Localization of the interaction site of HSV gD on the membrane fusion regulator, gH/gL

Tina M. Cairns[1*], Doina Atanasiu[1], Wan Ting Saw[1], Huan Lou[1], J. Charles Whitbeck[1], Noah T. Ditto[3], Birgitte Bruun[4], Helena Browne[4], Lucas Bennett[5], Chun Wu[5,6], Claude Krummenacher[5,7], Benjamin D. Brooks[3], Roselyn J. Eisenberg[2], and Gary H. Cohen[1]

[1]Department of Basic and Translational Science, School of Dental Medicine, [2]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA

[3]Carterra Inc., Salt Lake City, UT, USA

[4]Division of Virology, Department of Pathology, University of Cambridge, Cambridge, UK

[5]Department of Molecular and Cellular Biosciences, [6]Department of Chemistry and Biochemistry, [7]Department of Biological Sciences, Rowan University, Glassboro, NJ, USA

*Corresponding author

E-mail: tmcairns@upenn.edu (TMC)

Abstract (250 words max)

A cascade of protein-protein interactions between four herpes simplex virus (HSV) glycoproteins (gD, gH/gL, gB) drive fusion between the HSV envelope and host membrane, thereby allowing for virus entry and infection. Specifically, binding of gD to one of its receptors induces a conformational change that allows gD to bind to the regulatory complex gH/gL, which then activates the fusogen gB, resulting in membrane fusion. Using surface plasmon resonance and a panel of anti-gD monoclonal antibodies (MAbs) that sterically blocked the interaction, we previously showed that gH/gL binds directly to gD at sites distinct from the gD receptor binding site. Here, using an analogous strategy, we first evaluated the ability of a panel of uncharacterized anti-gH/gL MAbs to block binding to gD and/or inhibit fusion. We found that the epitopes of four gD-gH/gL blocking MAbs were located within flexible regions of the gH N-terminus and the gL C-terminus, while the fifth was placed around gL residue 77. Taken together, our data localized the gD binding region on gH/gL to a group of gH and gL residues within the front end of the heterodimer. Surprisingly, a second set of MAbs did not block gD-gH/gL binding but instead stabilized the complex by altering the kinetic binding. However, despite this prolonged gD-gH/gL interaction, "stabilizing" MAbs also inhibited cell-cell fusion, suggesting a unique mechanism of protection and further supporting targeting of this interaction to prevent fusion in both therapeutic and vaccine strategies against HSV.

Importance (150 words max)

Entry of HSV into target cells is driven by a cascade of protein-protein interactions involving four viral glycoproteins (gD, gH/gL, gB). Using a highly sensitive surface plasmon resonance

technique and a panel of anti-gD monoclonal antibodies (MAbs), we recently identified a domain on the receptor-binding protein gD that promoted direct interaction with the fusion regulator gH/gL. Here, using an analogous approach, we identified anti-gH/gL MAbs that block gD-gH/gL binding, thereby identifying the gD binding domain on gH/gL. Surprisingly, we also identified anti-gH/gL MAbs that stabilized the usually fleeting interaction between gD and gH/gL. As both "blocking" and "stabilizing" MAbs inhibited membrane fusion, our findings shed light on the function of gH/gL in the fusion pathway and support targeting this interaction for both therapeutic and vaccine strategies against HSV.

Introduction

The entry of herpes simplex virus (HSV) into a mammalian cell requires the coordinated action of four viral glycoproteins (gD, gH/gL, gB). However, unlike many other viruses, herpesviruses encode receptor-binding and membrane fusion functions on separate proteins (gD and gB, respectively). As such, fusion is driven by a cascade of protein-protein interactions that is initiated by the binding of gD to one of its cellular receptors (HVEM or nectin-1) (1-7).

HSV gH is a type I transmembrane protein, whereas HSV gL is not membrane-anchored and associates non-covalently with the gH ectodomain. On mature virions and on the surface of HSV-infected cells, gH and gL are found together in a stable 1:1 heterodimeric complex (8). The crystal structure of a modified form of HSV-2 gH/gL revealed an extensive interaction between the two proteins that explained their interdependence for folding, transport, and function (8-11). gH/gL work together as a unit to regulate the activity of the herpesvirus fusogen, gB (1, 9, 12, 13). However, the activity of gH/gL itself is regulated by another viral protein, gD.

Crystal structures of gD with and without HVEM and nectin-1 (14-17) reveal that the C-terminus of the gD ectodomain normally occludes the receptor binding site and must move away before it can interact with the receptor. We hypothesize that this conformational change in gD also promotes its physical interaction with gH/gL, thereby resulting in its functional activation and consequent promotion of the cascade of events leading to fusion (1, 12). A form of HSV gH/gL containing only the heterodimer ectodomain can trigger fusion of cells expressing gB, gD, and a gD receptor (albeit with lower efficiency than full-length gH/gL) (1), suggesting that the postulated physical interaction between gD and gH/gL occurs within the gH/gL ectodomain. Furthermore, by generating gH/gL chimeras encoding segments of HSV-1 and saimiriine herpesvirus-1 sequence, a gD interaction site on gH/gL was roughly mapped to the N-terminal half of the gH ectodomain (18).

In our previous study, we used surface plasmon resonance (SPR) to demonstrate binding between soluble forms of HSV-2 gD (gD2) and gH/gL (gH2/gL2) (19). We used a form of gD [gD2(285t)] with the C-terminus removed (20), so it would already be "primed" for interaction with gH/gL (21). The soluble gD was captured to a biosensor chip via an anti-gD monoclonal antibody (MAb) that presented the gH/gL binding face of gD, and then soluble gH/gL was added. We found that gH/gL bound directly to gD with a relatively fast on- and off-rate. Anti-gD MAbs were identified that blocked the binding of gH/gL and the detailed location of their epitopes outlined a potential gH/gL binding region on gD that spanned a face adjacent to, but distinct from, the predicted nectin-1 and HVEM binding sites.

Having identified the gH/gL binding site on gD, we next set out to determine the physical location of the reciprocal binding site on gH/gL. To accomplish this, we first characterized a panel of anti-gH/gL MAbs and grouped them into antigenic communities sharing similar

competitive and phenotypic traits. We then determined their ability to block the interaction of gD-gH/gL and assessed their function in a cell-cell fusion assay. The MAbs separated into three groups: 1) those that had no effect on either gD-gH/gL binding or cell-cell fusion; 2) those that blocked gD-gH/gL binding and inhibited cell-cell fusion; and 3) those that, surprisingly, stabilized the gD-gH/gL complex, a subset of which inhibited cell-cell fusion. By comparing the kinetics between gH/gL bound with IgG (dimeric binding) vs Fab (monomeric binding), we determined that the stabilization effect was due to the valence of IgG. However, valence had no impact on blocking gH/gL binding to gD, as both IgG and Fabs blocked equally well. Several of the MAbs that blocked the gD-gH/gL interaction localized to regions of gH/gL that were missing in the crystal structure; therefore, to visualize this potential binding region we developed a 3D structural model for the entire gH/gL ectodomain. We concluded that the gD binding region on gH/gL includes the very N-terminus of gH, the C-terminus of gL, and is centered around gL residue 77. Now that gD-gH/gH interaction domain has been identified, it can be targeted to prevent fusion for use in both therapeutic and vaccine approaches against HSV.

Results

Previously, we used a large panel of anti-gD MAbs (22) to screen for those that blocked gD-gH/gL binding, enabling us to determine a possible gH/gL binding region on gD (19). Here, we used a similar method to probe the corresponding side of the gD-gH/gL complex. For this, we first characterized a large panel of anti-gH/gL MAbs, and then screened them for their ability to block complex formation with gD. We hypothesized that the epitopes of anti-gH/gL MAbs that block binding to gD would locate a potential gD binding site on gH/gL. Furthermore, we

screened these MAbs for inhibition of fusion, theorizing that those that blocked gD-gH/gL binding would also have a functional impact.

For this study, we first used a high-throughput surface plasmon resonance (SPR) technique (22-24) to determine which of a panel of 36 MAbs compete for binding to gH/gL (Fig. 1A). MAbs (IgG) were covalently arrayed on an SPR sensor chip and then soluble forms of either gH1/gL1 or gH2/gL2 were injected across the chip surface followed by each of the 36 MAbs injected in series (24). Sensorgrams of the binding were analyzed to generate a heat map and dendrogram for each protein (Fig. S1). MAbs were arranged into bins to reflect competition vs. no competition (23-25), and grouped into a network plot, descriptive of which epitopes are engaged. The result is a graphical representation of the heat map (Fig. S1A, C). Finally, the data were depicted as MAb "communities" that reflect their competitive behavior (Fig. 1B, C).

The MAb community maps for gH1/gL1 and gH2/gL2 are shown in Fig. 1B and 1C, respectively. We color-coded each community: purple, green, orange, blue, and yellow for gH1/gL1 (Fig. 1B) and purple, green, yellow, cyan, and red for gH2/gL2 (Fig 1C). The purple community of gH2/gL2 was further sub-divided into purple, orange, and magenta based on competition and previous mapping data (26). The arrangement of the MAbs within and between communities reflects the extent of competition. For example, the red community in gH2/gL2 form a tight, overlapping cluster of MAbs that have a high degree of competition between all members (Fig. 1C).

Here, we provided the first analysis of cross-competition between MAbs that bind specifically to either gH or gL, as our previous study examined anti-gH and anti-gL MAbs separately (26). Thus, this study allowed us to examine the relationships of MAbs in the gH/gL

complex itself. Interestingly, the three MAbs against gL (26-28) were sorted into their own communities (CHL18 to the cyan community of gH2/gL2 and L4 and CΔ48L3 to the yellow communities of gH1/gL1 and gH2/gL2, respectively).

Epitope mapping MAbs that bind gH2/gL2.

Our next objective was to map the epitopes of key members of each community and position them onto the 3D structure of gH2/gL2 (Fig. 2). To account for residues that were not resolved by crystallography (9), we used a hypothetical model of the complete gH2/gL2 ectodomain (Fig. S2). Previous peptide binding data (26, 29) localizes the epitopes of yellow (CΔ48L3), cyan (CHL18), magenta (CHL17, CHL32), and orange (CHL29, CHL30, CHL31, CHL35) MAbs to specific stretches of amino acids (gL 173-183, gL 208-219, gH 19-38, gH 145-155 and 676-686, correspondingly). Likewise, monoclonal antibody resistant (*mar*) virus data for CHL2 localizes the red community around gH residue 116 (26). However, we had no information regarding the location of the purple (CHL21, CHL37) and green (CHL27, 53S) communities. Only 6 of the 36 anti-gH/gL MAbs bind type-common epitopes, i.e. bind both gH1/gL1 and gH2/gL2 (designated by asterisks in Fig. 1B, C). The type-common MAbs, which are found in purple, orange, and green communities, serve as a "bridge" between data generated on either gH1/gL1 or gH2/gL2 through the type-specific MAbs with which they compete.

(i) Epitope mapping of CHL21 and CHL37.

CHL21 and CHL37 (purple community) recognized type-common epitopes, but also competed with type-1 specific MAbs 46S, 52S, and BBH5 (Fig. 1B). CHL37 was previously characterized via Western blot analysis as a linear, type-2 specific anti-gH MAb (26). Here, we show that newly characterized MAb CHL21 behaves in a similar fashion by Western blot (Fig.

3A). In contrast to the Western data, both CHL37 and CHL21 bind to gH1/gL1 via SPR (Fig. 3B), suggesting that the epitopes of CHL21 and CHL37 in gH1/gL1 are more sensitive to the detergent used in both denaturing and "native" Western blot analyses (30). We previously used a panel of overlapping peptides to localize CHL37 to the N-terminus of gH (26). However, we found that it (and CHL21) can bind to gH2Δ48/gL2 (Fig. 3A,B), which contains an N-terminal gH deletion, suggesting that its epitope lies further downstream. This discrepancy may be due to amino acid similarity between the N-terminus of gH and the CHL37 epitope. Based on competition with 52S (31) and BBH5 (Materials and Methods) (Fig. 1B), we placed the epitopes of CHL21 and CHL37 within the C-terminal half of gH, where the 52S epitope has been located (Fig. 2A).

(ii) Epitope mapping of CHL27.

To determine the location of the CHL27 epitope, we used SPR to screen for binding to several forms of gH/gL (Fig. 4A). CHL27 bound to both gH1/gL1 and gH2/gL2 but was unable to bind to gH2Δ48/gL2 (Fig. 4A), indicating that its epitope lies within the gH2 N-terminus. Interestingly, MAb 53S, which competes with CHL27 for binding to gH/gL (Fig. 1B, C), bound to gH2Δ48/gL2 (Fig. 4A), effectively separating the location of these two MAb epitopes.

We next screened CHL27 for binding to gH peptides, particularly those within the N-terminal region. CHL27 bound to two gH2 peptides, one comprising residues 28-47 and the other comprising residues 37-56 (Fig. 4B), thus defining its epitope to within amino acids 37-47 (Fig. 2; Fig. 4C). We used Western blot analysis of five full-length gH point mutants (previously characterized in Cairns et al. (32)) to confirm the peptide data. Four mutations (R39A, Y41A,

R43A, D44A) abrogated CHL27 binding (Fig. 4D). Therefore, we conclude that the epitope for CHL27 encompasses at least these four residues within the gH N-terminus (Fig. 4C, green).

(iii) Epitope mapping of 53S.

Prior studies suggest that the conformation-dependent epitope of 53S is located in the N-terminal half of gH/gL and may contain gL residues (8, 33-37). 53S competes with CHL27 for gH/gL binding (Fig. 1B, C) yet these two MAbs bind to distinct regions of gH/gL, as removal of the gH N-terminus abrogates CHL27 binding but not 53S binding (Fig. 4A). To localize the 53S epitope, we combined our Carterra (Figs. 1 and S1) and BIACORE (data not shown) SPR competition data to develop a 53S "competition tree" showing its relationships with other MAbs (Fig. 5A). 53S competes for binding with CHL27, BBH3, and LP11, but not with BBH2 or BBH4. Interestingly, 53S and LP11 exhibited unidirectional competition, as inhibition was dependent on which MAb bound to gH/gL first; if LP11 bound first, binding of 53S was blocked but not vice versa. This result suggests that either a conformational change occurs upon binding of LP1 that prevents 53S from binding, or that binding of LP11 prevents a conformational change required for 53S to bind. BBH2 and BBH4, which compete with LP11, do not compete with 53S or CHL27 (Figs. 1B and S1). Using point mutations in gH1 known to abrogate the binding of these five MAbs (LP11, CHL27, BBH3, BBH2/BBH4) as a guide, we localized a potential region for the 53S epitope on gH2: "north" of LP11, "east" of BBH3, and "west" of CHL27 in the 3D model (Fig. 5B, dotted circle). Two previously characterized gL insertion mutants which show a decreased reactivity to 53S (P48 and R55) also lie in this region (37).

To further define the 53S epitope we chose nine surface-accessible amino acids [two gH residues (V161, T162) and seven gL residues (R46, D50, D51, P77, Q138, H142, P144)] to

mutate to alanine and test for 53S binding (Fig. 5B, green). Each mutation was placed into a mammalian expression plasmid containing full-length gH2. The two mutant forms of gH2 and seven of gL2 were co-transfected into C10 cells with WT-gL2 or gH2, respectively. Total cell lysates were tested for protein expression by reactivity to anti-gH2/gL2 polyclonal antibody (PAb) R176 using Western blotting (Fig. 5C). Although all proteins were expressed, mutant gH-V161A was present at reduced levels and contained a lower level of glycosylated gH (the top band of the gH doublet, Fig. 5C), indicative of altered protein processing and insufficient trafficking to the cell surface (10, 11, 32). However, with the exception of gH-V161A, all mutants had near-WT levels of cell-surface expression as detected by MAbs CHL27 (anti-gH) and CΔ48L3 (anti-gL) in a CELISA (Fig. 5D).

Due to 53S binding a conformational epitope (and being unreactive in Western blotting due to the presence of detergent), 53S reactivity was examined by CELISA. Only mutant gL-P77A was severely deficient in 53S binding (Fig. 5C). This reduction in 53S binding was not indicative of poor processing or lack of cell-surface protein expression (Fig. 5C,D).

We next used the split luciferase (cell-cell) fusion assay (SLA) (27, 38) to test each mutant for function. In the SLA, reconstitution of functional luciferase from its two "split" domains, $Rluc8_{(1–7)}$ and $Rluc8_{(8-11)}$, individually expressed in effector and target cells, reflects the level of fusion. Effector B78H1 cells were transfected with gB2, gH2, gL2 and $Rluc8_{(1–7)}$ plasmids, while target C10 cells expressing the gD receptor nectin-1 were transfected with $Rluc8_{(8–11)}$ plasmid (39). Effector cells were incubated with the SLA substrate for 1 h before target cells were added. Soluble gD2(306t) was added when effector and target cells were mixed to trigger fusion and activity was measured for 2 h. As expected due to its reduced expression, mutant gH-V161A exhibited a 60% decrease in fusion activity as compared to WT gH (Fig. 5E).

However, mutant gL-P77A was fully functional in cell-cell fusion (Fig. 5E). Thus, with its loss of 53S binding but retention of fusion function, mutant gL-P77A appeared to resemble a 53S MAb-resistant (*mar*) mutant protein (40).

Anti-gH/gL MAbs block or stabilize gD-gH/gL binding.

In our previously described SPR assay (Fig. 6A), we detected an interaction between gD2 and gH2/gL2 when gD was captured by certain anti-gD MAbs and presented on a biosensor chip and gH/gL was flowed (i.e., gD is the ligand and gH/gL is the analyte) (Fig. 6B), but not in the reverse orientation (19). This interaction was prevented by the binding of a subset of anti-gD MAbs bound to gD prior to addition of gH/gL, enabling us to identify the gH/gL binding "face" on gD (19). Having defined the epitopes of our anti-gH/gL MAb panel, our next goal was to exploit this technique to define the site on gH/gL that interacts with gD.

We adapted this experimental design to map the gD binding site on gH/gL (Fig. 7A). In this approach, we first incubated gH/gL with anti-gH/gL MAbs for 10 minutes prior to injection across a chip containing gD captured by 1D3. The control was to flow gH/gL alone (Fig. 7, black curves). The colored curves show gH/gL binding when pre-incubated with the indicated MAb, with each color signifying its community membership (Fig. 1C). Importantly, we found that a subset of anti-gH/gL MAbs blocked gD-gH/gL binding while others did not (Figs. 7B-E). MAbs in the green (53S, CHL27) and magenta (CHL17, CHL32) communities blocked the interaction with gD by 85-100%, and MAb CHL18 decreased gH/gL binding to gD by 50% (Fig. 7B). In contrast, MAbs in the orange community had no effect on gD-gH/gL binding (Fig. 7C).

We also found a third, unexpected, outcome. When MAbs from the purple (CHL21, CHL37), red (CHL2, CHL4) or yellow (CΔ48L3) communities were pre-incubated with gH/gL,

binding to gD was not blocked. Yet, the gH/gL binding kinetics were markedly different from that observed when pre-incubated with either orange community MAbs or no MAb (compare Fig. 7D to 7C). Whereas gH/gL binding normally exhibits a fast on- and off-rate (black curves) (19), its off-rate slowed considerably when it was pre-mixed with purple, red, or yellow MAbs (Fig. 7D,E). These results suggest that the red, purple, and yellow MAbs stabilize the gD-gH/gL binding complex.

The effects of anti-gH/gL MAbs on gD-gH/gL binding are dose-dependent.

Next, we tested if the effects of the anti-gH/gL MAbs on gD-gH/gL binding were dose-dependent. As in the previous figure, the level of gH/gL binding to gD in the absence of antibody is shown in each graph as a black curve. We used anti-gB MAb A22 (41), which does not bind to gD or gH/gL, as a control; as expected, it did not inhibit the binding of gH/gL to gD at any concentration tested (Fig. 8A).

In these studies, we found that MAb 53S inhibited gH/gL binding to gD in a dose-dependent manner, with binding partially reduced at the lowest concentration of IgG tested and binding was completely blocked by 0.2 mg/mL (Fig. 8B). MAb CHL17 blocked gD-gH/gL binding in a similar dose-dependent manner, with complete blocking by 0.4 mg/mL IgG (Fig. 8C). In contrast, blocking by MAb CHL18 was dose-dependent but not complete, even at the highest concentration tested (1mg/mL IgG) (Fig. 8D). One interpretation of these data is that the epitope of CHL18, which is at the C-terminus of gL (26), is only partially exposed (29) and therefore the MAb cannot fully access the epitope to prevent binding to gD.

MAb CHL2, which appeared to stabilize the gD-gH/gL interaction (Fig. 7E), did not block the interaction at any concentration tested (Fig. 8E). However, effects on the kinetics of

gH/gL binding to gD were dose-dependent, with the off-rate appearing to be slower at higher concentrations of IgG (Fig. 8E). This effect was also observed with MAb CΔ48L3 (Fig. 8F).

Stabilization of the gD-gH/gL complex requires bivalent IgG.

The subset of anti-gH/gL MAbs that stabilized the gD-gH/gL complex were found in three communities (purple, red, yellow) whose epitopes are spread across the gH/gL molecule (Fig. 2), therefore this "function" cannot be localized to a single domain on the heterodimer. As each individual MAb (IgG) can bind to two antigens simultaneously, we asked whether the gD-gH/gL complex was stabilized through cross-linking of two gH/gL molecules. To address this question, we generated antigen-binding fragments (Fabs) that are capable of binding only one antigenic molecule. Hence, if cross-linking was a factor, pre-incubation of gH/gL with Fab would not stabilize gD-gH/gL complexes.

We generated Fabs for six of the anti-gH/gL MAbs tested in Fig. 7, representing five of the six MAb communities (CHL17, magenta; CHL27 and 53S, green; CΔ48L3, yellow; CHL2, red; CHL37, purple) (Fig. 1C). All Fabs were tested using SPR for gH/gL binding; unfortunately, the Fab generated from CHL18 (cyan community) did not bind to gH/gL and was omitted from this study (data not shown). We found that MAbs CHL2, CHL37, and CΔ48L3, which stabilized the complex as IgG, failed to do so as Fabs, there was no change in the off-rates of gH/gL/Fab on gD, and the binding curves overlapped that of gH/gL alone on gD (Fig. 9). For comparison, MAbs that blocked the gD-gH/gL interaction (CHL17, CHL27, 53S) did so as both Fabs (Fig. 9) and IgG (Fig. 7). We can draw two conclusions from this data. First, the ability of MAbs to stabilize gH/gL binding to gD is due to the cross-linking of two separate gH molecules. Second, the ability of certain MAbs to block the gD-gH/gL interaction is not due to cross-

linking, but more likely is due to interfering with the interaction itself. As such, we suggest that the epitopes of these MAbs overlap the gD binding site on gH/gL.

The effect of anti-gH/gL MAbs on cell-cell fusion.

Having characterized our panel of anti-gH2/gL2 MAbs, our goal was to use this information to determine the functional site on gH/gL that interacts with gD, thereby enabling gH/gL to be triggered to activate the fusion protein gB to drive fusion. To monitor fusion, we again used the SLA (Fig. 5E) (27, 38). Specifically, effector cells were transfected with gB2, gH2, gL2 and $Rluc8_{(1–7)}$ plasmids, while target cells expressing the gD receptor nectin-1 were transfected with $Rluc8_{(8–11)}$ plasmid. Effector cells were incubated with the luciferase substrate, incubated with the indicated IgG for 1 h before target cells were added, and fusion was triggered by the addition of soluble gD2(306t) and target cells. Luciferase activity was measured for 2 h and data were normalized to the 2h reading of the no antibody sample (Fig. 10A, black bar).

We found that orange community MAbs, which did not block gH/gL binding to gD, also did not block fusion (Fig. 10A), as was reported previously (26). With one exception (CΔ48L3), all MAbs that stabilized the gD-gH/gL interaction also inhibited fusion. To test whether the fusion inhibition was due to stabilization of gD-gH/gL complex, we also tested Fabs of these MAbs. The CHL2 Fab could neither stabilize the complex (Fig. 9) nor inhibit fusion (Fig. 10B). Surprisingly, although the CHL37 Fab did not stabilize the complex (Fig. 9), it still inhibited fusion (Fig. 10B), suggesting the existence of a third, as yet undetermined, mechanism of inhibiting gH/gL activity. All MAbs that blocked the interaction of gH/gL with gD inhibited cell-cell fusion as both IgG and Fab (Fig. 10). Therefore, the blocking of gD-gH/gL complex formation has a functional consequence (the inhibition of fusion). Interestingly, Fabs 53S and

CHL32 did not inhibit fusion as well as their IgG counterparts (Fig. 10), perhaps because the smaller Fabs were less able to sterically interfere with binding to gD.

The epitopes of anti-gH/gL MAbs that block gD binding identify a potential binding site for gD on gH/gL.

Having mapped the location of the epitopes of the anti-gH/gL MAbs that block the gD-gH/gL interaction, we used this information to identify the gD binding site on gH/gL (Fig. 11). Because the gH/gL crystal structure lacks the gH N-terminus and gL C-terminus, we used our modeled gH/gL structure that includes these regions (Fig. S2). When we examine the left side of gH/gL, the epitope MAb 53S (centered around gL residue 77) is in close proximity to those of MAbs CHL17/32 and CHL27 (Fig. 11A), all of which block the gD-gH/gL interaction. Turning the gH/gL molecule 180 degrees to the right side (Fig. 11B), the CHL18 epitope is now visible, along with more of the CHL17/32 and CHL27 epitopes. By looking at the gH/gL molecule from a "top-down" viewpoint, the epitopes of all five gD-gH/gL blocking MAbs are seen clustered together (Fig. 11C). We propose that this region, which contains portions of the gH N-terminus and much of gL, comprises the gD binding site on gH/gL.

Discussion

HSV entry and cell-cell fusion occur in a cascade of events involving the four essential glycoproteins: gD (the receptor binding protein), gH/gL (the key regulator of fusion), and gB (the fusogen). Although it was theorized that gD transmitted the fusion triggering signal through direct interaction with gH/gL, the complex remained elusive and the binding sites hypothetical.

Studies showed largely indirect evidence for a gD-gH/gL binding complex (7, 18, 29, 42-45). However, in our previous study (19), we showed direct evidence that gD physically interacts with gH/gL. The gD-gH/gL interaction was shown in real time using SPR, and could be blocked by neutralizing and fusion-blocking anti-gD MAbs. By mapping the epitopes of anti-gD MAbs that either allowed or blocked gD-gH/gL binding, we identified the potential gH/gL binding site on gD (Fig. 11D), on the opposite face of the molecule as the receptor binding region.

In the present study, we turned our focus on gH/gL. gH/gL sits in the middle of the fusion cascade, interacting first with gD and subsequently with gB. gH/gL is the "bridge" between the receptor binding protein and the fusogen and has the essential job of regulating the fusion process. To examine its interaction with gD, we used SPR and a panel of anti-gH/gL MAbs to map those that blocked gD-gH/gL binding. The epitopes of MAbs that blocked gH/gL binding to gD mapped to two presumably flexible regions, the N-terminus of gH and the C-terminus of gL. Because neither region was present in the gH/gL crystal structure (9), we used 3D modeling in order to visualize them (Fig. S2). The blocking epitopes of CHL27 and CHL17/32 (gH N-terminus) and CHL18 (gL C-terminus) created a continuous "patch" on the molecule (Fig. 11C). Interestingly, these three MAbs do not prohibit each other from binding, signifying that their epitopes are adjacent but not overlapping. This group of epitopes neighbor that of MAb 53S, which is present on the "body" of gH/gL near gL residue 77 (Fig. 11C). We conclude that the epitopes of MAbs that block gD binding define the gD binding site on gH/gL, located at the N-terminus of the heterodimer and involving both gH and gL residues.

Our data on the location of the gD binding site on gH/gL are in agreement with several other studies using chimeric proteins in the literature. First, functional chimeras consisting of the

gD ectodomain fused to the N-terminus of gH exist for both HSV-1 (35) and pseudorabies virus (46-48). These chimeras can substitute for either gD or gH in virus-complementation and cell-cell fusion experiments, perhaps in part because gD is already positioned at the N-terminus of gH/gL. Second, gH/gL chimeras containing segments of HSV-1 and saimiriine herpesvirus-1 proteins suggested that the gD binding site on gH/gL was located within the N-terminal half of gH, as it was only when gD and this portion of gH were from the same virus that the proteins were functional for fusion (18).

Four of the five anti-gH/gL MAbs that block binding to gD have epitopes that are located on the flexible termini of gH (CHL17, CHL32, CHL27 on the N-terminus) or of gL (CHL18 on the C-terminus) (Table 1). It had previously been postulated that these flexible regions may move in response to gD binding, as a mutant form of gH missing the N-terminus (gHΔ48/gL) was partially activated for fusion in a gD-independent fashion (29). Furthermore, MAbs with epitopes on the C-terminus of gL that either did not inhibit or only weakly inhibited fusion gained activity when the gH N-terminus was removed. These data lead to a model where the gH N-terminus may partially occlude the gL C-terminus until the gH N-terminus is moved through binding to gD. Indeed, our 3D model of the complete gH/gL ectodomain places these two regions next to each other (Fig S2). Movement of the gH N-terminus and the gL C-terminus may be an important conformational step for the binding of gD and activation of gH/gL.

It is currently unknown if gH/gL has distinct "sides" for interacting with gD and gB and if it can bind these molecules simultaneously as gD does for receptor and gH/gL (19). Current evidence suggests that the gB binding region on gH/gL is located in the vicinity of the LP11 epitope (9), which is just below and adjacent to the potential gD binding site (Fig. 5B). With the

binding sites being close physically, it is possible that gH/gL would need to disengage from gD before binding gB. Unfortunately, soluble gB is in the post-fusion form and there is no evidence that this form can bind to gH/gL (2, 19, 49, 50) unless both soluble proteins are treated with low pH and incubated with liposomes (51). Perhaps a membrane-bound, pre-fusion gB (52) can be used to examine gH/gL-gB binding in future experiments.

A subset of MAbs in the red, purple, and yellow communities stabilize the gD-gH/gL binding complex by reducing the rate of disassociation. We found that these MAbs alter the kinetics of the complex by cross-linking gH/gL molecules. When these IgGs were converted to Fabs, each MAb lost the ability to stabilze the gD-gH/gL complex. The stabilization phenomenon may be explained by the notion that IgG (but not Fab) binding turns gH/gL into a bivalent analyte in our SPR system, thereby exhibiting an avidity effect - a slower off rate because both molecules of gH/gL bound by same IgG must disengage gD for the binding signal to disappear.

Numerous small-molecule inhibitors convey their physiological activity by stabilizing specific protein complexes (53). Stabilization of the gD-gH/gL complex does correlate with a functional phenotype in some cases. When CHL2 is converted from IgG to Fab, it loses its ability to inhibit fusion (Fig. 10), suggesting that stabilization may be a mechanism of fusion inhibition. If the gD-gH/gL complex, which normally comes together and dissociates rapidly (19), is forced to stay together, this may affect the subsequent interaction of gH/gL with gB and break the fusion cascade.

Interestingly, CHL37, which loses its ability to stabilize when converted to a Fab, is still able to inhibit cell-cell fusion (Fig. 10B). CHL37 may have a second mode of action beyond

stabilization. We have seen this with other viral MAbs (e.g., anti-gD MAb MC23 blocks gD's interaction with both its receptor nectin-1 and gH/gL) (19, 54). Since CHL37 Fab does not stabilize or block the gD-gH/gL interaction, what is its mechanism of action? One hypothesis is that it could be preventing a conformational change in gH/gL needed to act further down the line with gB. Since the epitope of CHL37 is thought to be on the opposite face of gH/gL as the gH/gL-gB interaction domain (9), at this time we do not believe that it is sterically blocking this interaction.

We previously hypothesized that the gD binding region on gH/gL centered around the 52S epitope (gH amino acid 536) due to the inability of 52S to inhibit cell-cell fusion in a gD-independent system using a mutant gH (29, 31). However, MAbs that inhibited gH/gL binding to gB were able to inhibit the fusion activity of the mutant gH. Therefore, it was theorized that 52S did not inhibit fusion because the step it blocked (which was thought to be gD binding) had been bypassed. Here, we show that MAbs CHL21 and CHL37, which compete with 52S, did not block gD-gH/gL binding. Yet, these MAbs both have an effect on gH/gL function and inhibit cell-cell fusion. If we assume that 52S, CHL21, and CHL37 have epitopes that are overlapping or in close proximity to each other and inhibit the same function of gH/gL, it would suggest that these MAbs impede an as-yet unknown function other than the binding of either gD or gB.

To further study this premise, 52S (which is type-1 specific) must be tested for its ability to block gD-gH/gL binding. Unfortunately, we are unable at this time to detect gD1-gH1/gL1 binding in our system (data not shown), most likely due to a difference in the nature of the binding between type-1 and type-2 glycoproteins. The kinetics of fusion are faster when type-2 gD and gH/gL are used as compared to type-1 (55). It may be that the gD1-gH1/gL1 interaction is even more fleeting than that of gD2-gH2/gL2 (19) and that is why it is not detected in the SPR

assay. Future experiments using stabilizing anti-gH/gL MAbs and chimeras of gH1/gL1 and gH2/gL2 (to further elucidate the type-difference in binding and fusion kinetics) are planned.

Several important questions remain to be answered about the gD-gH/gL complex. Is the complex required to be fleeting to allow for conformational changes to occur so gH/gL can in turn bind to gB? Or are the gB and gD binding sites close enough so that they cannot simultaneously bind to gH/gL, unlike receptor and gH/gL on gD (19)? Can we define the contact residues between gD and gH/gL? To do so would require visualization of the complex by cryo-electron microscopy or crystallography. Perhaps the stabilizing anti-gH/gL MAbs can be used to "lock" the molecules together in order to visualize the complex. Although these MAbs lose their ability to stabilize the complex as Fabs, longer F(ab)2 fragments could be tested for this purpose. Once we identify the contacts between gD and gH/gL, this information can be used for targeted therapies that disrupt this interaction and inhibit the viral fusion cascade and HSV infection.

Materials and Methods

Cells and soluble proteins. All soluble proteins used in this study were purified from baculovirus-infected insect (Sf9) cells. HSV type-2 gD(285t) and gD2(306t) were purified using a DL6 immunosorbent column as described previously (20, 54, 56). The hexahistidine -tagged proteins gH2/gL2 and gH1/gL1 (containing full-length gH ectodomains) and the N-terminal truncation mutant gH2Δ48/gL2 were purified via nickel affinity chromatography (9, 51). B78-H1 mouse melanoma cells were grown in Dulbecco modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS) and 100 μg/ml of penicillin-streptomycin. For B78-

C10 cells (stably expressing nectin-1 receptor), medium was supplemented with 500 µg/ml of Geneticin (G418) (57).

Antibodies. The following anti-gH/gL MAbs were used in this study: 46S, 52S, 53S (31, 33, 58); CHL2, CHL4-16, CHL17, CHL18, CHL29-31, CHL35, CHL37, CHL43 (26); LP11 (gift of A. Minson) (31, 59); H6 (8); L4 (28); and CΔ48L3 (29). CHL21 and CHL27 were generated in the same hybridoma fusion as the other CHL MAbs (26) but were previously uncharacterized. To generate anti-gH/gL MAbs BBH2,3,4,5, mice were immunized with HSV-1 strain SC16. Following fusion, hybridoma supernatants were screened by ELISA against soluble gH/gL protein (60). Monoclonal antibody resistant (*mar*) mutants were generated using the protocol described in Minson et al. (61). Anti-gD MAb 1D3 (54, 62, 63) was used to capture soluble gD2(285t) to the biosensor chip. Anti-gB MAb A22 (41) was used as a negative control. PAbs R137 and R176 were generated against purified gH1/gL1 and gH2/gL2, respectively (64, 65).

Plasmid DNAs. Plasmids pTC510 (gH2-WT), pTC579 (gL2-WT), pTC642 (gH2Δ48), pTC684 (gH2Δ64), pTC673 (gH2-R39A), pTC755 (gH2-T40A), pLF681 (gH2-Y41A), pTC712 (gH2-R43A), pTC756 (gH2-D44A), and pCAGGS/MCS (a gift of P. G. Spear) were previously described (32, 65-67). The remaining plasmids were generated by GenScript (Piscataway, NJ): plasmids pTC1065 (gH2-WT) and pTC1066 (gL2-WT) were generated with optimized HSV-2 (333) codons and placed into vector pcDNA3.1 (EcoRI/HindIII); plasmids pDA1069 (gH2-V161A), pDA1070 (gH2-T162A), pDA1071 (gL2-R46A), pDA1072 (gL2-D50A), pDA1073 (gL2-D51A), pDA1074 (gL2-P77A), pDA1075 (gL2-Q138A), pDA1076 (gL2-H142A), and

pDA1077 (gL2-P144A) were generated by site directed mutagenesis of pTC1065 (for gH2) or pTC1066 (for gL2). Full-length plasmids used in the fusion assay [$Rluc8_{(1–7)}$, $Rluc8_{(8–11)}$, and WT glycoprotein constructs pTC580 (gB2), pTC578 (gD2), pTC510 (gH2), pTC579 (gL2)] have all been described previously (39, 65).

Generating gH/gL MAb community maps using the continuous flow microspotter (CFM)/ surface plasmon resonance imaging (SPRi). Epitope binning experiments of 36 anti-gH/gL MAbs were performed on soluble gH2t/gL2 and gH1t/gL1 using the Carterra CFM/SPRi system. We used a method described previously (22-24). Briefly, a CFM 2 was used to create a 48-spot microarray of amine-coupled mAbs on a CDM200M sensor chip (Xantec GmbH). Upon docking the printer chip into the SPR imager (IBIS MX96), the chip was blocked with ethanolamine and the system primed with a running buffer of PBS-0.01% Tween 20. Epitope binning was performed in a classical sandwich assay format using 100 nM soluble gH/gL as antigen, 100 nM per MAb as analyte, and 1M glycine pH 2.0 for regeneration. All MAbs were tested in the role of both analyte (in solution) and ligand (on chip). However, several MAbs were inactive as ligands so their competitive profiles were determined solely from their performance as analytes. SPRi data were processed in SPRint software and analyzed using Carterra's Epitope Binning 2.0 software for heat map generation, sorting, and network plotting. Binary sorting routines were used to organize the heat maps and epitope bins were viewed as community network plots. All experiments were performed at room temperature.

Modeling of the full-length ectodomain of gH2/gL2. The full length ectodomain structure of HSV-2 gH was constructed using the Zhang lab's ITASSER web server (68) by inputting the gH2 sequence (P89445) (69). The gH2/gL2 crystal structure (PDB ID: 3M1C) (9) was used as the template to model the missing residues. gH2 residues 19-48,116-136 and 797-803 were now visible in the 3D model. The same process was repeated to construct the full-length structure of gL2 using the sequence (P28278) and the same structure (PDB ID: 3M1C) as the template to fill-in the missing residues 17-23 and 166-224. The superimposed images of the model and crystal structure of gH2, gL2, and the gH2/gL2 complex (Fig. S2 C, D, E) show that all missing loops have been filled.

Split luciferase assay (SLA). The SLA protocol has been described previously (27, 38, 70). Briefly, 5×10^4 B78 cells (effector cells) were seeded on white, cell culture-treated 96-well plates; 4×10^5 C10 cells (target cells) were seeded on 6-well plates. Transfection was performed the following day. A master mix containing 125 ng each of the gB, gH, gL, and $Rluc8_{(1-7)}$ plasmids was split over three wells of effector cells. Target cells were transfected with 1 µg of $Rluc8_{(8-11)}$ plasmid/per well. Twenty-four hours post-transfection, effector cells were preincubated for 1 h at 37°C with both EnduRen substrate (Promega) diluted 1:1,000 in fusion medium (DMEM without phenol red supplemented with 50 mM HEPES and 5% FBS) and 20 µg/ml of MAb. Fusion was triggered by the addition of 50ug/ml soluble gD(306t) and target cells. Luciferase production was monitored over a 2 h period, with measurements taken every 5 min using a BioTek plate reader. Add in how we add MAbs to inhibit fusion here. Define "inhibition of fusion."

Glycoprotein binding using the BIACORE 3000. Experiments were carried out on a BIACORE 3000 optical biosensor at 25°C following previous guidelines (54, 71, 72). All injections were performed at a flow rate of 5 μL/min using HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20). After each experiment, the chip surface was treated with brief pulses of 0.2 M Na_2CO_3 (pH 10) until the RU signal returned to baseline (regeneration). First, the gH/gL binding-permissive anti-gD MAb 1D3 (IgG) (19) was amine-coupled to a CM5 sensor chip (GE Healthcare Bio-Sciences, Pittsburgh, PA). Second, 0.05 mg/mL of soluble, purified gD2(285t) was injected across the chip surface until approximately 150-300 RU was captured. Lastly, purified gH2t/gL2 (0.2 mg/ml) was injected for 240 s (analyte) and the binding was recorded. To test for blocking of gD-gH/gL binding via anti-gH/gL MAbs, soluble gH2/gL2 was pre-incubated with 0.6 mg/mL IgG or Fab at RT for 10 min before flowing the mixture across the captured gD2(285t).

Western blotting. B78-C10 cells were transfected with the desired plasmids according to the GenePORTER protocol (Gene Therapy Systems, Inc.). At 24 h post-transfection, cells were lysed in 10 mM Tris (pH 8)-150 mM NaCl-10 mM EDTA-1% NP-40-0.5% deoxycholic acid-1 mM phenylmethylsulfonyl fluoride. Typically, 5% of the total cell extract (from a single well of a 6-well plate) was separated by electrophoresis on a sodium dodecyl sulfate (SDS)-12% polyacrylamide gel. Proteins were detected by Western blotting onto nitrocellulose and probing with the desired counter-antibody.

Peptide ELISA. Synthetic 20-mer peptides were purchased from Mimotopes Pty. Ltd. (Melbourne, Australia) and described previously (26). Fifty microliters of an approximately 1 μM concentration (in PBS) of each peptide was placed in each well of a 96-well Reacti-bind high-binding-capacity streptavidin-coated plate (Pierce) and incubated for 1 h. The plate was then blocked with 200 μl of 5% milk-PBS-T for 30 min and probed with 50 μl of 20 μg/ml MAb for 1 h in milk-PBS-T. All steps were performed at room temperature. Bound IgG was visualized with goat anti-mouse IgG-horseradish peroxidase.

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B.D.B. and N.T.D. are employed by Carterra, Inc.

Table 1: Properties of αgH/gL MAbs

MAb	Community/ Sub-community	Type	Epitope Residues	Competes with	Blocks gD-gH/gL binding	Stabilizes gD-gH/gL	Blocks fusion (SLA)
CHL27	green	TC	gH 37-47	53S	Yes	No	Yes
53S	green	TC	gL 77	CHL27, LP11, BBH3	Yes	No	Yes
CHL17	magenta	T2S	gH 19-38[a]	CHL32	Yes	No	Yes
CHL32	magenta	T2S	gH 19-38[a]	CHL17	Yes	No	Yes
CHL21	purple	TC	ND[b]	CHL37, 52S, 46S, BBH5	No	Yes	Yes
CHL37	purple	TC	ND	CHL21, 52S, 46S, BBH5	No	Yes	Yes
CHL29	orange	TC	gH 676-686[a]	CHL30	No	No	No
CHL30	orange	TC	gH 676-686[a]	CHL29	No	No	No
CHL2	red	T2S	gH 116[a]	CHL4-16	No	Yes	Yes
CΔ48L3	yellow	T2S	gL 173-183[c]	-	No	Yes	No[d]
CHL18	cyan	T2S	gL 209-218[a]	-	Yes (50%)	No	Yes (50%)

[a] Cairns et al. (26)

[b] ND, not determined

[c] Atanasiu et al. (29)

[d] blocks gD-constitutive fusion for gH2Δ48 & gH2Δ29 >50% but not WT-gH2 (29)

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