Using Antibodies and Mutants To Localize the Presumptive gH/gL Binding Site on Herpes Simplex Virus gD

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ABSTRACT HSV virus-cell and cell-cell fusion requires multiple interactions between four essential virion envelope glycoproteins, gD, gB, gH, and gL, and between gD and a cellular receptor, nectin-1 or herpesvirus entry mediator (HVEM). Current models suggest that binding of gD to receptors induces a conformational change that leads to activation of gH/gL and consequent triggering of the prefusion form of gB to promote membrane fusion. Since protein-protein interactions guide each step of fusion, identifying the sites of interaction may lead to the identification of potential therapeutic targets that block this process. We have previously identified two “faces” on gD: one for receptor binding and the other for its presumed interaction with gH/gL. We previously separated the gD monoclonal antibodies (MAbs) into five competition communities. MAbs from two communities (MC2 and MC5) neutralize virus infection and block cell-cell fusion but do not block receptor binding, suggesting that they block binding of gD to gH/gL. Using a combination of classical epitope mapping of gD mutants with fusion and entry assays, we identified two residues (R67 and P54) on the presumed gH/gL interaction face of gD that allowed for fusion and viral entry but were no longer sensitive to inhibition by MC2 or MC5, yet both were blocked by other MAbs. As neutralizing antibodies interfere with essential steps in the fusion pathway, our studies strongly suggest that these key residues block the interaction of gD with gH/gL.

IMPORTANCE Virus entry and cell-cell fusion mediated by HSV require gD, gH/gL, gB, and a gD receptor. Neutralizing antibodies directed against any of these proteins bind to residues within key functional sites and interfere with an essential step in the fusion pathway. Thus, the epitopes of these MAbs identify critical, functional sites on their target proteins. Unlike many anti-gD MAbs, which block binding of gD to a cellular receptor, two, MC2 and MC5, block a separate, downstream step in the fusion pathway which is presumed to be the activation of the modulator of fusion, gH/gL. By combining epitope mapping of a panel of gD mutants with fusion and virus entry assays, we have identified residues that are critical in the binding and function of these two MAbs. This new information helps to define the site of the presumptive interaction of gD with gH/gL, of which we have limited knowledge.

KEYWORDS HSV, glycoproteins, neutralizing antibodies, cell-cell fusion, *mar* residues, escape mutants, herpes simplex virus

Herpes simplex virus (HSV) is a complex human pathogen that infects epithelial cells before spreading to the peripheral nervous system and establishing a lifelong latent infection. Multiple interactions between the four essential virion envelope glycoproteins, gD, gB, gH, and gL, and between gD and a cellular receptor, nectin-1 or

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FIG 1 Location of antibody communities on gD. A surface representation of gD crystal structure is shown in gray. Antibody communities were previously defined based on competition. (A) Red, pink, and yellow (better defined on the second side) communities contain antibodies that block receptor binding. (B) On the opposite face of the receptor binding site, three communities are visible. The brown community was defined by both competition and peptide mapping (262 to 279); the green community was positioned based on MAb 12S, which is affected by a linker insertion at position 243. In the blue community, MAb VID has a *mar* mutation at residue 54. (C) Ribbon representation of gD with point and linker insertion mutations (shown as spheres) used in this study to map MC5 and MC2. Residues that were mutated are color-coded based on their ability to affect binding and function of MAbs MC2 and MC5. Residues 54 and 77 (blue spheres) are essential for the binding and function of MC5. Residue 67 (bright green) is essential for both binding and function of MC2. Residues in light green (64, 243, 245, 246, and 248) are implicated in anchoring, while light gray residues (63, 185, 186, and 244) form the periphery of continuous region that we define as the epitope for this MAb. The black spheres represent residues that when mutated had no effect on MC5 or MC2 binding or function in cell-cell fusion or virus entry.

herpesvirus entry mediator (HVEM), are necessary for HSV entry into all permissive cell types (1, 2). HSV-induced fusion (and virus entry) consists of several sequential steps: (i) binding of gD to a receptor (nectin-1 or HVEM), followed by (ii) a conformational change in gD that allows it to activate the regulatory protein gH/gL, leading to (iii) activation of gB into a fusogenic state (3, 4). Critical information regarding this sequence of events has come from crystallographic structures, as well as the influence of mutants and monoclonal antibodies (MAbs) on fusion events.

In prior studies, we and others showed that virus-neutralizing antibodies are directed at gD, gH/gL, and gB (5–10). We hypothesized that the MAbs that neutralize virus infectivity do so by binding to an epitope in their target protein that is near a key functional site, thereby interfering with one of the sequential steps in the fusion pathway. A large number of the MAbs directed at gD neutralized virus infection by interfering with receptor binding, and their epitopes cluster in three communities (11) on what we define as the receptor side or “face” of the three-dimensional (3D) structure of gD (Fig. 1A). Some of these MAbs blocked binding of gD only to HVEM (e.g., 1D3 as a representative of the neutralizing MAbs from the yellow community [Fig. 1A; see also Table 1]). Others block gD binding only to nectin-1 (e.g., MC23 from the red community), and still others block gD binding to both receptors (e.g., DL11 from the pink community) (8, 12–14).

We discovered another group of MAbs exemplified by MC2 (green community [Fig. 1B]) and MC5 (blue community [Fig. 1B]) that can neutralize HSV infection but do not block the ability of gD to bind either receptor (13). In that study, we presented preliminary evidence that placed portions of the epitopes for these MAbs on a different face of gD (Fig. 1B), separate and distinct (180° away) from the receptor binding face (Fig. 1A). We postulated that MC2 and MC5 interfere with another function of gD, namely, its ability to activate gH/gL. Given the possibility that these MAbs might interfere with an interaction between gD and gH/gL, we decided to more precisely

determine their epitopes. Several attempts to do so were confounded by the fact that both MAbs recognize conformation-dependent epitopes, as defined by their reactivity in a Western blotting assay against gD protein carried out under native conditions only (13). Furthermore, although there are monoclonal antibody-resistant (*mar*) mutants for many MAbs directed against gB, gD, or gH/gL (5–7, 15–20), we were unable to isolate *mar* viruses for either MC2 or MC5 (13).

In this study, we took a two-pronged approach to map the MC2 and MC5 epitopes. The first prong involved detailed epitope mapping with a large panel of gD mutants, and the second utilized a kinetic fusion assay (split luciferase assay [SLA] [21, 22]) and virus neutralization to assess the function of the mutants and their sensitivity to inhibition by MC2 and MC5. Using these approaches, we mapped the footprint of both MC2 and MC5 on the gD structure and identified key residues for both. Out of the 26 gD mutants used in this study, 2, the R67A and P54Q mutants, stood out. The R67A mutant was unable to bind MC2, while the P54Q mutant was unable to bind MC5, as assessed by either Western blot analysis or cell-based enzyme-linked immunosorbent assay (ELISA). Additionally, we found that both the gD R67A and gD P54Q mutants were functional for fusion and infection, even in the presence of the neutralizing MAbs MC2 and MC5, respectively. Our data show that neither the R67A nor P54Q mutation altered other antigenic regions of gD, and therefore, both mutants closely resemble *mar* mutant proteins. In addition, we found that the epitopes for both MC2 and MC5 are indeed situated on the second face of gD (Fig. 1C) (11) that we previously postulated to be involved in its interaction with gH/gL (13). Our data provide detailed information about the residues near or in the probable site on gD that likely interacts with gH/gL and how these two antibodies function in neutralization.

RESULTS

Mapping of the binding location of MAb MC2. Earlier studies indicated that a portion of the MC2 epitope, an HSV-2-specific neutralizing MAb, resides between residues 234 and 250 of gD (13). Because the only residue within this stretch that differs between HSV-1 gD (gD₁) and HSV-2 gD (gD₂) is at position 246 (Pro in gD₂ and Ala in gD₁), we reasoned that this residue is likely part of the MC2 epitope.

Using the crystal structure of gD as a guide, we mutated surface accessible residues in the vicinity of P246 (Fig. 1C). The 19 mutant forms of gD₂ (see Materials and Methods) were transfected into B78-C10 cells that express the receptor nectin-1 (23). Total cell lysates were tested for reactivity to gD polyclonal antibody (PAb) R7 and MAb MC2 by Western blotting under native conditions (limited SDS and no reduction or boiling). Empty pCAGSS vector and wild-type (wt) gD₂ were used as controls. We found that gD was present in the lysates of all mutants as detected by R7 PAb (Fig. 2A). In contrast, assessment of MC2 binding (Fig. 2B) revealed a variety of phenotypes. Five mutants showed no reactivity (R67A, G243A, K245A, P246A, and Y248A), and four exhibited low levels of reactivity (E63A, A185V, R186, and P244A). The other 10 mutants bound MC2 with medium to full wt reactivity. This experiment confirmed that the MC2 epitope is discontinuous, and more importantly, it identified nine residues potentially associated with it.

Cell surface expression of a mutant form of gD is a requisite for any possible functional activity in fusion and for assessing the ability of MC2 to block that function. Thus, our next task was to determine cell surface expression of each mutant as a surrogate for presentation on the virion envelope. For this, we again used the PAb R7 in a cell-based ELISA (CELISA) (22). All 19 mutants were expressed on the cell surface at roughly equivalent levels (Fig. 3A). Among these mutants, the R67A mutant showed little reactivity with MC2 and two, the R64A and Y248A mutants, had an intermediate binding phenotype (Fig. 3B, light green bars). Although there were several discrepancies in the results of the CELISA and Western blot assays, it is important to note that both identified R67 and Y248 of gD as critical for MC2 binding.

To ensure that none of these mutations globally affected the gD structure, we examined the binding of multiple MAbs from either the same competition community

FIG 2 Point mutations to identify the MC2 epitope. Cells transfected with wt or mutant gD₂ constructs were analyzed by Western blotting under native (nondenaturing) conditions of SDS-PAGE, using the polyclonal gD antibody R7 (A) or Mab MC2 (B). Total extracts from cells transfected with empty plasmid (pCAGGS) or wt gD were used as controls.

of MAbs (green) as MC2 or from flanking communities (representative MAbs MC4 and MC5 from the brown and blue communities, respectively [Fig. 1B]) (11). Mab 12S (dark green in Fig. 3B), which competes with MC2 (11), recognized all mutants, including the R67A and Y248A mutants, at levels similar to those for wt gD. This suggests that although MC2 and 12S are in the same community and compete for similar sites (11), their epitopes differ. This difference is also reflected in their virus neutralization profiles: MC2 is HSV-2 specific for both binding and neutralization, while 12S, which is type common for binding, is HSV-1 specific for neutralization (11, 13, 24).

With one exception (the R64A mutant), all of the mutants reacted at wt levels with MC4 (brown bars in Fig. 3C) or MC5 (blue bars). Therefore, the changes induced by R67A and Y248A were specific for MC2. We suggest that mutation R64A had a global effect on the structure of gD, as the mutant exhibited reduced reactivity to all the MAbs tested. The fact that PAb R7 was able to react with these mutants is expected, given that it recognizes both native and denatured gD (25). We conclude that both R67 and Y248 are key residues specific to the MC2 epitope.

Using the SLA to test the function of mutant proteins. Our identification of gD mutants, the R67A and Y248A mutants, that appear to identify residues in the MC2 epitope can be likened to the identification of mutant proteins in *mar* viruses, which can no longer bind the MAb used to select the virus it comes from. To identify the equivalent of *mar* mutant forms of gD for MC2 among the constructs presented here, we first sought to determine if the split luciferase assay (SLA) could be used as a measure of function. As a proof of principle, we tested the known *mar* mutants DL11 *mar* (S140N [17, 26]) and MC23 *mar* (T213A [13]). We sought to determine (i) if they trigger levels of fusion similar to those of the wt construct in the SLA, (ii) if their fusion phenotype is resistant to the generating antibody (in which case the mutant S140N [DL11 *mar*] should be resistant to DL11 blocking and T213A [MC23 *mar*] should be resistant to MC23), and (iii) if they are sensitive to other neutralizing MAbs. Indeed, we found that the fusion activity induced by the mutant S140N and T213A gDs was very similar to that of wt gD (Fig. 4A). While DL11 (Fig. 4B, pink) and MC23 (red) each inhibited fusion of cells expressing wt gD, DL11 had no effect on fusion using gD S140N (compare black and pink in Fig. 4C) and MC23 had no effect on fusion by T213A (compare red and black in Fig. 4D). We conclude that the S140N and T213A mutants confirm our expectations of proteins from *mar* viruses. Thus, their phenotypes measured by a neutralization assay of virus infection are identical to what we found in the kinetic assay (SLA) of cell-cell fusion. We conclude that SLA can be used to identify gD residues that likely lie within the MC2 epitope and are critical for its blocking function.

FIG 3 Surface expression and antibody reactivity of mutant gDs (CELISA). C10 cells seeded onto a 96-well plate were transfected with wt or mutant gDs. Total levels of expression were determined with R7 Pab (A). (B) Reactivity of mutants to MC2 and 12S, both from the same community. (C) Representative MAb MC4 and MC5 from neighboring communities were used to determine the impact of these mutations on the gD antigenic structure. Data are averages from at least three independent experiments, each done in duplicate.

Effect of gD point mutations on fusion in the presence and absence of MC2.

Having validated the SLA, we next used it to evaluate the fusion capacity of the 19 gD mutants potentially associated with the MC2 epitope in the absence or presence of MC2 MAb. According to our binding data (Fig. 2 and 3), two of these, the R67A and Y248A mutants, had no or limited ability to bind MC2. If these mutants functioned in the same way as did “true” *mar* mutants (exemplified by the S140N mutant [Fig. 4]), then we would expect them to be as viable as wt gD for fusion. Moreover, we would expect that the level and kinetics of fusion by these mutants would be insensitive to the presence of MC2 but would remain sensitive to other neutralizing MAb whose epitopes are distinct from MC2.

Using the SLA, we found that all of the mutants, including the R67A and Y248A mutants, were capable of driving fusion at levels that were the same as or close to wt levels, suggesting that none of the mutations significantly impaired the ability of gD to

FIG 4 Using existing *mar* mutations to test functional properties. (A) Plasmids encoding wt, DL11 *mar* (S140N), and MC23 *mar* (T213A) proteins were tested in a split luciferase fusion assay. (B) Comparison of abilities of DL11 and MC23 MAbs to block cell-cell fusion mediated by full-length wt gD. (C and D) Effect of DL11 (C) or MC23 (D) *mar* mutations on the ability of MAbs to block cell-cell fusion. Data from a representative experiment are shown.

trigger fusion over a 2-h time course (Fig. 5A). Moreover, testing of each mutant in the presence of MC2 (Fig. 5B) revealed that all mutants, except for the R67A mutant, were as sensitive to the inhibitory effects of MC2 as wt gD. As a negative-control MAb, we used anti-Myc (included with all but shown for wt gD only). Surprisingly, the Y248A mutant was also sensitive to MC2, suggesting that while both R67 and Y248 are important for the level of gD binding to MC2, only R67 is essential for the inhibitory effect of MC2 on fusion. As the R67A mutant maintained the sensitivity to MAbs from other communities, such as MC4 (brown curves in Fig. 5C) or MC5 (blue), we define R67 as a key residue in the MC2 epitope. Since the R67A mutant behaved in fusion like the *mar* mutants for DL11 and MC23, we consider R67A to represent a true *mar* mutation for MC2.

Epitope mapping of MC5. MC5 is a type-common neutralizing MAb directed at a conformational epitope of gD that is located upstream of residue 234 (13). Using a panel of linker insertion mutants (27), we found that binding of MC5 was inhibited when gD had a 4-amino-acid insertion at position 77 (i77) (13) (Fig. 1C). We examined a total of seven mutants, including the i77 (EDLP linker), i83 (GRSS linker), and i84 (GKIFP linker) (27) mutants and the P54Q, N94R, T96A, and S123Q point mutants (28, 29). We found that all were expressed on the cell surface at or close to wt levels, as detected by CELISA with R7 PAb (Fig. 6A). In contrast, we found that MC5 showed little or no activity against the P54Q and i77 mutants (Fig. 6B, dark blue). We found that binding to a related MAb in the blue community (H162) was also impacted by these two mutations (Fig. 6B, light blue). To determine whether these two mutations introduce broader antigenic charges in this community of epitopes, we tested several other competing MAbs within the blue community: H106, H193, 3D5, and 11S (11). Each of

FIG 5 Effect of point mutations on function (SLA). (A) Ability of gD₂ point mutations to trigger fusion in a kinetic fusion assay. Data are presented as percent fusion by wt gD at the 2-h time point, when the rate of fusion was at its maximum. (B) Blocking of fusion with MC2 MAb. Cells transfected with gB, gH, gL, and the indicated plasmids were treated with 20 μ g/ml of MC2 or a control Myc MAb or left untreated (no Ab). Fusion was triggered by the addition of receptor cells (C10). The dotted line represents the effect of MC2 on fusion by wt gD at 2 h. (C) Effect of MC4 and MC5 MABs on fusion by wt, R67A, or Y248A gDs. Experiments were done in triplicate; curves from a representative experiment are shown.

FIG 6 Mapping of MC5 epitope. (A) Expression levels of wt and mutant gD at the cell surface by CELISA using R7 PAb for detection. (B) Ability of MAbs from the blue community to recognize wt and mutant gDs. Two mutants show specific changes to MC5 and H162 but not to 11S. (C) Sentinel MAbs from neighboring red (MC23) and green (12S) communities were used to determine the impact of these mutations on the general gD antigenic structure. Data are averages from at least three independent experiments, each done in duplicate.

these MAbs recognized all mutant gD proteins at levels similar to those of the wt (including the P54Q and i77 mutants). Figure 6B shows the data for 11S (cyan) in a representative fashion. These results extend the mapping data previously reported for this community (11) and show that residue P54 and the region disrupted by i77 are key for both the MC5 and H162 epitopes but not for epitopes recognized by other MAbs in this group. Furthermore, binding of MC23 (representative MAbs from the red competition communities) and 12S (from the green community [Fig. 1]) was not

FIG 7 Effect of linker insertion and point mutations on the ability of MAbs to block cell-cell fusion. (A) Ability of gD point and linker mutations in a kinetic fusion assay. Data are presented as percent fusion by wt gD at the 2-h time point, when the rate of fusion is at its maximum. (B) Cells transfected with gB, gH, gL, and the indicated gD constructs were treated with 20 μ g/ml of MC5, H162, or 11S or left untreated (no Ab). To determine the integrity of other epitopes, sentinel MAbs from the green (12S) and pink (DL11) communities were used. Fusion was triggered by the addition of receptor-expressing cells and was followed in live cells for 2 h. Each sample was normalized to the activity of each construct in the absence of any MAb. Experiments were done in triplicate; curves from a representative experiment are shown.

affected by these mutations (red and green bars in Fig. 6C, respectively). This indicates that the effects of these mutants were specific for the MC5 and H162 epitopes and that there were no global changes in the gD structure.

Effect of linker insertion and point mutations on fusion in the presence and absence of neutralizing MAbs. Testing of the seven mutants described above by SLA revealed that all functioned at or near wt levels over a 2-h time course (Fig. 7A). While MC5 blocked the fusion activity of cells expressing gD i83, i84, N94R, T96A, and S123Q mutants (Fig. 7B, dark blue), it failed to block fusion by cells expressing the gD P54Q or i77 mutant (Fig. 7B). The results for MAb H162 mirrored those for MC5 (blue in Fig. 7B). As expected, the inhibitory activity of MAbs from different communities (12S, green curves, and DL11, pink curves), or even from the same community as MC5 (11S, cyan), was unaffected by any of the mutations. Thus, we conclude that residue 54 in gD is essential in the binding of both MC5 and H162. Additionally, we note that residue 77 is normally buried in the 3D structure of gD but that some of the residues that surround it (75 to 79) are surface accessible and thus accessible to antibody. We hypothesize that

FIG 8 Complementation of gD-null HSV-1. (A) FgDβ virus was complemented with wt gD (gD₁ or gD₂) or gD mutant gD₁-P54Q or gD₂-R67A. Each sample was assayed in 2 or 3 separate experiments, and the average value (with error bars depicting SEs) was plotted. The percentage of wt activity was calculated as follows: (sample titer/wt titer) × 100. (B to E) Neutralization of FgDβ-gD₁ (B), FgDβ-gD₂ (C), FgDβ-P54Q (D), and FgDβ-gD₂ (E) via MAbs MC2, MC5, and MC23. In each experiment, 25 mg/ml of MAb IgG was mixed with complemented virus and then incubated for 30 min at room temperature before infecting VD60 cells. The virus-antibody mixtures were incubated with the cells at 37°C, and plaques were allowed to develop. The results are expressed as percentages compared to the no-MAb control. Error bars depict SEs; a minimum of 2 experiments were performed.

they are part of the MC5 and H162 epitopes, a suggestion made previously for MC5 (13).

Functional analysis of gD mutants in the context of virus. We next employed a complementation assay (17, 27) to assess the ability of the gD₁ P54Q and gD₂ R67A mutant proteins to function in virus entry when incorporated in the virion envelope of a gD-null virus (FgDβ) (30, 31), as described in Materials and Methods. Similar results in both the cell-cell fusion and virus entry assays would add significantly to the importance of our findings and validate our current approach to identify *mar* residues. Plasmids containing wild-type gD₁ (pRE4) or gD₂ (pCW357) were used as positive controls, and empty pCAGGS vector was used as a negative control. We found that wt gD₁ and gD₂ constructs (Fig. 8A, black bars), as well as gD₁ P54Q (white bar) and gD₂

R67A (gray bar) mutants, complemented the FgD β null virus at equivalent levels, suggesting that the two mutations did not affect the ability of the virus to enter cells.

We next performed neutralization (blocking of entry) assays (13, 32, 33) with the different MAbs to determine whether we could in fact, designate R67A and P54Q as true *mar* mutations that would render the viruses resistant to neutralization by MC2 or MC5. When the null virus was complemented with wt gD₁ or wt gD₂, MAbs MC5 and MC23 blocked the entry of the resulting viruses (FgD β -gD₁ and FgD β -gD₂, respectively) (Fig. 8B and C). Because it is an HSV-2 specific MAb, MC2 blocked the entry of FgD β -gD₂ but not the entry of FgD β -gD₁ (green bars in Fig. 8B and C), thus recapitulating the results obtained using the SLA. Entry of the FgD β -P54Q virus was blocked by the type-common MAb MC23 (red bars in Fig. 8D) but not by MC5 (blue bars in Fig. 8D). MC2 did not block entry of FgD β -P54Q virus (green bar), and this was expected because the P54Q mutation was in an HSV-1 background and MC2 is HSV-2 specific. When we complemented FgD β with gD R67A (FgD β -R67A), virus entry was completely resistant to blocking by MC2 (Fig. 8E, green bar) but was blocked by other unrelated MAbs, such as MC5 (blue bar) or MC23 (red bar). Based on our results, we conclude that FgD β -R67A and FgD β -P54Q viruses have all the features of escape or *mar* mutants for MC2 and MC5, respectively. Thus, the virus complementation data corroborate the data for the cell-cell fusion assay.

DISCUSSION

Our overall goal is to understand the mechanism by which four glycoproteins, gD, gB, gH, and gL, carry out membrane fusion essential for both virus entry and cell-cell spread and to define the regions involved in these interactions.

The published crystal structures of gD, free (34) or bound to either HVEM (35) or nectin-1 (36, 37), showed that binding of either receptor requires movement of the C-terminal portion of the gD ectodomain. We proposed that this form triggers downstream events beginning with a change in gH/gL to an activated state which, in turn, drives the prefusion form of gB (4) through intermediate states to a final form and so effects and regulates fusion (3, 38, 39). We suggested that gD plays two roles in the fusion pathway, based on two major observations. First, we found that an engineered gD mutant that contains an additional disulfide bond that constrains the motion of the C terminus binds both HVEM and nectin-1 as well as wt gD, but it does not trigger cell-cell fusion, nor does it complement the infectivity of a gD-null virus (40). Thus, its phenotype separates receptor binding from downstream functions of gD that are required for fusion and virus entry. Second, we isolated two gD-neutralizing MAbs, MC2 and MC5, that bind to gD and neutralize HSV infection but do not block binding of gD to either receptor (13). Thus, these MAbs must block a second gD function. We postulated that this step involves an interaction between gD and gH/gL and that the presumptive gH/gL binding site must be outside the receptor-binding region. This led us to propose that gD has two faces, one for each function (Fig. 1), with the MC2 and MC5 epitopes being involved in the second function, namely, an interaction between gD and gH/gL. In an earlier study (13), we found part of the MC2 epitope to include residue 246 and a portion of the MC5 epitope to a region near amino acid 77. However, further mapping was incomplete, because we were unable to generate *mar* mutants by growing the virus in the presence of either of these neutralizing MAbs.

In this investigation, we studied the properties of 26 mutants in the vicinity of either residue 246 (for MC2) or residue 77 (for MC5). We tested each mutant for surface expression, as well as changes in the antigenic structure of gD induced by the mutations. We used two methods to test the effect of the mutations on gD function: a kinetic assay of fusion (SLA) and complementation of virus entry.

Figure 1C summarizes the data for the MC2 and MC5 epitopes. Six residues comprise the MC2 epitope. Of these, R67 is critical for MC2 binding and for the MAb to block cell-cell fusion and virus entry and therefore has all the characteristics of a *mar*-like residue. While additional gD point mutants (the R64, G243, P245, P246, and Y248 mutants) showed no MC2 binding by Western blotting only, these mutants all bound

MC2 in CELISA, suggesting that the small amounts of detergent present in the Western blot were enough to disrupt the weaker protein-protein interaction between the mutant gD epitope and the MC2 paratope. We propose that mutants that showed reduced binding (the E63A, A185V, R186A, and P244A mutants) define the periphery of the binding site. The remaining mutants (the A65V, R184A, A187A, S188A, K190A, Y191A, A192V, H242A, and P247A mutants) had no effect on MC2 and thus are not part of the MC2 epitope. Thus, our data have defined the MC2 epitope to include R67 and five adjoining amino acids.

We applied the same strategy to map MC5 and found P54 to be a key *mar*-like residue, based on its behavior in two functional assays. Particularly interesting was the fact that residue 54 is not only essential for the binding and blocking activity of MC5 but also essential for other MAbs from the same community as MC5, specifically H162 (Fig. 7B) and a human derived MAb, VID (11, 28, 41, 42).

The designation of *mar* residues for the P54Q and R67A mutants in the SLA was confirmed with a complementation assay of virus entry. The FgD β -P54Q and FgD β -R67A complemented viruses exhibited the functional characteristics of escape mutants, showing resistance to neutralization by MC5 and MC2, respectively (Fig. 8). The complete agreement between the cell-cell fusion and virus complementation assay highlights the importance of the SLA as a screening tool before the more laborious task of creating viruses that carry mutations is employed.

The fact that we failed to isolate MC2 and MC5 antibody escape viruses (13) is likely a technical issue. An intriguing possibility is based on the fact that viruses become resistant to neutralizing antibodies at a rate determined by the competition between the supply of beneficial escape mutants relative to the supply of lethal ones (43). Perhaps the viral population for these mutants became extinct before an escape mutant could emerge.

Although both MC2 and MC5 block a step that occurs after gD binds its receptor, our data show that they bind in different locales but on the same face of gD. We propose that one or both interfere with the ability of gD to bind to and activate gH/gL. Even though an interaction between gD and gH/gL has been shown by bimolecular complementation (44, 45), its biological significance has not been established, as the enhanced yellow fluorescent protein (EYFP)-linked complex formed was not compatible with fusion in the presence of gB and nectin-1. Clearly, our hypothesis can be validated only by a demonstration of direct binding. Such studies are currently under way. Nevertheless, our results have defined the probable location of such site. Once a direct demonstration of a gD-gH/gL interaction is established, some of the mutants should prove to be critical in defining the gD side of the interaction.

MATERIALS AND METHODS

Cells. B78-H1 mouse melanoma cells were grown in Dulbecco modified Eagle medium (DMEM) containing 5% fetal calf serum (FCS) and 100 μ g/ml of penicillin-streptomycin. For B78-C10 cells (stably expressing nectin-1 receptor), medium was supplemented with 500 μ g/ml of Geneticin (G418) (23).

Plasmids. Rluc8₍₁₋₇₎, Rluc8₍₈₋₁₁₎, and wt glycoprotein constructs pEP 98 (gB₁), pEP99 (gD₁), pEP100 (gH₁), pEP101 (gL₁), pTC580 (gB₂), pTC578 (gD₂), pTC510 (gH₂), pTC579 (gL₂), pSC390 (gD₁), and pRE4 (gD₁) have all been described previously (46–51). Mutant plasmids encoding monoclonal antibody-resistant (*mar*) gD1 pMM80 (S140N for DL11) and pSC542 (T213A for MC23) have also been characterized (17, 50).

For mapping of MC5, previously created mutants were used: gD₁ linker insertions D₁i77 (linker EDLP), D₁i83 (linker GRSS), and D₁i84 (linker GKIFP) and point mutations pD590 (P54Q), pD591 (T96A), pD5121 (N94R), and pD592 (S123Q) (27–29). For mapping of MC2, mutations were introduced in full-length gD₂ using the QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems). Primers designed to mutate individual residues were used to amplify the gD₂ gene of plasmid pCW357 (52) by PCR. The mutations were confirmed by sequencing of the entire gene. The 19 plasmids encoding the gD₂ substitutions were named as follows: pEL893 (E63A), pEL894 (R64A), pEL895 (A65V), pEL896 (R67A), pEL897 (R184A), pEL898 (A185V), pEL899 (R186A), pEL900 (A187V), pEL901 (S188A), pEL902 (K190A), pEL903 (Y191A), pEL904 (A192V), pEL905 (H242A), pEL906 (G243A), pEL907 (P244A), pEL908 (K245A), pEL909 (P246A), pEL910 (P247A), and pEL911 (Y248A).

Antibodies. The properties of antibodies used in this study are listed in Table 1. gD MAbs DL11, H162 (9, 53), and 12S (24) and the MC series (13) were previously described (11).

TABLE 1 Properties of MABs used in this study^a

Antibody	DL11	MC23	MC2	12S	MC4	MC5	H162	11S
Location/ <i>mars</i>	140 ^b	213 ^c	67 ^d	Not found	262–279 ^c	54 ^d	54 ^d	Not found
Binding specificity	Common	Common	HSV-2	Common	Common	Common	Common	Common
Neutralization specificity	Common	Common	HSV-2	HSV-1	None	Common	Common	HSV-1

^aDL11 belongs to the pink community, MC23 belongs to the red community, MC2 and 12S belong to the green community, MC4 belongs to the brown community, and MC5, H162, and 11S belong to the blue community.

^bFrom reference 20.

^cFrom reference 13.

^dFrom this study.

SLA. The dual split luciferase fusion assay (SLA) has been described previously (21, 22, 54). 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 *gD*_(1–7) plasmids and 30 ng of either pCAGGS (mock) or wt or mutant *gD* plasmid was split over three wells of effector cells. Target cells were transfected with 1 μ g of *gD*_(8–11) plasmid/per well. Twenty-four hours posttransfection, effector cells were preincubated for 1 h at 37°C with EnduRen substrate (Promega) diluted 1:1,000 in fusion medium (DMEM without phenol red supplemented with 50 mM HEPES and 5% fetal bovine serum [FBS]). Target cells were transferred to effector cells. Luciferase production was monitored over a 2-h period, with measurements taken every 5 min using a BioTek plate reader.

Blocking of fusion. Effector cells transfected as described above were preincubated with both EnduRen substrate and 20 μ g/ml of MAB (22). Fusion was triggered by the addition of target cells. A negative control (effector cells transfected with gB, gH, and gL but not *gD*) was also included.

CELISA. Using a previously described assay (21, 22), B78 cells were seeded on clear 96-well plates (5×10^4 cells per well) and transfection was done as described above for effector cells. Twenty-four hours posttransfection, cells were assayed for surface expression following a previously described protocol (22).

Western blotting. A total of 4×10^5 C10 cells were seeded on 24-well plates and transfected with 125 ng of pCAGGS or wt or mutant *gD*. Six hours posttransfection, cells were refed with DMEM plus 5% FBS. Forty-eight hours posttransfection, cells were lysed (100 mM Tris [pH 8], 1% NP-40, 0.5% deoxycholate, and $1 \times$ protease inhibitor). Total cell lysates were run on Novex 10% Tris-glycine gels under native conditions (55). After transfer, nitrocellulose blots were probed with 1 μ g/ml of the desired antibodies. Secondary anti-rabbit/mouse IgG horseradish peroxidase (HRP)-linked antibodies (Cell Signaling Technology) were used (diluted 1:2,500). The blots were developed with ECL Western blotting substrate or SuperSignal West Femto maximum-sensitivity substrate, both from Pierce. Images were captured with an Odyssey imaging system (Li-Cor Biosciences).

Virus complementation assay. Mutant forms of *gD* were tested for the ability to rescue the infectivity of FgD β virus in Vero cells (27, 31, 34, 35, 40, 49). Briefly, Vero cells were transfected with an expression plasmid containing the coding sequence for the wild-type (pRE4 or pCW357) or mutant *gDs*. After 24 h, the cells were infected with 10^6 PFU of FgD β virus. One hour postinfection, the virus was inactivated by incubation with 40 mM sodium citrate (pH 3.0)–10 mM KCl–135 mM NaCl. The acid was removed, fresh medium was added, and cells were incubated overnight. At 24 h postinfection, free and cell-associated virus was harvested by disrupting the cells with four freeze-thaw cycles. Each mutant was tested at least three times. Virus titers were determined by titration on VD60 cell monolayers (30, 31). One hundred percent complementation was defined as the total amount of virus obtained (extracellular plus intracellular) after infection of cells transfected with wild-type plasmid (pRE4 or pCW357).

Neutralization of complemented viruses. Using a standard plaque assay, 25 μ g/ml of MABs were mixed with the complemented viruses for 30 min at room temperature. The mixture was added to cells for an additional hour at 37°C. Monolayers of VD60 cells were overlaid with 1% methylcellulose and incubated with the MAB-virus mixture for 72 h. Cells were fixed with 5% formaldehyde solution and plaques were visualized by crystal violet staining.

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