A site of varicella-zoster virus vulnerability identified by structural studies of neutralizing antibodies bound to the glycoprotein complex gHgL

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Varicella-zoster virus (VZV), of the family Alphaherpesvirinae, causes varicella in children and young adults, potentially leading to herpes zoster later in life on reactivation from latency. The conserved herpesvirus glycoprotein gB and the heterodimer gHgL mediate virion envelope fusion with cell membranes during virus entry. Naturally occurring neutralizing antibodies against herpesviruses target these entry proteins. To determine the molecular basis for VZV neutralization, crystal structures of gHgL were determined in complex with fragments of antigen binding (Fab) from two human monoclonal antibodies, IgG-94 and IgG-RC, isolated from seropositive subjects. These structures reveal that the antibodies target the same site, composed of residues from both gH and gL, distinct from two other neutralizing epitopes identified by negative-stain electron microscopy and mutational analysis. Inhibition of gB/gHgL-mediated membrane fusion and structural comparisons with herpesvirus homologs suggest that the IgG-RC/94 epitope is in proximity to the site on VZV gHgL that activates gB. Immunization studies proved that the anti-gHgL IgG-RC/94 epitope is a critical target for antibodies that neutralize VZV. Thus, the gHgL/Fab structures delineate a site of herpesvirus vulnerability targeted by natural immunity.

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Varicella-zoster virus (VZV) is an enveloped, double-stranded DNA virus of the family Alphaherpesvirinae. Primary VZV infection initiates at the respiratory mucosa. T cells are infected in regional lymph nodes and disseminate varicella to the skin, causing varicella (chickenpox) (1). VZV reaches sensory ganglia by the hematogenous route or by antegrade axonal transfer from skin lesions and establishes latency in neurons (2). VZV reactivation can lead to herpes zoster (shingles), which is particularly prevalent in the elderly and in immunocompromised individuals (1, 3). An estimated one third of people in the United States will develop herpes zoster and are at risk for the chronic pain syndrome referred to as postherpetic neuralgia.

Live attenuated vaccines derived from the Oka strain are safe and effective against varicella and herpes zoster in healthy individuals but are contraindicated in most immunocompromised patients (4, 5). Subunit vaccines have the potential to be safe in these populations (6). High-titer VZV immunoglobulin given shortly after exposure decreases the incidence of varicella (7), indicating that antibodies against the VZV envelope glycoproteins can interfere with the initiation of infection and cell-to-cell spread. Thus, subunit vaccines made from one or more of these proteins could be an alternative to live attenuated VZV vaccines. Indeed in a phase 1/2 clinical trial, AS01b-adjuvanted envelope glycoprotein gE has been reported to be more immunogenic than a live attenuated VZV vaccine in healthy individuals in the age groups of 18–30 or 50–70 y. Advances are occurring in the development of this gE-based vaccine candidate against herpes zoster (6).

Similar to other herpesviruses, VZV entry into host cells is presumed to be initiated by virion attachment to the cell surface, followed by fusion between the virus envelope and the plasma membrane of the target cell. The envelope glycoproteins gB and the gHgL heterodimer (8) are highly conserved components of the herpesvirus entry machinery. VZV gB and gHgL are necessary and sufficient to induce cell fusion, which is considered a surrogate for virion envelope fusion (9, 10).

The crystal structures of the gHgL heterodimer ectodomain for herpes simplex virus 2 (HSV-2) and Epstein–Barr virus (EBV) showed that gH and gL cofold to form a tightly packed complex with no similarity to any known viral fusion proteins (11, 12). In contrast, the structures of HSV-1 and EBV gB are highly similar to the vesicular stomatitis virus membrane fusion glycoprotein G, indicating that gB is the herpesvirus fusion protein (13–15). Fluorescence bimolecular complementation studies with HSV-1 envelope glycoproteins imply that gHgL interacts with gB during membrane fusion (16, 17). This interaction was inhibited by the anti-gHgL neutralizing monoclonal antibody (mAb) LP11 (11). How gHgL facilitates the conformational changes in gB required for membrane fusion and viral entry remains elusive.

A homology model of VZV gH based on the HSV-2 gHgL structure was used to identify gH residues involved in membrane

Significance

Mapping neutralizing epitopes on viral entry glycoproteins allows the identification of potentially important functional regions. The structure of varicella-zoster virus (VZV) gHgL bound to two antibodies isolated from immune donors reveals a common binding site. Functional experiments demonstrate that the two antibodies neutralize VZV infection and inhibit glycoprotein gB/glycoprotein complex gHgL-mediated membrane fusion. Immunization experiments in mice demonstrate that VZV gHgL elicits potent neutralizing antibodies and confirm the key role of this antigenic site in antibody-mediated virus neutralization. This manuscript sheds light on the molecular mechanism of herpesvirus cell entry and will guide the design of subunit-based vaccines against VZV.

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fusion (9). Incorporating gH mutations into recombinant VZV showed the importance of the gH ectodomain and cytoplasmic domain for viral replication and regulation of cell fusion in vitro and for infection of human skin xenografts in the SCID mouse model of VZV pathogenesis, as are gL mutations (9, 19, 20).

The contribution of VZV gHgL to viral entry and spread was also demonstrated by the capacity of an anti-gH mouse mAb, mAb206, to neutralize VZV in vitro and to severely impair infection of human skin xenografts (20–22). Human neutralizing mAbs generated from a library of pooled B cells obtained from naturally immune individuals also target gHgL (23). Two of these, designated IgG-24 and IgG-94, and their respective fragment of antigen-binding (Fab), had the most VZV-neutralizing activity. Recently, a VZV-neutralizing human mAb (rec-RC IgG), which recognizes the gHgL heterodimer in VZV infected cells, was isolated from plasmablasts recovered from an individual who was immunized with a zoster vaccine (24).

The present study provides the first report, to our knowledge, on the structure of a herpesvirus gHgL heterodimer in complex with human mAbs and delineates the location of clinically relevant neutralizing epitopes on VZV gHgL. Structural and binding studies demonstrated that Fab-RC and Fab-94 target the same epitope formed by gH and gL residues, whereas Fab-24 binds to a distinct site on gH. Immunization studies proved that the gHgL Fab-RC/Fab-94 epitope is a critical neutralizing Ab against VZV infection. Compared with postfusion gH, gHgL elicited Abs in mice that more potently inhibited cell fusion and neutralized VZV. Vulnerability to Ab-mediated inhibition suggests the gHgL complex could be a component of an effective subunit vaccine.

Results

Binding of Human and Mouse Anti-gHgL Neutralizing mAbs to VZV gHgL. The interactions between VZV gHgL and the Fab fragments of IgG-RC, IgG-94, and IgG-24 were studied by surface plasmon resonance (SPR). Each of the three Fabs binds to gHgL, with comparable dissociation constants (K_D) in the subnanomolar range. The mouse mAb206 bound gHgL at ~100-fold lower affinity than the others (Table S1 and Fig. S1A and B). Additional binding experiments confirmed that Fab-94 and Fab-24 were able to bind VZV gHgL simultaneously, whereas Fab-94 and Fab-RC competed with each other (Fig. S1C). Immunoprecipitation experiments confirmed that Fab-24 does not compete with IgG-RC for gHgL binding. Furthermore, neither Fab-RC nor Fab-24 competed with mAb206, suggesting an independent epitope for mAb206 (Fig. S1D).

Because mutations in residues 38–41 of gH abolished mAb206 binding (9), the epitopes of the four Abs were further investigated using a truncated form of gHgL (ΔN-gHgL; aa 46–795 of gH) lacking the first 28 residues of the gH ectodomain (Fig. 1A and Fig. S2). The binding affinities of ΔN-gHgL for Fab-RC and Fab-24 did not differ from wild-type (WT) gHgL, whereas mAb206 did not bind the truncated complex (Fig. S1A and B). Thus, the gH N terminus is part of the mAb206 epitope that differs from the epitopes recognized by IgG-24, IgG-94, and IgG-RC.

Structure Determination of VZV gHgL/Fab Complexes. To delineate the molecular details of VZV gHgL and epitopes recognized by neutralizing mAbs, the gHgL/Fab-RC and gHgL/Fab-94 complexes were crystallized and their structures were determined by molecular replacement at 3.1- and 3.9-Å resolution, respectively (Fig. S2 and Table S2). Crystals could not be grown for gHgL/Fab-24 alone, and only poorly diffraacting crystals were obtained for the gHgL/Fab-94/Fab-24 ternary complex (Fig. S2). Therefore, negative-stain electron microscopy (EM) reconstruction was used to identify the Fab-24 binding site. Despite the relatively low-resolution diffraction data for the gHgL/Fab-24 complex, electron densities of Fab-94 and gHgL residues at the complex interface were clearly defined in simulated annealed omit maps, allowing unambiguous tracing and model building (Fig. S3A). In contrast, the electron densities for the Fab-94 constant domains lacked connectivity in several regions, even at the final stage of the refinement. Electron density was not observed for residues N-terminal to A36 of gH in either the gHgL/Fab-RC or the gHgL/Fab-94 structures, suggesting the gH N terminus is flexible.

VZV gHgL Has an Extended N-Terminal β-Sheet Compared with Herpesvirus Homologs. The gHgL/Fab-RC crystal structure was used for the structural analysis of gHgL because of its higher resolution. VZV gHgL assumes a boat-like shape reminiscent of HSV-2 gHgL, but different from the straight domain packing of the EBV gHgL or pseudorabies virus (PrV) gH (Fig. 1) (11, 12, 25). Similar to other herpesviruses, VZV gL cofolds with gH. The structural core of VZV gL has a chemokine fold comprising a three-stranded β-sheet (Lβ4/Lβ5/Lβ6), two α-helices (Lα2/Lα3), and a structurally conserved disulfide bond involving residues C49 and C80 (26). VZV gL was more similar to the alphaherpesviruses HSV-2 gL (Cor m:sd: 8.7 Å) than the gammaherpesviruses EBV gL (Cor m:sd: 19.7 Å), as anticipated from homology modeling (9) (Fig. 1B).

VZV gH can be divided into three domains: H1A/B, H2, and H3, as described for HSV-2 gH (Fig. 1) (11). The H1 domain of VZV gH differs most significantly from HSV and EBV gH. The six-stranded antiparallel β-sheet in the N terminus of the HSV-2 gHgL heterodimer, with four strands from gL (Lβ2/Lβ6/Lβ5/Lβ4) and two strands from gH H1 domain (Hβ1/Hβ2 in HSV-2, Hβ3/Hβ4 in VZV gH), are extended in VZV gH by two antiparallel β-strands, Hβ1/Hβ2 (Fig. 1B). Hβ1 (aa 36–45) bridges the interaction between Hβ2 and the remainder of the hybrid β-sheet. Unlike VZV Hβ1/Hβ2, the equivalent N terminus of HSV-2 gH adopts a coil conformation. EBV gHgL forms only a five-stranded β-sheet, with two strands contributed by gH and three by gL (Fig. 1B). In addition to the eight-stranded β-sheet, VZV gH and gL also interact through multiple loops of the gH H1B domain and two conserved α-helices of gL, Lα2/Lα3. A thumb-like loop in gH (loop A; aa 285–298) lies on the surface of Lα2 and Lα3, whereas a second loop in gH (loop B; aa 154–161) and the remainder of H1B elements function as a supporting “palm” for these two α-helices.

The H2 domain is mostly α-helical, and the H3 domain is composed of a 10-stranded β-sandwich. The first four α-helices in H2 domain have been alternatively assigned as part of H1B domain because of their interaction with the extended β-sheet in H1B, leading to the formation of a “syntaxin-like bundle” (12, 25). The sequence of the central H2 and C-terminal H3 domains

Xing et al.

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BIOCHEMISTRY
is more conserved than that of the N-terminal H1 domain across herpesvirus gHs, and this similarity is reflected in their structure. With the exception of an α-helix (aa 600–609) in the VZV H2 domain that replaces a loop in HSV-2 (aa 611–614), EBV (aa 497–502), and PrV (aa 447–451), secondary structural elements in H2 and H3 domain are well conserved (Fig. 1B). In particular, VZV and HSV-2 H2 and H3 domains are very similar, with a rmsd of 1.56 and 1.49 Å, respectively. This observation suggests that the functions of the H2 and H3 domains are conserved across the Herpesviridae.

**Structural Characterization of Neutralizing Epitopes on VZV gHgL.** The mAbs -RC and -94 belong to independent clonal lineages (24, 27). Using IMGT V-QUEST and IMGT junction analysis on V, D, and J germline segments (28), the closest homologous germline genes were identified as IGHV3-30*3901 F, IGHJ6*02 F, and IGHD3-10*01 F for Fab-94 heavy chain (HC) and IGKVI1-2*01 F and IGKJ4*01 F for Fab-94 light chain (LC). The germline genes of Fab-RC are IGHV1-2*04 F and IGKVI1-9*01 F, based on a partial nucleotide sequence of its HC and LC. Remarkably, the crystal structures of gHgL bound to Fab-RC and Fab-94 revealed that the two Fabs share a common epitope, which spans a surface composed of residues from both the gH-H1 domain and gL (Fig. 2). The buried surface of gHgL is larger in the complex with Fab-RC compared with Fab-94 (1036 and 822 Å², respectively), consistent with the slightly higher affinity of the gHgL/Fab-RC complex (Table S1).

The main structural components of the epitope are two loops in gH, loop A and loop B, and Lx3 of gL. Both Fab-RC and Fab-94 recognize essentially the same epitope, but assume different orientations, being rotated almost 180° (Fig. 2A). Although the HC complementarity determining region 3 (HCDR3) fit into the same cleft on gHgL, the other CDRs occupy opposite positions. Thus, the surface area of gHgL that contacts the LC CDRs (LCDRs) of Fab-RC interacts with the Fab-94 HCDRs, whereas the HCDRs of Fab-RC and the LCDRs of Fab-94 bury a similar surface of gHgL (Fig. 2A).

The Fab-24 binding site was identified using a negative-stain EM 3D reconstruction of the gHgL/Fab-94/Fab-24 ternary complex at a resolution of 15 Å. Unlike Fab-RC and Fab-94, Fab-24 contacts the gH H1 domain and part of H1 domain, and its epitope is composed of residues within amino acids 192–6GLSHI197–172, ERFGVSSLPRPTV186, 207–TFFSAEAIT216, 278–HTVK281, and 382–MGRTTIEY388 (Fig. 2B and C and Fig. S3B). Although bound to gHgL at a distinct location from the Fab-RC/Fab-94 epitope, Fab-24 appears to interact with structural elements directly linked to loops A and B. Thus, the structural data identified the epitopes of Fab-RC, Fab-94, and Fab-24, and together with binding data (Fig. S1D), confirmed that these human mAbs do not compete with mouse mAb206 for gHgL binding (Fig. 2C).

**The Fab-RC and Fab-94 Epitopes Have Common Molecular Features.** Residue W291 in the gH loop A plays a prominent role in both gHgL/Fab-RC and gHgL/Fab-94 interactions. In the gHgL/Fab-RC complex, the indole ring of W291 protrudes into a hydrophobic pocket formed by residues from LCDR1–LCDR3 and HCDR3 (Fig. 3A). The interaction is further enhanced by additional hydrophobic and charge–charge interactions around loop A, including F292 pointing toward LCDR2 and D288 forming hydrogen bonds with the main chain amides of HCDR3 to hold it in position (Table S3). In the complex with Fab-94, a backbone rearrangement of gH-loop A allows the aromatic ring of W291 and F292 to occupy a hydrophobic pocket formed by HCDRs 1–3 and LCDR3. In particular, the W291 side chain becomes sandwiched between the phenyl ring of HCDR2–Y399 and gL-F292 (Fig. 3B and Fig. S4A and B).

A six-amino-acid stretch in the HCDR3 of either Fab-RC or Fab-94 interacts with a surface cleft defined on one side by loop B 156–PLVY159 and on the opposing side by loop A 296–LNP297 (Fig. 3). In the gHgL/Fab-RC complex, this cleft is more open and occupied by the HCDR3 M102 side chain. The HCDR3 of Fab-94 inserts less deeply and uses the aromatic side chains of 163–HYV166 to contact residues in the upper part of the cleft (Fig. 3 and Table S3). Y103H is the only somatic mutation found in the Fab-94 variable domain that plays a role on the interface of gHgL/Fab-94 (Fig. S4C).

Fab-94 and Fab-RC both form specific interactions with the gL Lx3. E114 in the loop between Lx2 and Lx3 and R121 at the N-terminal end of Lx3 form H-bonds with Fab-94 HCDR2 S52 and N57, respectively. At the Fab-RC interface, LCDR2 R56 forms salt bridges with both D122 and D126 of Lx3. This arginine is one of the three somatic mutations (S56R) identified on the gHgL/Fab-RC interface in the variable domain nucleotide sequence of IgG-RC. The other two are S306D, which contributes to a charge–charge interaction with gH, and Y327F, which is part of the hydrophobic pocket in Fab-RC, accommodating gH-W291 (Fig. 3 and Fig. S4C and Table S3). Therefore, comparison of the two gHgL/Fab interfaces revealed a number of shared features, consistent with the competition of Fab-94 and Fab-RC for gHgL binding.

**Loop A Residues 295–WF322 in gH Are Critical for the RC94 Epitope.** Mutations were incorporated into gH loop A to identify residues that are critical for Fab-94 and Fab-RC binding. Affinity pull-down experiments demonstrated that single or double mutations of loop A 291–WF322 to alanines substantially reduced the interaction of gHgL with Fab-RC or Fab-94 (Fig. 4 and Fig. S2A).

Mutation of loop A residues 289–DTTWQF324 to 289–AGGAADD34 abolished binding to both Fabs. All the mutant gH proteins formed stable complexes with gL and retained their interaction with Fab-24.
Immunization with gHgL Elicits VZV Neutralizing Abs that Inhibit Membrane Fusion. To determine whether recombinant gHgL can elicit functional Abs in vivo that recognize the epitopes mapped by Fab-RC/Fab-94 or mAb206, BALB/c mice were immunized with equimolar amounts of MF59-adjuvanted gHgL, gHgL/Fab-RC, ΔNgHgL, or the gB ectodomain at two different concentrations. VZV Ab titers measured by ELISA were highest in sera collected from mice in the high-dose group at day 14 after the third immunization (Fig. S6). About tenfold more antigen-specific Abs were detected in sera from mice immunized with gB compared with gHgL in both dose groups.

Mice immunized with gHgL or ΔNgHgL developed neutralizing Abs that significantly reduced cell-associated VZV titers in melanoma cells by log_{10} 1.2 or 0.9, respectively, compared with the control mouse group (Fig. 5E). In contrast, gHgL/Fab-RC induced much lower levels of neutralizing Abs compared with the gHgL complex. These results suggest the Fab-RC epitope contributes to the induction of a significant fraction of the total VZV-neutralizing Abs that target gHgL. The mice immunized with gB did not produce neutralizing Abs even though the gB-specific Ab titers were higher than those obtained with the gHgL antigens by ELISA. It is known that recombinantly expressed ectodomain of herpesvirus gB tends to fold in the postfusion conformation, and it remains possible that a stabilized prefusion gB would elicit more potent neutralizing Abs (14, 15, 29, 30).

When pooled sera were tested in the membrane fusion assay, sera from all groups of gHgL immunized mice inhibited membrane fusion (Fig. 5F). Tenfold dilutions of gHgL, gHgL/Fab-RC, and ΔNgHgL sera retained the ability to inhibit fusion, whereas the gB sera only produced a 20% reduction in fusion at the same dilution. Inhibition of fusion was reduced significantly when all sera were tested at a 1:100 dilution. Inhibition by sera from mice given gHgL/Fab-RC indicates that the IgG-24 and mAb206 epitopes are sufficient to elicit fusion inhibitory Abs. Thus, gHgL was a more effective antigen than postfusion gB for eliciting fusion-inhibiting Abs in mice.

Discussion

The structural analysis of VZV gHgL in the present study identified epitopes targeted by mAbs that interfere with gB/gHgL-mediated membrane fusion and that have neutralizing activity against VZV. The serum Ab responses of mice given the gHgL, gHgL/Fab-RC, and ΔNgHgL immunogens demonstrated the role of the Fab-RC/Fab-94 epitopes in generating neutralizing Abs to VZV. Together, these data suggest that VZV gHgL could be used alone or in combination with other viral envelope glycoproteins, such as gE, to induce Abs that inhibit VZV infection. Antigen
neutralization compared with those mediated by the other mutants. The results of these experiments are shown in Fig. 5, which illustrates the percentage of fusion inhibition for various conditions.

**Fig. 5.** The role of VZV gHgL epitopes in gB/gHgL-mediated cell fusion and VZV neutralization. (A and B) gHgL binding Fabs (A) and IgGs (B) inhibit VZV cell fusion. Percentage of fusion was plotted relative to that observed in the absence of the Ab (No Ab). Fab-RC and IgG-RC are control Fab/IgG that bind an unrelated antigen. Transfection with empty vectors (vector) with no gHgL or gB was used as a negative control. (C and D) gHgL binding Fabs (C) or IgGs (D) neutralize VZV. Error bars show standard errors of the mean (SEM). One-way ANOVA was performed to establish significant differences in fusion or VZV neutralization compared with the control (black asterisks) and IgG-RC (red asterisks). (E) Sera from immunized mice inhibit VZV cell fusion. Undiluted, 10-fold (1:10) and 100-fold (1:100) diluted sera were analyzed. Error bars show SEM. NMS was used as a control.

Design strategies aimed at eliciting Abs specifically targeting the Fab-RC/Fab-94 epitope could be exploited to induce a potent neutralizing Ab response against VZV infection (31). Inhibition of gB/gHgL-mediated membrane fusion reflects one mechanism to neutralize cell-associated VZV. Abs to gH may be internalized by VZV-infected cells (21) and might restrict VZV replication not only by inhibiting fusion/entry but also by interfering with intracellular events necessary for the production of progeny virions. These complementary neutralization mechanisms could contribute to the differing capacities of human mAbs/Fabs or sera from immunized mice to neutralize VZV compared with their inhibition of gB/gHgL-mediated fusion.

The analysis of the VZV gHgL crystal structures showed that the N-terminal 18 residues (aa 18–35) are flexible, and that this region is followed by two β-strands (Hβ1/Hβ2) that are absent in HSV-2 gH. Deletion of residues 18–45 from the VZV gH N terminus, including the flexible N terminus and Hβ1, abrogated binding to the murine neutralizing mAb206 without affecting binding to Fab-RC or Fab-24. These data are consistent with a previous study in which substitution of residues 38 LREY 41 in Hβ1 to 38 GRGG 41 significantly reduced the binding affinity of mAb206, along with two other murine neutralizing mAbs, 258 and SG3 (9). The neutralization potency of these mAbs against a recombinant VZV mutant containing the 38 GRGG 41 substitutions was significantly decreased. In addition, the same mutant had impaired replication in human skin. Our crystal structures revealed that

**Fig. 6.** A conserved gB activation site on herpesvirus gHgL for membrane fusion is in proximity to the Fab-RC/94 epitope. (A) Side chains of corresponding residues mapped as part of the mAb LP11 epitope that was suggested as a gB binding site on HSV-1 gHgL are shown as spheres on VZV gHgL. (B) Side chains of residues important for VZV membrane fusion and gB activation are shown as spheres on EBV gHgL.
In conclusion, this study identified a site of vulnerability in VZV gHgL, which is recognized by neutralizing mAbs from VZV immune human subjects. Immunization studies further support that this site contributes significantly to inducing potently neutralizing Abs against VZV. These findings are relevant for the design of gHgL-based VZV subunit vaccines to prevent or reduce the severity of both primary and recurrent VZV infections.

**Materials and Methods**

Details are provided in SI Materials and Methods. It describes construct generation, protein expression, and purification, binding analysis, X-ray and EM analysis, immunization and ELISAs, and VZV neutralization assays and cell fusion assays.

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