Structure-Based Analysis of the Herpes Simplex Virus Glycoprotein D Binding Site Present on Herpesvirus Entry Mediator HveA (HVEM)

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Binding of herpes simplex virus (HSV) envelope glycoprotein D (gD) to a cell surface receptor is an essential step of virus entry. We recently determined the crystal structure of gD bound to one receptor, HveA. HveA is a member of the tumor necrosis factor receptor family and contains four characteristic cysteine-rich domains (CRDs). The first two CRDs of HveA are necessary and sufficient for gD binding. The structure of the gD-HveA complex reveals that 17 amino acids in HveA CRD1 and 4 amino acids in HveA CRD2 directly contact gD. To determine the contribution of these 21 HveA residues to virus entry, we constructed forms of HveA mutated in each of these contact residues. We determined the ability of the mutant proteins to bind gD, facilitate virus entry, and form HveA oligomers. Our results point to a binding hot spot centered around HveA-Y23, a residue that protrudes into a crevice on the surface of gD. Both the hydroxyl group and phenyl group of HveA-Y23 contribute to HSV entry. Our results also suggest that an intermolecular β-sheet formed between gD and HveA residues 35 to 37 contributes to binding and that a C37-C19 disulfide bond in CRD1 is a critical component of HveA structure necessary for gD binding. The results argue that CRD2 is required for gD binding mainly to provide structural support for a gD binding site in CRD1. Only one mutant, HveA-R75A, exhibited enhanced gD binding. While some mutations influenced complex formation, the majority did not affect HSV entry, suggesting that most contact residues contribute to HveA receptor function collectively rather than individually. This structure-based dissection of the gD-HveA binding site highlights the contribution of key residues within HveA to gD binding and HSV entry and defines a target region for the design of small-molecule inhibitors.

In humans, herpes simplex virus (HSV) typically causes mucosal lesions and then spreads to the peripheral nervous system, where it establishes latent infections in sensory neurons. HSV encodes at least 11 membrane glycoproteins, and four of these (gD, gH, gL, and gB) are essential for entry of virions into mammalian cells. Expression of these four glycoproteins alone can facilitate cell fusion (4, 33, 37, 44).

The current model for virus entry posits the following series of events. Initially gC and/or gB binds cell surface heparan sulfate proteoglycans (41). Although binding of gC is a high-affinity interaction (KD = 10−8 M) (38), it is not essential for virus entry (41, 42). This gC binding step is followed by binding of gD to one of several cell surface receptors. This essential step leads to pH-independent membrane fusion of the viral envelope with the cell plasma membrane, a process facilitated by gH/gL heterodimer and/or gB (41). Some experiments suggest that the gH/gL heterodimer and/or gB is the viral fusogen, but how their fusogenic activity is triggered by the gD-receptor interaction is unknown (18, 22).

Several cellular receptors for HSV have been identified through expression cloning and homology searches, including herpesvirus entry mediator A (HveA), nectin-1 (HveC), nectin-2 (HveB), and a modified form of heparan sulfate (3-OST-3) (5, 41). The focus of this study is on HveA (also called HVEM, TNFRSF-14, ATAR, and TR2), a member of the tumor necrosis factor receptor (TNFR) protein family (24, 28, 32). HveA has four cysteine-rich domains (CRDs) that are characteristic of the TNFR family (Fig. 1 and 2A). HveA binds directly to HSV gD and mediates entry of most HSV-1 and HSV-2 strains (32, 47).

Biochemical studies showed that CRD1 and CRD2 of HveA are necessary and sufficient to bind gD (46). A truncated form of HveA containing both CRD1 and CRD2 binds gD and blocks entry of HSV, but forms of HveA consisting of CRD1 or CRD2 alone neither bind gD nor block virus entry. Furthermore, a monoclonal antibody (MAb), CW3, that recognizes a conformational epitope located on CRD1 blocks gD binding to HveA and partially blocks HSV entry (46). This observation led to the suggestion that gD interacts primarily with CRD1. It was hypothesized that the requirement of CRD2 for gD binding was due either to the presence of a few critical binding residues within CRD2 or to an indirect effect of CRD2 on the presentation of a gD binding site within CRD1.

In a series of structure-function studies with purified forms of gD and HveA proteins, we found that the ectodomain of gD truncated at residue 306, gD(306), binds HveA with a KD of 3.2 × 10−6 M. A shorter form of gD truncated at residue 285,
HveA (Fig. 1) (35). Other gD contact residues lie within a previously described epitope (residues 11 to 17) for gD(285t) expressed by recombinant baculovirus-infected insect cells and CRD2 (8, 34).

In accordance with the biochemical data, the crystal structure reveals that gD makes direct contact with amino acids in both CRD1 and CRD2 of HveA, but the majority of contacts lie within CRD1. All of the HveA amino acids that contact gD are within residues 17 to 26 and 30 to 39 of CRD1 and residues 49 and 74 to 76 of CRD2 (Table 1, Fig. 2B). At the gD-HveA interface, an intermolecular antiparallel β-sheet is formed between a β-strand consisting of HveA residues 35 to 37 and a short β-strand in gD consisting of residues 27 to 29 (Fig. 2C). The gD-HveA interface centers around the phenol ring of HveA-Y23, which protrudes into a cleft on the surface of gD (Fig. 2D and E) (8).

The gD contact residues are contained within only two short segments of gD (residues 7 to 15 and 24 to 32) in the N-terminal hairpin loop. Some of these gD contact residues lie within a previously described epitope (residues 11 to 17) for neutralizing group VII MAbs that block binding of gD to HveA (Fig. 1) (35). Other gD contact residues lie within the previously described functional region 1 (FR1) of gD (residues 27 to 43) (Fig. 1) (10). Mutations in FR1 prevent gD from binding HveA and prevent HSV from infecting cells (10, 27, 48).

In this study, we examined the contribution of each HveA contact residue to gD binding, virus entry, and HveA oligomerization. By use of site-directed mutagenesis, we changed each HveA contact residue to alanine and examined the phenotypes of the mutated gD receptors. Our results show that HveA-Y23 plays a key role in gD binding and HSV entry. Three mutations in CRD1 eliminate HveA function as a gD and HSV entry receptor. The results suggest that CRD2 is required for gD binding mainly to provide structural support for a gD binding site present in CRD1. Although eight mutants showed reduced gD binding and one mutant, HveA-R75A, exhibited enhanced binding, mutation of the majority of contact residues had little effect on the ability of HveA to act as an entry receptor. This result suggests that the majority of contact residues contribute to binding collectively rather than individually.

**Materials and Methods**

**Cells and viruses.** B78-H1 mouse melanoma cells (30) and African green monkey kidney (Vero) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS). 293T cells were grown in DMEM supplemented with 10% FCS. HSV-1 hrR3 carries lacZ under the ICP4 promoter and was kindly provided by P. G. Spear (32). HSV Rid1 TK12 was puriﬁed on a sucrose gradient (23).

**PAbs and MAbs.** Rabbit serum R7 polyclonal antibody (PAB) was raised against HSV-2 gD and cross-reacts with HSV-1 gD (25). Rabbit serum R140 PAB was raised against HveA(200t) (43). MAB CW3 was raised against HveA(200t) (43). R166 PAB was raised against nectin-1 [HveC(346t) lacking a His tag] (26) and was used here as a negative control. Immunoglobulin G (IgG) was puriﬁed from rabbit serum or mouse ascites ﬂuid with HiTrap protein G 1-mL columns (Amersham Pharmacia Biotech). R140 PAB and R166 PAB IgGs were directly labeled with Alexa Fluor 488 with the Alexa Fluor 488 protein labeling kit (Molecular Probes).

**Production and puriﬁcation of HSV-1 gD.** Procedures for production and puriﬁcation of gD(285t) expressed by recombinant baculovirus-infected insect cells have been described elsewhere (39).
The N- and C-terminal residues observed in the crystal structure and the location of HveA CRDs are indicated. The HveA molecule is shown partially in blue, and the HveA contact residues found within CRD1 and CRD2 are shown in green. The gD molecule is shown in gray, with the contact residues located in the N-terminal loop of gD shown in red. Contact residues were defined as amino acids containing atoms that come within 4 Å of the partner molecule (see Table 1). Some of the contact residues are numbered for reference. (B) An enlarged view of the gD-HveA interface shown in the same orientation as in panel A. HveA contact residues are displayed in green, and gD contact residues are red. The boxed area indicates a region shown at higher magnification in panel D. (C) Magnification of a disulfide bond within HveA CRD1 and three β-strands within the gD-HveA binding site. HveA-C37 (yellow) forms a disulfide bond with HveA-C19 (purple). Residues 35 to 37 within a β-strand of HveA (residues 35 to 40, shown in green) form hydrogen bonds with a short β-strand on gD (gD residues 27 to 29, shown in red) to form an intermolecular, antiparallel β-sheet. This augments a two-stranded β-sheet in HveA CRD1 formed by residues 22 to 26 (blue) and residues 35 to 40 (green). (D) Interactions between HveA-Y23 and gD amino acids. Carbon (green), oxygen (red), nitrogen (purple), and sulfur (yellow) atoms are shown. The blue dotted lines indicate hydrogen bonds between HveA-Y23 and gD. The black lines indicate other interactions, defined as distances of 4 Å or less (see Table 1). The hydroxyl group of HveA-Y23 interacts with gD-A12 and gD-L25. (E) Space-filling model of the gD binding site on the surface of HveA. The gD-HveA interface from panel B is rotated so that the N-terminal loop of gD lies on top of the HveA binding surface. The N-terminal loop of gD is shown as a gray ribbon, with the gD contact residues colored red. The HveA contact residues are numbered. The space-filling spheres represent atoms of HveA contact residues from category 1 (blue), category 2 (purple), category 3 (yellow), and category 4 (green) (see Table 1). HveA-Y23 (green) is located at the center of the interface.
dilutions of R140 PAb IgG or CV3 MAb IgG to detect HveA cell surface expression. All antibodies were prepared in DMEM-5% FCS. Cells were washed with PBS three times and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Ig antibodies for 30 min at 25°C. Following another three PBS washes, cells were rinsed with 20 mM citrate buffer (pH 4.5). ABTS peroxidase substrate (Moss, Inc.) was added, the absorbance at 405 nm was recorded at multiple time points with a microtiter plate reader, and the mean slopes were recorded.

**Virus entry assay.** To detect HSV entry mediated by HveA, we used a modification of a published entry assay (36). Briefly, B78-H1 cells transfected with HveA plasmids as above were plated in 96-well plates and grown to confluence overnight at 37°C. Serial dilutions of HSV-1 hrR3 were added, and the cells were incubated for 60 min at 4°C and then shifted to 37°C for 3 h. Following another 6 h, the cells were washed with PBS and lysed in DMEM containing 0.5% NP-40. β-Galactosidase activity in the lysate was measured by adding substrate (chlorophenol red-β-D-galactopyranoside; Boehringer Mannheim) and measuring absorbance at 570 nm.

**RESULTS**

**Production and expression of HveA mutants.** To determine the contribution to receptor function of the 21 HveA amino acids that contact gD, we individually mutated each of these HveA contact residues. HveA contact residues were determined by use of the crystal structure of HveA bound to gD (PDB identification number 1JMA) and were defined as those amino acids containing atoms located within 4 Å of atoms of the gD structure (Table 1; Fig. 2B). Site-directed mutagenesis was used to substitute alanine for each of the HveA contact residues. This amino acid was chosen as a generic substitute because its presence removes the majority of the original residue’s side chain atoms. Alanine is also commonly found in many secondary structures in both exposed and buried positions (45). The final plasmid constructs were cloned and sequenced, and the phenotypes of the mutated receptors in cells were compared to wild-type HveA.

Surface expression was used as a first indication that each of the mutant HveA molecules was properly folded and transported to the plasma membrane. Plasmids carrying each of the HveA mutants were transfected into B78-H1 cells. This mouse melanoma cell line does not express any gD receptors, but it is used as a reporter of a published entry assay (36). Briefly, B78-H1 cells were transiently transfected with plasmids carrying each of the HveA mutants and evaluated for the expression of HveA after 48 h. (A) Cells were stained with fluorescently labeled anti-HveA PAb IgG and visualized by immunofluorescence microscopy to detect cell surface expression. Results for the transfection of wild-type HveA (wtHveA) and three representative HveA mutants are shown. (B) HveA expression was quantitated by CELISA. Cells were plated on 96-well plates and treated with anti-HveA PAb IgG and analyzed by flow cytometry (FACS). Sample data for wild-type HveA and three representative HveA mutants show the level and range of positive staining. (D) Flow cytometry data for all of the HveA mutants are shown to indicate the percentage of cells staining positive for HveA. The dotted line represents 50% of the cells staining positive for HveA. Mutants were divided into four categories based on their ability to bind gD (see Fig. 4 and Table 1).

the other 18 HveA mutants were expressed on the cell surface, as judged by immunofluorescence assay.

Two methods were used to quantify the relative level of HveA on the cell surface. In the first, expression was measured by CELISA (19). B78-H1 cells were transiently transfected

![Figure 3](http://jvi.asm.org/)

**FIG. 3.** Expression of HveA mutant proteins on transfected cells. B78-H1 cells were transiently transfected with plasmids carrying each of the HveA mutants and evaluated for the expression of HveA after 48 h. (A) Cells were stained with fluorescently labeled anti-HveA PAb IgG and visualized by immunofluorescence microscopy to detect cell surface expression. Results for the transfection of wild-type HveA (wtHveA) and three representative HveA mutants are shown. (B) HveA expression was quantitated by CELISA. Cells were plated on 96-well plates and treated with anti-HveA PAb IgG and analyzed by flow cytometry (FACS). Sample data for wild-type HveA and three representative HveA mutants show the level and range of positive staining. (D) Flow cytometry data for all of the HveA mutants are shown to indicate the percentage of cells staining positive for HveA. The dotted line represents 50% of the cells staining positive for HveA. Mutants were divided into four categories based on their ability to bind gD (see Fig. 4 and Table 1).
with each of the HveA mutants. The transfected cells were fixed with paraformaldehyde and incubated with various concentrations of R140, followed by secondary antibody conjugated to horseradish peroxide. Signal levels were essentially the same regardless of whether the cells were fixed before or after the addition of antibody (data not shown). Data are shown for wild-type HveA and the three representative HveA mutants (Fig. 3B). HveA expression by cells transfected with plasmids carrying these three mutants was identical to that of cells transfected with the wild-type HveA plasmid. Similar CELISA results were obtained for the other 18 HveA mutants (data not shown).

As a second method, flow cytometry was used to determine both the level and percentage of transiently transfected B78-H1 cells expressing the HveA mutant proteins. Transiently transfected B78-H1 cells were fixed with paraformaldehyde and stained with fluorescently labeled R140 to detect HveA surface expression. Data for wild-type HveA and the three representative HveA mutants showed that signal intensity varied over a broad range (Fig. 3C). For wild-type HveA and 20 of the HveA mutants, approximately 50% of the cells displayed detectable levels of HveA (Fig. 3D). For HveA-C37A, only about 30% of cells stained positive for cell surface expression of the receptor. Thus, flow cytometry profiles similar to those for wild-type HveA were observed for 20 of the 21 mutants.

**Binding of gD to HveA mutants.** We used CELISA to determine the ability of gD to bind to the various HveA mutant proteins expressed on the cell surface. Various concentrations of gD(285t) were added to B78-H1 cells that had been transfected with plasmids expressing the HveA mutants. gD binding was detected with an anti-gD polyclonal antibody. By use of this approach, the 21 HveA mutants were initially categorized based on their gD binding phenotype. Sample data for wild-type HveA and the three representative HveA mutants are shown (Fig. 4A). No gD bound to cells transfected with HveA-Y23A. gD bound less well to cells transfected with HveA-Y23A. gD bound less well to cells transfected with HveA-S74A than it did to those transfected with wild-type HveA. We were surprised to find that gD showed enhanced binding to cells transfected with HveA-R75A.

To categorize the gD binding phenotypes of all the HveA mutants (Table 1), we calculated the amount of gD bound to each mutant as a percentage of the amount of gD bound to wild-type HveA by using two different gD concentrations. At 100 nM gDt, nine HveA mutants bound gD at wild-type levels (category 1), one bound gD at increased levels (category 2), and 11 bound gD at reduced levels (category 3). Of the 11 mutants showing reduced binding at 100 nM gDt, 8 bound at reduced levels when the gDt concentration was increased to 1 μM (category 4), while the remaining 3 mutants did not bind gD at all (category 4) (Fig. 4C). For each HveA mutant, these experiments were repeated at least three times, and the pattern of gD binding remained consistent.

The three HveA mutations that led to a complete loss of gDt binding are located in CRD1 (HveA-Y23A, HveA-G22A, and HveA-C37A). The eight mutations that produced reduced gDt binding are distributed in both CRD1 (HveA-P17A, HveA-K26A, HveA-G34A, HveA-T35A, HveA-V36A, and HveA-P39A) and CRD2 (HveA-S74A and HveA-T76A). The mutation that resulted in significantly increased gDt binding, HveA-R75A, is in CRD2. The remaining nine mutations had only modest effects on gDt binding and are located in both CRD1 (HveA-K18A, HveA-S20A, HveA-P21A, HveA-G30A, HveA-E31A, HveA-L32A, HveA-T33A, and HveA-E38A) and CRD2 (HveA-L49A).

These results indicate that mutations in some contact residues negatively affect HveA binding to gD more than others.
i.e., some contact residues are more critical for gD binding than are others. Some of these phenotypes are readily understood based on the structure of the gD-HveA complex. For example, in the crystal structure, HveA-Y23 lies at the center of the interface between gD and HveA, protrudes into a pocket on the surface of gD, and makes extensive contacts to seven atoms of gD (Fig. 2D and E, Table 1). The loss of gD binding by HveA-Y23A is most likely due to the loss of direct gD interactions with the Y23 side chain. In contrast, HveA-R75 makes multiple contacts with gD, and we might have expected mutation to alanine to reduce gD binding. The observed increased gD binding by HveA-R75A suggests that the side chain of HveA-R75 may instead hinder complex formation.

HveA mutants mediating entry of HSV. We wanted to determine whether the observed changes in gD binding correlate with the ability of HveA to function as an entry receptor. B78-H1 cells were transiently transfected with plasmids expressing each of the 21 HveA mutants and infected with HSV carrying lacZ under the control of the ICP6 promoter. After 6 h of infection, cells were lysed, and the level of β-galactosidase activity was determined and used as a measure of entry (32). The HveA mutants fell into two groups, those that mediated HSV entry and those that did not. Data for wild-type HveA and the three representative HveA mutants are shown in Fig. 5A. HveA-R75A and HveA-S74A were as effective as wild-type HveA for HSV entry, while HveA-Y23A was non-permissive.

A comparison of entry data for all of the HveA mutants at a single HSV concentration is shown in Fig. 5B. The three HveA mutants that did not bind gD, HveA-Y23A, HveA-G22A, and HveA-C37A, were not permissive for HSV entry (Table 1). All of the remaining HveA mutants were able to mediate HSV entry at or near wild-type levels, regardless of their level of gD binding. Thus, the effect of mutations on HveA function as a viral receptor was less apparent in the entry assay than in the gD binding assay. Some subtle effects of mutation on entry were noted, but the differences were small.

Additional HveA mutations. We constructed an additional series of HveA mutants to address questions raised by the previous results. HveA expression levels were determined by CELISA, and the ability of the mutants to mediate HSV entry was determined with the protocol described previously.

(i) HveA-Y23. We have shown that HveA-Y23 is critical for HveA function as a gD receptor. This amino acid interacts with gD through its hydroxyl group as well as its phenyl ring (Fig. 2D). To address the relative importance of the hydroxyl group and phenyl ring of HveA-Y23, we constructed HveA-Y23F. CELISA data indicate that HveA-Y23F is expressed on cells at wild-type levels (data not shown). We found that HveA-Y23F permits entry of HSV, although the efficiency of entry is reduced approximately 50% compared to wild-type HveA (Fig. 5C). The properties of HveA-Y23A and HveA-Y23F indicate that while the hydroxyl group is not required for HSV entry,

![FIG. 5. HveA mutant proteins mediating HSV entry into transfected cells. Transiently transfected B78-H1 cells expressing the HveA mutant proteins were seeded on 96-well plates, incubated with an HSV β-galactosidase reporter virus for 6 h, and assayed for β-galactosidase activity as a measure of virus entry. (A) Sample data show the ability of wild-type HveA (wHveA) and three representative HveA mutants to mediate entry of increasing amounts of HSV. Assays detecting HveA expression and gDt binding were run in parallel (Fig. 3 and 4). (B) Data show the original panel of 21 HveA mutants mediating entry at one concentration of HSV. Mutants were divided into the categories based on gD binding phenotype (see Fig. 4 and Table 1). Data are expressed as a percentage of entry mediated by wild-type HveA. Results from one representative experiment are shown. (C) The ability of additional HveA mutants to mediate entry of increasing amounts of HSV is shown. (D) Sample data show the inability of wild-type HveA and three representative HveA mutants to mediate entry of increasing amounts of a mutant β-galactosidase reporter virus, HSV Rid1, after 6 h. Vero cells were included as a positive control for HSV Rid1 entry.](http://jvi.asm.org/)
both the phenyl ring and the hydroxyl group of HveA-Y23 contribute to HveA function as an entry receptor.

(ii) HveA-C37. The inability of HveA-C37A to bind gD could be due to either the loss of direct contacts between HveA-C37 and gD or the loss of a disulfide bond between HveA-C37 and its partner HveA-C19 (Fig. 2C). To address this issue, we constructed the mutant HveA-C19A. HveA-C19A was expressed on cells at wild-type levels (data not shown) but did not permit entry of HSV (Fig. 5C). We conclude that the failure of HveA-C37A to bind gD and mediate HSV entry is due to the loss of a disulfide bond between HveA-C37 and HveA-C19 that likely affects HveA conformation.

(iii) HveA CRD2. Mutations of contact residues in CRD2 increased (HveA-R75A), decreased (HveA-S74A, HveA-T76A), or did not affect (HveA-L49A) gD binding, but none eliminated HSV entry. We wanted to determine whether HveA function would be affected when three contiguous CRD2 residues were mutated in the same molecule. We constructed the HveA triple mutant HveA-74/75/76A, containing alanine substitutions for HveA-S74, -R75, and -T76. HveA-74/75/76A was expressed on the cell surface at wild-type levels and functioned as an HSV receptor as well as wild-type HveA did (Fig. 5C). Thus, none of these three CRD2 residues that contact gD are absolutely required for HveA function in HSV entry.

Do any of the HveA mutants mediate entry of HSV Rid1? HSV Ridley is a virus with a single amino acid mutation in gD (Q27P). This virus is unable to use human HveA for entry, and gD from this virus exhibits a 10-fold higher affinity for a second HSV receptor, nectin-1 (16, 20, 27, 32). The gD-HveA crystal structure suggests that the Rid1 mutation might distort the intermolecular antiparallel β-sheet formed between HveA-T35, HveA-V36, and HveA-C37 and gD residues 27 to 29 (8).

We asked whether any of the original panel of 21 HveA mutants were able to compensate for the HSV Rid1 entry defect. Dilutions of HSV-Rid1 carrying lacZ under control of the thymidine kinase promoter (32) were added to B78-H1 cells that had been previously transfected with each of the HveA mutants. β-Galactosidase activity was determined as a measure of entry after 6 h. As a positive control, Vero cells, which are permissive for HSV Rid1, were also infected with the virus. Results for wild-type HveA and three representative mutants are shown in Fig. 5D. Neither wild-type HveA nor any of the HveA mutants were able to mediate entry of HSV Rid1 (data not shown).

HveA oligomerization. Many members of the TNFR family oligomerize in the absence of ligand via pre-ligand association domains (PLADs) present in CRD1 (9). It has been proposed that TNFR trimerization is required for ligand binding and subsequent signaling events in the cell. The TNFR-1 ectodomain crystallizes as a dimer with multiple monomer-monomer contacts in CRD1 (34). The crystal structure of the ligand-receptor tumor necrosis factor alpha–TNFR-1 complex revealed a tumor necrosis factor alpha trimer bound to three receptors, with no direct contacts among the three receptors (1). In contrast, the crystal structure of the gD(285t)-HveA complex shows a monomer of HveA bound to a monomer of gD. However, nondenaturing Western blot studies and gel filtration analyses indicate that HveA forms oligomers via CRD1, and several biochemical assays indicate that gD is a dimer (32, 46, 48).

Thus, it is possible that mutations in HveA may disrupt HveA oligomerization and thereby affect gD binding and HSV entry. To address this possibility, plasmids carrying the original 21 HveA mutants were transfected into 293T cells to obtain sufficient amounts of receptor for Western blotting. Cell extracts were electrophoresed on SDS gels under non-denaturing conditions (12), and Western blots were probed with R140, a polyclonal antibody that recognizes both monomeric and dimeric forms of HveA. All of the HveA mutants were expressed in 293T cells. Sample data for wild-type HveA and four selected HveA mutants are shown in Fig. 6. The monomer form of wild-type HveA was present at 55 kDa, and a dimer form of HveA was present at 110 kDa. The amount of HveA in these cell extracts was not equilibrated, and differences in signal levels do not indicate differences in levels of expression. Each of the mutant forms of HveA was detected in both monomeric and dimeric forms to the same extent as the wild type, including HveA-K18A. An alignment of HveA with TNFR-1 shows that HveA-K18 is homologous to TNFR-K32. In contrast to HveA-K18A, the mutation TNFR-K32A prevented TNFR-1 oligomerization (9). The apparently reduced molecular weight of HveA-S74A (Fig. 6) is likely due to the fact that this mutation eliminates an N-glycosylation site at HveA-N72. Loss of this N-glycosylation does not prevent the receptor from mediating HSV entry (Fig. 5A). The other 17 HveA mutants also exhibited a normal pattern of oligomerization (data not shown). These results indicate that the inability of HveA-G22A, HveA-Y23A, and HveA-C37A to bind gD and mediate HSV entry is probably not due to defects in dimerization. The gD binding site and the HveA dimerization site in CRD1 are not the same.
DISCUSSION

The crystal structure of the gD-HveA complex shows that gD directly contacts residues in both CRD1 and CRD2 of HveA. In this study, we used site-directed mutagenesis to determine the contribution of each of the HveA contact residues to gD binding, HSV entry, and HveA oligomerization. We found that some mutations had a greater effect on HveA function than did others. Thus, the HveA contact residues do not contribute equally to gD binding. We determined that HveA-Y23 is critical for gD binding. Twenty-one HveA contact residue mutants were classified into four categories (Table 1). Nine mutants displayed wild-type HveA function (category 1); one bound gDt at increased levels and permitted HSV entry (category 2); eight bound gDt at reduced levels but permitted HSV entry (category 3); and three did not bind gDt or mediate HSV entry (category 4).

Mutations that do not affect HveA function (category 1). gD binding and HSV entry were not greatly affected by alanine substitutions in 9 of the 21 contact residues (Table 1). Most of these residues interact with gD solely through main chain atoms, and thus it is not surprising that substituting alanine for these residues has little effect on HveA function. HveA-S20 and HveA-E38 have multiple side chain interactions with gD (Table 1), yet HveA-S20A and HveA-E38A function as well as wild-type HveA. Thus, analysis of the number of side chain versus main chain interactions for a residue does not always predict the effect of a mutation in that residue on HveA function.

Mutation that increases gDt binding and permits HSV entry (category 2). HveA-R75A permits HSV entry and binds gDt better than does wild-type HveA. This result suggests that the side chain of HveA-R75 partially interferes with gD binding instead of promoting gD binding, as might be expected from the crystal structure. The negative effect of the HveA-R75 side chain on gD binding may be due to its presence in a region of positively charged gD residues (gD-R16, gD-R18, and gD-K20) and subsequent unfavorable electrostatic interactions across the protein interface. The availability of a presumably high-affinity form of HveA, such as HveA-R75A, may prove valuable for future studies of HveA, just as the high-affinity form of gD, gD(285t), has been fundamental to our studies of gD.

Mutations that reduce gDt binding but permit HSV entry (category 3). Alanine substitutions in 8 of the 21 contact residues resulted in substantially reduced gD binding without greatly affecting the level of HSV entry (Table 1). Thus, reduced levels of gD binding do not necessarily affect HveA function in HSV entry. Reduced gD binding may affect the rate of HSV entry, but preliminary experiments suggest that this is not the case (data not shown). Only those mutations that were totally negative for gD binding (category 4) failed to function for HSV entry.

Five of the residues in category 3 (P17, K26, G34, T35, and V36) cluster near a β-strand consisting of gD residues 27 to 29 (Fig. 2E). This β-strand participates in an intermolecular antiparallel β-sheet with HveA residues 35 to 37 and augments a two-stranded β-sheet in HveA CRD1 (Fig. 2C) (8). Results for category 3 mutants suggest that the intermolecular β-sheet is important for gD binding because substituting alanine for any of these five residues diminishes gD binding, possibly by directly or indirectly disrupting this β-sheet.

Mutation of residues that contact gD through side chain atoms resulted in reduced gD binding more frequently than mutation of residues that contact gD through only main chain atoms (Table 1). However, some results for mutants in category 3 were different from what might be predicted from the crystal structure. For example, HveA-V36A shows reduced gD binding despite the fact that only the main chain atoms of HveA-V36 contact gD. The HveA-V36A mutation most likely reduces gD binding indirectly by causing a local change in conformation.

The results for category 2 and 3 mutants suggest that HSV entry tolerates a wide range of affinities between HveA and gD. Similarly, a wide range of affinities between gD and nectin-1 are tolerated for entry of HSV, bovine herpesvirus, and pseudorabies virus (13, 27, 31). Future optical biosensor analysis of gD binding to mutant HveA proteins will define more precisely the binding kinetics and affinity for gD of these HveA mutants.

Why were differences in levels of HSV entry generally more subtle than differences in the levels of gD binding? One possible explanation is that the high levels of HveA expression associated with transient transfection may mask differences in the efficiency of entry mediated by the mutant receptors. Additionally, the interaction between gD and HveA may be different when gD is presented in the context of an intact virion rather than in a soluble form. Studies of influenza virus hemagglutinin (HA) have shown virus infection to be a highly sensitive assay for protein function, in that HA mutants displaying severely diminished fusion capacity were able to facilitate virus entry (14). Binding to an abundant receptor, such as sialic acid in the cases of influenza virus and polyomavirus, may permit the virus to contact multiple receptors, and this avidity may facilitate efficient virus entry. In fact, in the case of polyomavirus, mutants that bind receptor more weakly are more virulent in the host (2).

Mutations in HveA that abolish function (category 4). We identified four mutations in HveA that result in the complete loss of gD binding and ability to mediate HSV entry. Two of these mutations involve HveA-Y23 (HveA-Y23A and HveA-G22A), and two (HveA-C37A and HveA-C19A) eliminate a disulfide bond in CRD1.

(i) HveA-Y23 is critical for HveA function. The inability of HveA-Y23A to bind gD was predicted from the gD-HveA crystal structure (8). HveA-Y23 lies at the center of the gD-HveA interface and contacts gD residues on both sides of the N-terminal gD loop (Fig. 2D, E). This amino acid is sandwiched between the side chains of gD-M11 and gD-P14 and makes extensive contacts with seven atoms on three gD residues (Fig. 2D, Table 1). The hydroxyl group of HveA-Y23 is hydrogen bonded to gD-A12 and gD-L25. Previous studies have shown that substitution of gD-L25 with proline permits HSV to overcome gD-mediated interference to virus entry, alters HSV gD receptor usage, and has been postulated to prevent the interaction of gD with HveA (6, 29, 41).

To determine the contribution to HveA function of the hydroxyl group versus the phenyl group of HveA-Y23, we constructed mutant HveA-Y23F. The ability of HveA-Y23F to mediate HSV entry indicates that interactions with the hy-
hydroxyl group of HveA-Y23 are not critical for HveA function. This result is supported by the fact that the primate HveA homolog functions as an HSV entry mediator and has phenylalanine in place of tyrosine at position 23 (17). From this result, we predict that gD-M11 is important for gD binding to HveA because gD-M11 interacts with the HveA-Y23 phenyl ring (Fig. 2D) and lies within a known epitope for neutralizing anti-gD MAb (group VII) (35). However, the reduced capacity of HveA-Y23F to mediate HSV entry indicates the HveA-Y23 hydroxyl group does contribute to HveA function, possibly through the hydrogen bond with gD-L25 (Fig. 2D).

The inability of HveA-G22A to bind gD may be due to a disruption of interactions between the adjacent residue HveA-Y23 and gD. The introduction of an extra methyl group into HveA-G22 may cause a shift in the position of HveA-Y23. Alternatively, replacing glycine with the conformationally more restricted amino acid alanine may cause a structural change in HveA and thereby impair gD binding. This alternative possibility is supported by the observation that G22 is a structurally conserved residue present in many TNFR-like molecules (6, 29, 41).

Studies in other laboratories have indicated that single residues can contribute a large fraction of the binding energy at protein-protein interfaces (3). These residues are located at “hot spots” of binding and are generally found at the center of the interface. A hot spot on HveA for gD binding lies at HveA-Y23. Tyrosine is frequently found in binding hot spots and is three times more likely to be in a hot spot than phenylalanine, suggesting that the ability of tyrosine to hydrogen bond may be important (3).

Structure-based mutagenesis of human growth hormone has shown that a subset of contact residues account for complex formation with the human growth hormone receptor (11, 15). The conclusion that the human growth hormone functional binding epitope is much smaller than the structural epitope suggested that design of smaller hormone mimics may be possible. Likewise, the results of the present study indicate that a small-molecule inhibitor could be designed to prevent gD interactions with the phenol ring of HveA-Y23 (8) and thereby abolish the ability of HveA to permit HSV entry. Two synthetic peptides isolated from phage display libraries have been shown to bind HveA and inhibit gD binding (40). The mechanism of this inhibition is unknown. In future studies we plan to test the ability of these peptides to bind HveA-Y23A, HveA-Y23F, and HveA-G22A. The results may indicate whether the peptides inhibit gD binding by interfering with interactions between HveA-Y23 and gD.

(ii) A disulfide bond in CRD1 is critical for HveA function. The loss of gDt binding by both HveA-C37A and HveA-C19A is probably due to the loss of a disulfide bond that supports CRD1 structure and the gD binding site (Fig. 2C). However, indirect evidence suggests that HveA-C37A and HveA-C19A retain much of the wild-type HveA conformation. HveA-C37A is expressed on the cell surface and is able to form dimers. Additionally, both HveA-C37A and HveA-C19A are recognized by the MAb CW3 (data not shown), a conformation-dependent MAb that binds HveA CRD1 and blocks HSV entry (46).

An alternative explanation for the loss of gDt binding by both HveA-C37A and HveA-C19A is that the absence of the disulfide bond disrupts an intermolecular β-sheet formed between HveA residues 35 to 37 and gD residues 27 to 29 (Fig. 2C). Mutations in gD-Q27 (Rid mutations) are predicted to disrupt this β-sheet interaction and eliminate an intermolecular hydrogen bond between gD-Q27 and HveA-C37 (8), yielding one explanation for the inability of the HSV Rid viruses to use HveA for entry.

CRD2 contact residues are not critical for HveA function. Previously, analysis of a series of HveA truncations localized the gD binding domain to CRD1 and CRD2 of HveA (46). These findings were validated by the crystal structure of the gD-HveA complex (8). In the present study, none of the individual mutations in CRD2 contact residues completely eliminated HveA function in gD binding or HSV entry. In fact, even when residues S74, R75, and T76 were all mutated to alanine in the same molecule (HveA-74/75/76A), the receptor still functioned in entry. These results suggest that CRD2 is required for gD binding mainly to provide structural support for the gD binding site in HveA CRD1. The fact that HveA binds gD primarily via residues in CRD1 explains why MAb CW3, an antibody that binds entirely within CRD1, is able to efficiently block gD binding to HveA (46).

None of the HveA mutations compensate for the entry deficiency of HSV mutant strain Rid1. The mutant strain HSV Rid1 carries the single gD mutation Q27P and was selected for its resistance to gD-mediated interference to infection (16). Although human HveA does not serve as an entry receptor for HSV Rid1, the murine HveA homolog has been reported to mediate entry of HSV Rid1 (41), suggesting that conservative changes in HveA can compensate for the block to entry. Two of the mutations made in this study (HveA-E38A and HveA-L49A) have changes identical to changes present in the murine HveA homolog (24). The inability of these two mutations to mediate HSV Rid1 entry indicates that the E38A and L49A changes are not solely responsible for the ability of murine HveA to mediate HSV Rid1 entry. In fact, none of the mutations introduced in this study resulted in forms of HveA that permit entry of HSV Rid1. Even the increased gD binding of HveA-R75A was insufficient to overcome the HSV Rid1 block to HveA-mediated entry. The ability of murine HveA to mediate HSV Rid1 entry is probably due to multiple changes in the gD binding region.

HveA oligomerization is not affected by the HveA mutations. Truncations of the HveA ectodomain have indicated that CRD1 plays a major role in HveA oligomerization (46). Many members of the TNFR family oligomerize via a domain present in CRD1 called a PLAD (9). A single alanine substitution at lysine-32 within TNFR-1 CRD1 prevents trimers from forming, suggesting that this residue is a required element of the TNFR-1 PLAD (9). Our Western blot analysis of proteins electrophoresed on nondenaturing gels indicates that a mutation in HveA homologous to TNFR-K32A, HveA-K18A, does not affect HveA oligomerization. Although it is possible that this analysis of HveA in cell extracts does not accurately reflect the state of the HveA on the cell surface, the results suggest that if HveA contains a PLAD, it differs from the PLAD of TNFR-1.

Changes in the ability of some HveA mutants to bind gD and mediate HSV entry cannot be attributed to a lack of HveA dimerization. None of the HveA mutants were deficient in...
dimerization, indicating that the gD binding site does not directly overlap the HveA dimerization site in CRD1.

Although HveA crystallizes as a monomer bound to gD (8), gel filtration analyses have suggested that a dimer of HveA binds to a monomer of gD (47). HveA mutants that dimerize but do not permit HSV entry, such as HveA-Y23A, could be used to examine whether HveA oligomerization plays a role in entry. If so, HveA-Y23A may dominantly interfere with the function of wild-type HveA during HSV entry. Such studies are anticipated as a follow-up to this initial report.

In summary, we have determined the contribution of specific HveA residues within the gD binding site to HveA function as a gD and HSV entry receptor. HveA-Y23 plays a key role in gD binding and HSV entry due to a large number of interactions with gD. An intermolecular β-sheet formed between gD and HveA residues 35 to 37 also contributes to binding. Our results suggest that the functional gD binding site lies primarily within HveA CRD1. Most of the other HveA contact residues contribute collectively rather than individually to the interaction between HveA and gD. The binding hot spot containing HveA-Y23 may be a good target for the design of an inhibitor of gD-HveA complex formation. Similar mutagenesis studies to examine the properties of gD contact residues are currently under way.

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