfected cells [see text]).

exception of the $\Delta 240-242$ deletion mutant, all of the mutated proteins were transported to the surface of transfected cells, as detected by immunoperoxidase staining (data not shown). Cytoplasmic extracts were then prepared from COS cells 40 h after transfection. As controls, plasmids pRE4, which expresses wild-type gD-1, and pWW17, which expresses the $\Delta 234-244$ deletion mutant (12), were included. To quantitate relative gD expression, equal volumes of each extract were electrophoresed on a denaturing polyacrylamide gel, followed by transfer to nitrocellulose and probing with MAb DL6 (26). Based on this result, the volumes of extract loaded on subsequent gels were normalized so as to give approximately equal signals with DL6 (Fig. 6A). No protein could be detected for mutant $\Delta 240-242$, even after repetition of the mutagenesis, so it was omitted from further analysis. Each extract was then electrophoresed on a native polyacrylamide gel with no comb, and strips of nitrocellulose were cut from the resulting Western blot and probed with various MAbs (Fig. 6B to D). MAbs HD1 (panel B) and ABD (panel C), which recognize discontinuous epitopes in antigenic sites Ia and III, respectively (41-43), bound to all of the mutant proteins. The binding of DL11 (panel D) was eliminated by deletion of residues 222 to 224, 225 to 227, and 228 to 230 (lanes 1 to 3, respectively), suggesting that residues 222 to 230 contribute to antigenic site Ib.

Activity of deletion mutants in a complementation assay. Each mutant was tested for its ability to complement the production of infectious F-gD β virus in COS cells by using quantities of plasmid DNA that result in similar numbers of gD-expressing cells. F-gD β lacks a gD gene and produces infectious virus only when functional gD protein is provided *in trans* (35). With the exception of $\Delta 252-254$, none of the mutants was able to complement F-gD β , as found previously for the $\Delta 234-244$ mutant (42) (Fig. 5). To address the possibility that lack of complementation was due to failure of the mutated proteins to be incorporated into virions, extracellular complemented F-gD β virus was centrifuged through a 10% sucrose cushion at $84,000 \times g$ for 2 h at 4°C. The pellet was solubilized in denaturing SDS-PAGE sample buffer, electrophoresed on a 7.5% polyacrylamide gel, Western blotted, and probed with

polyclonal antibodies against gD and gB (Fig. 7). Although each of the mutant proteins was detected in virions, the $\Delta 225-227$ and $\Delta 243-245$ proteins were incorporated inefficiently and may explain their complementation-negative phenotype. Taken together, these results suggest that a region encompassing at least residues 222 to 251 is required for gD function.

Expression of gD-1($\Delta 222-224$) and gD-1($\Delta 231-233$) as soluble forms in the baculovirus system. In order to examine the receptor-binding properties of a subset of the 3-amino-acid gD deletion mutants, we constructed two baculovirus recombinants expressing gD-1 truncated after residue 306 and lacking residues 222 to 224 (DL11 negative) or residues 231 to 233 (DL11 positive). While both proteins were detected in recombinant baculovirus-infected insect cells, only the $\Delta 222-224$ protein [gD-1($\Delta 222-224$, 306t)] was secreted. The baculovirus-produced gD-1($\Delta 222-224$, 306t) was purified by nickel-agarose chromatography (Fig. 1), and its reactivity with a panel of MAbs was analyzed by native Western blot (Fig. 8). As antic-

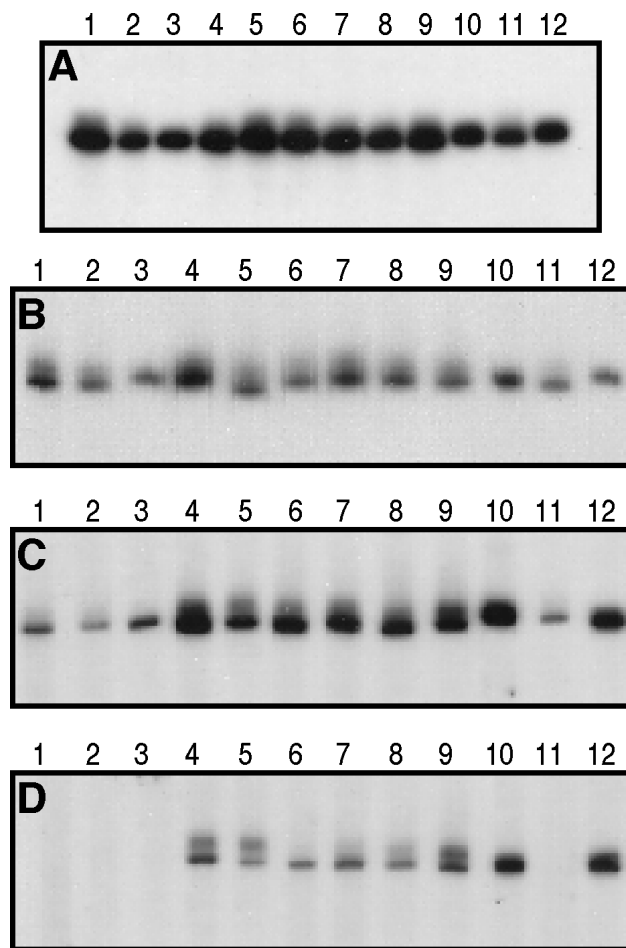


FIG. 6. Antigenic analysis of HSV-1 gD 3-amino-acid deletion mutants. Replicate cultures of COS cells were transfected separately with plasmid constructs expressing wild-type gD, gD lacking residues 234 to 244, and each of the 3-amino-acid deletion mutants. Detergent extracts were prepared from transfected cells after 40 h, subjected to SDS-PAGE, and transferred to nitrocellulose (Western blot). Blots were then probed with MAbs against HSV gD. (A) Blot probed with DL6. (B) Blot probed with ABD. (C) Blot probed with HD1. (D) Blot probed with DL11. Lane 1, gD-1($\Delta 222-224$); lane 2, gD-1($\Delta 225-227$); lane 3, gD-1($\Delta 228-230$); lane 4, gD-1($\Delta 231-233$); lane 5, gD-1($\Delta 234-236$); lane 6, gD-1($\Delta 237-239$); lane 7, gD-1($\Delta 243-245$); lane 8, gD-1($\Delta 246-248$); lane 9, gD-1($\Delta 249-251$); lane 10, gD-1($\Delta 252-254$); lane 11, gD-1($\Delta 234-244$); lane 12, wild-type gD-1.

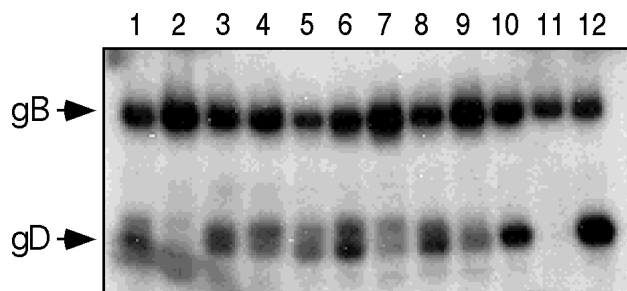


FIG. 7. Detection of gD in F-gD β virus complemented with the 3-amino-acid gD deletion mutants. Extracellular, F-gD β virus which had been complemented separately with each of the 3-amino-acid deletion mutants was prepared as described in the text and analyzed by SDS-PAGE followed by Western blotting. The resulting blot was probed with a mixture of two polyclonal antibodies, R7 (raised against gD) and R69 (raised against gB). Lane 1, virus complemented with gD-1(Δ 222–224); lane 2, virus complemented with gD-1(Δ 225–227); lane 3, virus complemented with gD-1(Δ 228–230); lane 4, virus complemented with gD-1(Δ 231–233); lane 5, virus complemented with gD-1(Δ 234–236); lane 6, virus complemented with gD-1(Δ 237–239); lane 7, virus complemented with gD-1(Δ 243–245); lane 8, virus complemented with gD-1(Δ 246–248); lane 9, virus complemented with gD-1(Δ 249–251); lane 10, virus complemented with gD-1(Δ 252–254); lane 11, cells transfected with salmon sperm DNA; lane 12, virus complemented with wild-type gD-1.

ipated from the data shown in Fig. 5, this mutant form of gD reacted with MAbs ABD and HD1. The deletion mutant also reacted as well as gD-1(306t) with MAbs DL6 and DL2, further validating its structural integrity. In contrast, gD-1(Δ 222–224, 306t) failed to react with either of two group Ib MAbs tested, DL11 and D2. These results were consistent with the antigenic properties of the full-length form of this protein expressed in mammalian cells (see Fig. 6).

gD-1(Δ 222–224, 306t) binds poorly to HveA and HveC and fails to block HSV infection. To assess the receptor-binding properties of gD-1(Δ 222–224, 306t), we examined its ability to bind to HveA(200t) and to HveC(346t) by ELISA. As shown in Fig. 9A, the binding of gD-1(Δ 222–224, 306t) to HveA(200t) was barely detectable, even at concentrations as high as 6 μ M. The binding to HveC(346t) was diminished by ca. 10-fold relative to that of gD-1(306t). Consistent with its reduced binding to both HveA and HveC, gD-1(Δ 222–224, 306t) failed to block virus infection of HeLa or Vero cells (Fig. 10). The failure of gD-1(Δ 222–224, 306t) to bind well to either of two known HSV receptors most likely explains the inability of the full-length form of this protein to block virus infection or to complement the infectivity of a gD-null virus.

DISCUSSION

During the past decade, numerous studies have examined how the structure of HSV gD relates to its function. Some

studies focused on a discontinuous antigenic site which was bound by several type-common, complement-independent neutralizing MAbs (antigenic site I). On the basis of additional characteristics, group I MAbs were subdivided into subgroups Ia and Ib (41). For example, the group Ia MAb, HD1, binds to gD truncated at amino acid residue 233, whereas the group Ib antibodies, such as DL11, do not. Separate studies demonstrated the involvement of residues upstream of 233 in antigenic site Ib as well (39, 43). Single-amino-acid changes were identified which allowed gD to function during virus replication while conferring resistance to neutralization by certain group I antibodies. Specifically, two group Ib antibodies failed to neutralize a virus expressing gD with a Ser-to-Asn change at residue 140, and a third group Ib antibody failed to neutralize a virus expressing gD with a Gln-to-Leu change at residue 132 (Fig. 11).

Evidence of an overlap between antigenic site Ib and a putative functional region of gD was provided by Muggeridge et al. (42), who examined seven gD mutants containing N-terminal, internal, or C-terminal amino acid deletions for their ability to complement a gD-null virus. gD lacking residues 234 to 244 was expressed on the surface of transfected cells and, although not globally altered in structure, failed to rescue the infectivity of a gD-null virus. Interestingly, this mutant protein failed to react with DL11, suggesting that antigenic site Ib, as well as a functional region of gD, was disrupted by this 11-amino-acid deletion. In a similar study, Feenstra et al. (17) found that deletion of residues 231 to 235 resulted in a protein which also failed to complement a gD-null virus but retained reactivity with DL11. More recently, Nicola et al. (44) showed that DL11 blocked the interaction of soluble HveA with gD or with HSV virions, and Krummenacher et al. (31) showed that DL11 blocked the interaction of soluble HveC with HSV virions. Finally, gD truncated at residue 234 was bound by DL11 weakly and bound HveA with a markedly lower affinity (K_D) than molecules truncated at residue 275, 285, or 306 (47).

Linker-scanning mutational analysis of HSV gD (7) identified four distinct functional regions within the gD primary structure wherein linker insertions did not cause global structural alterations but greatly diminished or eliminated the protein's ability to complement the infectivity of a gD-null virus (shaded areas designated I through IV in Fig. 11). This study also revealed a relationship between antigenic site Ib and regions II and III. First, linker insertions within region II abolished or greatly diminished binding by DL11. Second, region II (residues 125 to 161) encompasses residues previously shown to affect the binding of group Ib antibodies (residues 132 and 140; see Fig. 11). Third, region III (residues 225 to 246) includes residues required for group Ib antibody binding. These observations suggest that regions II and III may be closely

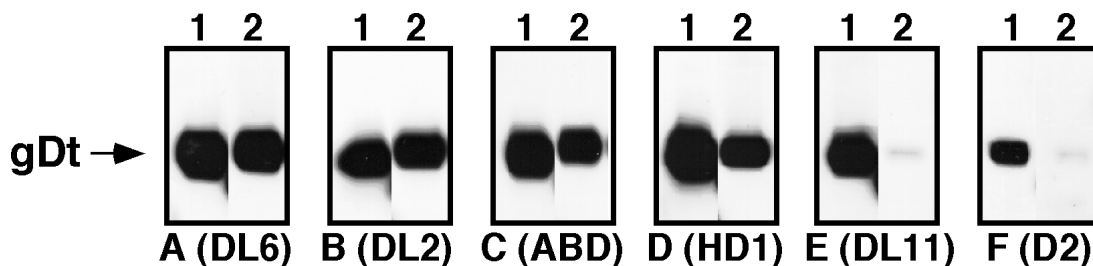


FIG. 8. Antigenic analysis of gD-1(Δ 222–224, 306t) produced in recombinant-baculovirus-infected cells. Purified proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the gD-specific MAbs indicated below each panel. Lane 1, gD-1(306t); lane 2, gD-1(Δ 222–224, 306t). The gD-1(306t) bands shown in each panel correspond to those shown in Fig. 2A to F.

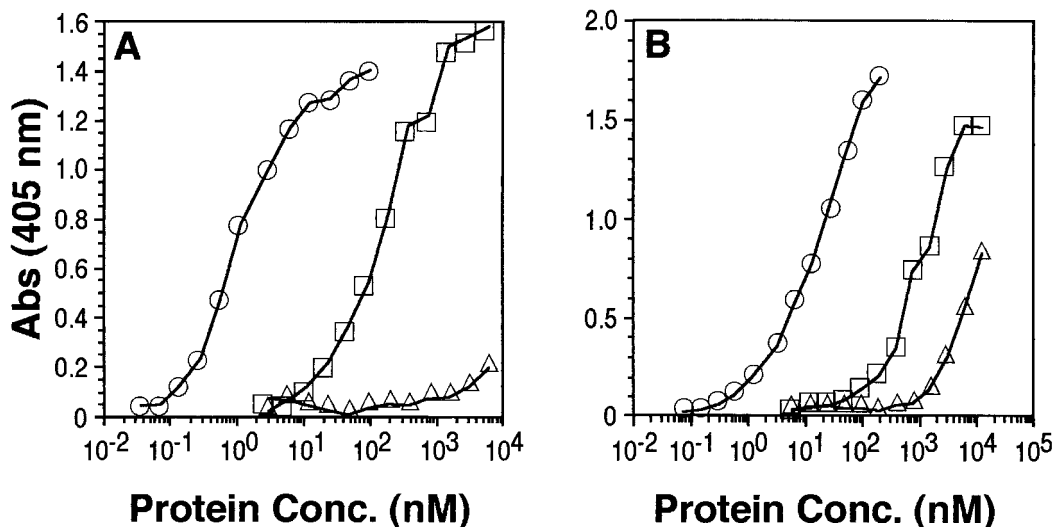


FIG. 9. Analysis of gD-1(Δ 222-224, 306t) for receptor binding by ELISA. The wells of an ELISA plate were coated with an excess of HveA(200t) or HveC(346t) and incubated with increasing concentrations (shown on the x axis) of gD-1(306t), gD-1(250t), or gD-1(Δ 222-224, 306t). Bound gD was detected by incubating sample wells with a rabbit antiserum raised against gD (R7), followed by peroxidase-conjugated goat anti-rabbit antibody and then peroxidase substrate. (A) Binding to HveA(200t). (B) Binding to HveC(346t). Symbols: \square , gD-1(306t); \circ , gD-1(250t); \triangle , gD-1(Δ 222-224, 306t).

positioned within the folded structure of gD and may, together, form a functional (receptor-binding) domain.

In the present study we addressed the contribution of gD residues between 222 and 275 to the formation of both antigenic site Ib as well as a receptor-binding domain. We constructed three baculovirus recombinants expressing gD truncated after residues 240, 250, and 260 and analyzed them along with previously described gD truncations (after residues 234, 285, and 306) for antigenic structure, receptor binding, and virus-blocking activity. All of the truncated proteins were bound by several MABs recognizing discontinuous epitopes. In contrast, reactivity with DL11 was not exhibited by all of the truncation mutants. Although gD-1(250t) reacted strongly with DL11, gD-1(240t) and gD-1(234t) had significantly diminished

reactivity. Thus, we conclude that the C-terminal limit for full DL11 binding occurs between residues 240 and 250.

Analysis of the C-terminal truncation mutants for receptor binding revealed a pattern somewhat similar to that seen for DL11 binding. Using the activity of gD-1(306t) as a reference point, gD-1(285t), gD-1(260t), and gD-1(250t) exhibited enhanced binding to both HveAt and HveCt (Fig. 11), a property previously demonstrated for gD-1(285t) and gD-1(275t) (31, 47). The higher affinity of gD-1(285t) and gD-1(275t) for HveA was shown by optical biosensor studies to result from a faster k_{on} . Here we found that gD-1(260t) and gD-1(250t) bound to both HveA and HveC with kinetics and K_D values very similar to those of gD-1(285t). The calculated K_D values for the interactions of gD-1(240t) with HveA and HveC were quite similar

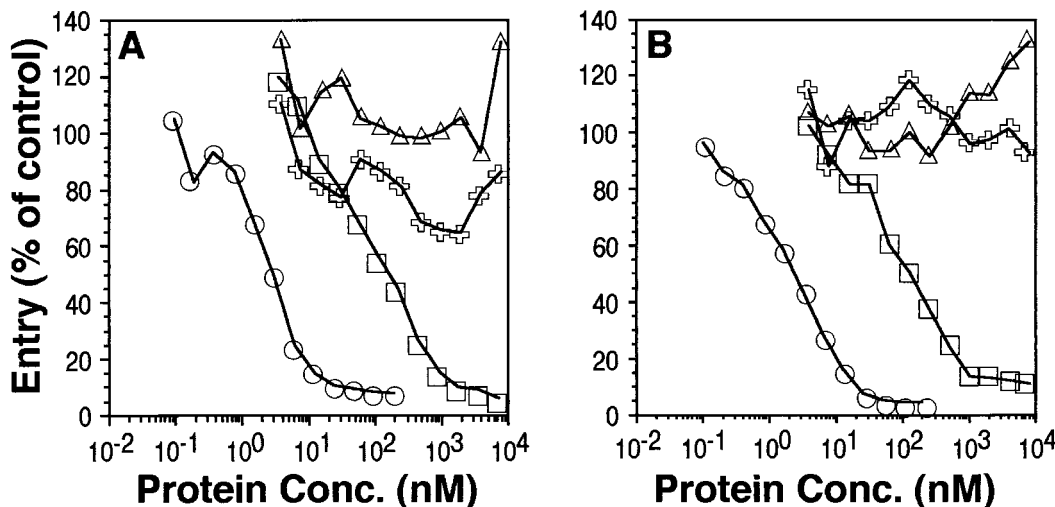


FIG. 10. Analysis of gD-1(Δ 222-224, 306t) for blocking of HSV entry. Cells in 96-well tissue culture plates were incubated with increasing concentrations (shown on the x axis) of gD-1(306t), gD-1(250t), or gD-1(Δ 222-224, 306t) prior to infection with HSV-1(KOS) carrying a β -galactosidase reporter gene (*hrrR3*). At 5 h postinfection, cells were lysed and assayed for β -galactosidase activity. (A) HeLa cells. (B) Vero cells. Symbols: \square , gD-1(306t); \circ , gD-1(250t); \triangle , gD-1(Δ 222-224, 306t); \oplus , bovine serum albumin.

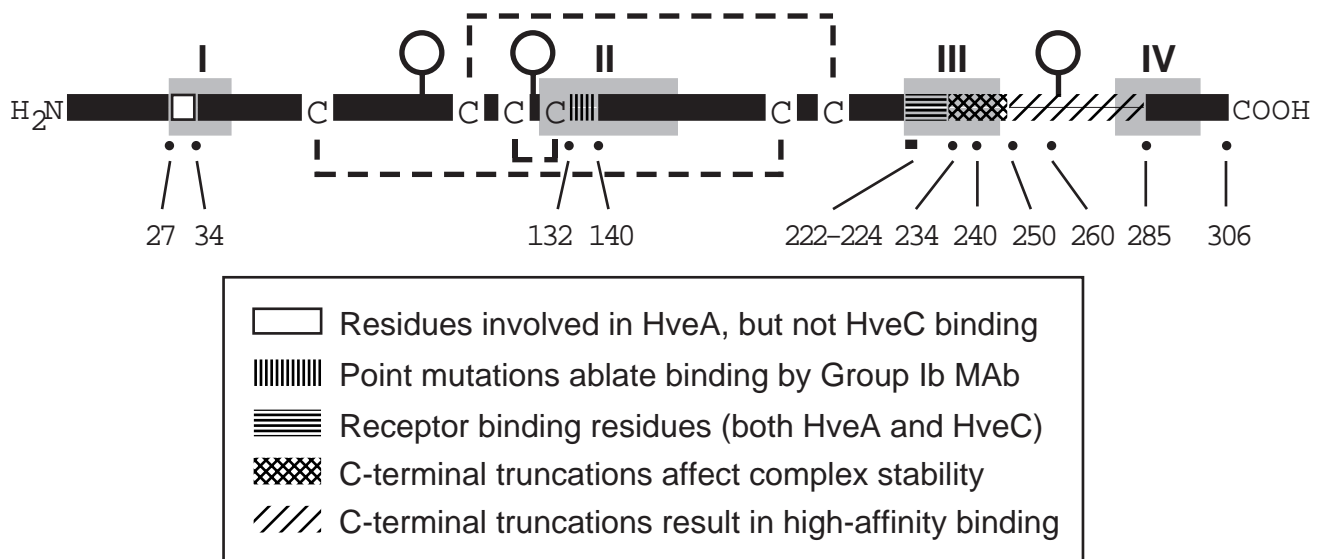


FIG. 11. Stick diagram of HSV gD showing features relevant to this study. The amino- and carboxy-terminal ends of the gD ectodomain are indicated by H₂N and COOH, respectively. Cysteine residues are denoted by C, and the disulfide bond pattern (36) is indicated by dashed lines. Functional regions I to IV as defined by Chiang et al. (7) are shaded in gray. The positions of individual residues relevant to the present study are marked by a dot and labeled with the residue number.

to those for gD-1(306t). Interestingly, the k_{on} and k_{off} values obtained for the interaction of gD-1(240t) with HveC were significantly faster than those obtained for gD-1(306t). From these experiments it is clear that the loss of high-affinity receptor binding by gD is first evident in gD-1(240t), the same truncation point at which full DL11 reactivity is lost. The shorter truncation, gD-1(234t), exhibited an approximately 10-fold faster k_{off} for both HveA and HveC relative to gD-1(306t), perhaps due to deletion of gD residues which stabilize the complexes (Fig. 11).

The ability of soluble gD to bind receptor molecules should theoretically correspond to its ability to block HSV infection of cells bearing those receptors. In the case of both HeLa and Vero cells, the blocking activities of all but one of the proteins closely matched their receptor-binding properties as seen by ELISA. Interestingly, gD-1(240t), which bound both HveA and HveC with a K_D very similar to gD-1(306t), was much less effective in blocking HSV infection than gD-1(306t). Perhaps the membrane-bound forms of HveA and HveC recognize gD somewhat differently than the truncated forms used in the ELISA and biosensor studies. This result might also suggest that there is yet another receptor in HeLa and Vero cells to which gD-1(240t) binds with lower affinity than gD-1(306t). This explanation seems unlikely, at least for HeLa cells, since Cocchi et al. (8) showed that a MAb to HIgR (HveC) effectively blocks HSV infection of this cell line. Alternatively, the difference in blocking ability between gD-1(306t) and gD-1(240t) may reflect the different rates of gD-HveC complex formation and/or dissociation observed in the optical biosensor studies discussed above.

The observation that gD-1(234t) bound HveA, albeit in an unstable manner, indicated that at least some receptor-binding residues were present upstream of 234. To extend our analysis of the group Ib epitope and functional region III, we generated a panel of plasmid constructs expressing full-length HSV-1 gD with sequential, nonoverlapping, 3-amino-acid deletions extending from residue 222 through residue 254. The altered gD molecules expressed in transiently transfected cells were first analyzed for function by using a complementation assay. Only one of the 3-amino-acid gD deletion mutants ($\Delta 252-254$) com-

plemented the infectivity of a gD-null virus, confirming the conclusions of Chiang et al. (7). All forms of gD retained the folded structure necessary for reactivity with group III and group Ia MAbs, and each was detected on the surface of transfected cells. However, gD lacking residues 222 to 224, 225 to 227, or 228 to 230 failed to react with DL11, indicating that even small deletions in this region of gD disrupt antigenic site Ib. Earlier results, examined in connection with data presented here, suggest that gD residues 222 through 230 are critical for proper formation of the DL11 epitope, whereas residues between positions 231 and 250 may be important for proper presentation of the DL11 epitope but are not directly involved in antibody binding.

To examine the receptor-binding properties of some of the 3-amino-acid deletion mutants, we cloned and expressed two of these proteins ($\Delta 222-224$ and $\Delta 231-233$) as truncated forms in the baculovirus system. Although both of these molecules were detected in recombinant-baculovirus-infected cells, only gD-1($\Delta 222-224$, 306t) could be purified from the culture medium. gD-1($\Delta 222-224$, 306t) reacted with several MAbs (but not DL11, as expected), bound weakly to HveA and HveC, and failed to block HSV infection of mammalian cells. This result showed that gD-1($\Delta 222-224$) is nonfunctional due, at least in part, to its greatly diminished binding to cellular receptors. Once again, these data support the concept of an overlap between a receptor-binding domain of gD and the DL11 epitope.

Antibodies directed to viral proteins can neutralize virus infectivity by binding to and occupying the site on a virion protein which interacts with a cellular receptor during virus attachment and entry. This mechanism of neutralization by certain MAbs has been demonstrated for several different viruses, including influenza virus (3, 51). Whether DL11 (and other group Ib MAbs) neutralizes HSV infectivity by occupying part of its receptor binding domain has yet to be conclusively demonstrated. The data presented here and in previous publications are clearly consistent with this interpretation, although it is formally possible that the receptor-binding site on gD is spatially distinct from the group Ib antigenic site. To address this and other questions, we are currently attempting

to determine the crystal structure of gD alone, as well as gD complexed with each of its two known receptors or complexed with the DL11 MAb.

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