Differential Accessibility of a Rotavirus VP6 Epitope in Trimers Comprising Type I, II, or III Channels as Revealed by Binding of a Human Rotavirus VP6-Specific Antibody

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Previous human antibody studies have shown that the human V_H1-46 antibody variable gene segment encodes much of the naturally occurring human B cell response to rotavirus and is directed to virus protein 6 (VP6). It is currently unknown why some of the V_H1-46-encoded human VP6 monoclonal antibodies inhibit viral transcription while others do not. In part, there are affinity differences between antibodies that likely affect inhibitory activity, but we also hypothesize that there are differing modes of binding to VP6 that affect the ability to block the transcriptional pore on double-layered particles. Here, we used a hybrid method approach for antibody epitope mapping, including single-particle cryo-electron microscopy (cryo-EM) and enhanced amide hydrogen-deuterium exchange mass spectrometry (DXMS) to determine the location and mode of binding of a V_H1-46-encoded antibody, RV6-25. The structure of the RV6-25 antibody–double-layered particle (DLP) complex indicated a very complex binding pattern that revealed subtle differences in accessibility of the VP6 epitope depending on its position in the type I, II, or III channels. These subtle variations in the presentation or accessibility of the RV VP6 capsid layer led to position-specific differences in occupancy for binding of the RV6-25 antibody. The studies also showed that the location of binding of the noninhibitory antibody RV6-25 on the apical surface of RV VP6 head domain does not obstruct the transcription pore upon antibody binding, in contrast to binding of an inhibitory antibody, RV6-26, deeper in the transcriptional pore.

Rotaviruses (RVs) are nonenveloped, triple-layered icosahedral viruses that belong to the *Reoviridae* family, and they are the leading cause of severe diarrheal illness in infants and young children worldwide (1). The inner capsid layer of virus protein 2 (VP2) encloses 11 segments of double-stranded RNA that each encode a single protein, except for segment 11, which codes for two proteins. The VP2 layer is surrounded by 780 molecules of virus protein 6 (VP6). VP6 is arranged into 260 trimers and forms the intermediate viral capsid layer. The VP2 and VP6 layers form the transcriptionally active double-layered particle (DLP). The outer capsid layer is composed of virus protein 7 (VP7) with spikes of virus protein 4 (VP4) and forms the transcriptionally inactive mature infectious virion particle (2–8).

RV VP6 is folded into two distinct domains: an α -helical base domain with a triangular cross section and a jelly-roll head domain that forms a roughly hexagonal cross section. The T=13 icosahedral symmetry of the VP6 and VP7 layers defines three types of channels that exist within the viral architecture: type I, II, and III channels. Twelve type I channels are located down the icosahedral 5-fold axes and serve as egress points of nascent viral mRNA during viral transcription (6). Sixty type II channels are arranged to surround the type I channels, and 60 type III channels are positioned around the icosahedral 3-fold axes (5, 9–12). While the VP6 layer has a T=13 symmetry, the inner VP2 layer of the DLP has a T=1 architecture. This mismatch in symmetry between the VP2 and VP6 layers leads to five distinct VP6 trimer positions on the DLP outer layer, designated P (pentad), P' (peripentonal), T (triad), T' (adjacent to triad), or D (dyad) (4, 13). The P trimers surround the icosahedral 5-fold axes, the T trimers are located directly on the 3-fold axes, and the D trimers are adjacent to the 2-fold axes. The P' trimers are the only

trimers that contact the pentad trimers, and the T' trimers are the only ones to contact the triad trimers.

Antibody repertoire studies in our laboratory identified V_H1-46 as the dominant antibody heavy chain variable gene segment used for the natural human B cell response to RV VP6 in infants and adults (14–17). Representative antibodies encoded by the V_H1-46 gene segment were expressed and characterized (18). For example, the monoclonal antibody (MAb) RV6-26 was shown to inhibit virus replication when introduced into cells, whereas MAb RV6-25, another human VP6-specific antibody encoded by V_H1-46, was unable to do so. Previously, we elucidated the structural basis for viral inhibition by RV6-26 (19). In the studies presented here, we sought to understand why the closely related MAb RV6-25 was unable to inhibit virus, even though it was derived from the same donor, bound VP6, and also was encoded by the identical V_H gene segment. We employed a hybrid method approach for epitope mapping, including single-particle cryo-electron microscopy (cryo-EM) and hydrogen-deuterium exchange

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mass spectrometry (DXMS) techniques. The data revealed that antibody bound to the location of the RV6-25 epitope on the apical surface of the RV VP6 head domain and does not obstruct the transcription pore upon binding, in contrast to binding of the RV6-26 antibody deeper in the type I channel on DLPs. Interestingly, the cryo-EM structure of the RV6-25 antibody-DLP complex indicated an intricate binding pattern that revealed subtle differences in accessibility of the VP6 epitope depending on its position in the type I, II, or III channels. These variations in antigenic architecture of the RV VP6 capsid layer led to position-specific differences in occupancy for binding of the antibody.

MATERIALS AND METHODS

Expression and purification of recombinant RV6-25 Fab. The isolation of the genes encoding the human VP6-specific MAb RV6-25 from a single B cell was described previously (14), and the antibody gene sequences are available in GenBank under accession numbers [AF452995](#) and [AF453155](#) for the variable heavy and light chains, respectively. The RV6-25 heavy and light chain variable region genes were sequence optimized and synthesized as cDNAs (GeneArt). These synthetic genes were cloned into the pEE6.4 expression vector (Lonza) in frame with a mouse kappa chain leader sequence at the 5' end and optimized C_L and C_{H1} constant domain sequences at the 3' end, to encode a fully human Fab antibody fragment without affinity tags. The separate plasmids carrying heavy or light chain genes each were transformed into *Escherichia coli* strain DH5 α cells for large-scale plasmid DNA preparation (PureYield; Promega). Heavy- and light-chain-encoding plasmid DNAs were cotransfected transiently into a high-producing clonal variant of the HEK-293 cell line (FreeStyle 293-F cells; Invitrogen) using Polyfect reagent (Qiagen). The 293-F cells were grown in FreeStyle 293 serum-free expression medium (Invitrogen) in shaker flasks at 125 rpm and 8% CO₂ for 7 days. The culture supernatant was collected on day 7 and purified by fast protein liquid chromatography (FPLC) using an Äkta FPLC device and a HiTrap KappaSelect column (GE Healthcare) in Dulbecco phosphate-buffered saline (D-PBS) and then concentrated with 30-ml Amicon Ultra centrifugal filter units with a 30-kDa-molecular-mass cutoff (Millipore).

Preparation and purification of rotavirus double-layered virus particles (DLPs). A strain of rhesus rotavirus (RRV) was kindly provided by Susana López (Universidad Nacional Autónoma de México). MA-104 cells (CRL-2378.1; ATCC, Manassas, VA) were used to propagate rotavirus to make DLPs. These cells were grown in complete medium (CM) consisting of Dulbecco modified Eagle medium (DMEM) with 4.5% glucose (Mediatech) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen), 0.1 mM minimum essential medium (MEM) nonessential amino acids (Invitrogen), 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate (all from Mediatech). Infection medium (IM), used when cells were inoculated with rotavirus and cultured, contained all of the above supplements except serum, and trypsin-EDTA (Invitrogen) was added to a final concentration of 1 μ g/ml. RRV was inoculated onto cell culture monolayers of the MA-104 cells at a low multiplicity of infection (MOI). When cell monolayers exhibited significant cytopathic effect (typically after 48 h), the supernatant and cell fractions were collected and virus was isolated by ultracentrifugation in a Sorvall Discovery 90SE centrifuge with a Surespin 630 rotor at 20,000 rpm for 1.5 h at 4°C. The resulting pellet was resuspended in Earle's balanced salt solution (Gibco), and the cellular debris was removed by addition of 1,1,2-trichloro-trifluoroethane (EMD Chemicals) and blending. The suspension then was centrifuged at 2,000 rpm for 0.5 h at 4°C to separate virus from cell debris. Virus particles then were collected and purified by ultracentrifugation through a CsCl cushion at 20,000 rpm for 1.5 h. To prepare concentrated DLPs, the collected viral particles were treated with 10 mM EGTA (Lonza) for 5 min at room temperature to remove the outer VP4 and VP7 protein layers of the rotavirus triple-layered particles. The resulting DLPs were purified further by CsCl density gradient ultracentrifugation at 29,700 rpm for 20 h. The visible band containing DLPs was collected and concentrated further by ultracentrifugation in the same CsCl density gradient at 29,700 rpm for 2 h. The band then was collected and dialyzed into Tris-buffered saline (Mediatech).

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Cryo-electron microscopy. Purified DLPs were mixed with a 5-fold molar excess of purified RV6-25 Fab protein for 1 h on ice. Excess antibody was removed using centrifugal filters packed with Sephadex G-50 resin (molecular mass exclusion of 200,000 Da) and immediately plunge-frozen into liquid ethane cooled by liquid nitrogen on fenestrated carbon transmission electron microscopy (TEM) grids. All data were collected on an FEI Tecnai Polara microscope operating at 300 kV in nanoprobe mode. Images were recorded digitally on a Gatan UltraScan 4000 (4k-by-4k) charge-coupled device (CCD) camera. Micrographs were collected with a defocus range of 0.5 to 5 μ m and with an absolute magnification of $\times 199,000$.

Image processing and reconstruction. Individual particle images were centered, cropped, and binned so that each pixel was 6.0 Å by using in-house scripts that utilized IMAGIC (20) and EMAN (21) image processing subroutines. A total of 1,062 particle images were picked and processed in this data set. Individual particle images were used for initial contrast transfer function (CTF) parameter determination using CTFFIND3 (22). Orientation, magnification, and defocus parameter refinement were carried out using FREALIGN (23). Image processing was carried out through 10 rounds of FREALIGN refinement. The resolution was determined by the Fourier shell correlation (FSC) 0.5 criterion to be 21.5 Å, with an additional peak reaching the 0.5 threshold at 18 Å. Icosahedral symmetry was imposed during data processing.

VP6f expression and purification. A DNA copy of the VP6 sequence encoding residues 147 to 339 was amplified from a full-length VP6 DNA construct by PCR with *Pfu* Ultra polymerase (Stratagene) using the following primers: forward, 5'-GGAAGGccatggCCCGGACCGGCTTCACCTTCCAC-3', and reverse, 5'-GTGGTGctcgagGCTGGCGTCGGCCAGCACGC-3' (lowercase letters indicate the restriction endonuclease recognition site). The PCR product was cloned into the pET28a vector (Novagen) using the NcoI and XhoI restriction sites. For expression and purification, the pET28a vector containing the head domain construct was transformed into *E. coli* strain BL21 (DE3). Cells were grown in 1 liter of LB medium to an optical density at 600 nm (OD₆₀₀) of 0.6, protein expression was then induced by addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, and cells were allowed to grow overnight at 20°C. Cells were harvested by centrifugation and disrupted with a French pressure cell in 50 mM sodium phosphate buffer, pH 8.0. The soluble fraction was clarified and applied over a nickel-nitrilotriacetic acid (Ni-NTA) column. The VP6 protein then was eluted with the above buffer with 400 mM imidazole. The eluate was concentrated in Amicon Ultra filter tubes (Millipore) and passed through a Superdex column for additional purification. Purified VP6 fragment (VP6f) was run on an analytical Superdex 200 10/300 GL column (GE Healthcare) at 0.5 ml/min, in 10 mM Tris, 150 mM NaCl, pH 7.5, and compared to Bio-Rad gel filtration standards. The elution curve was used to demonstrate that the purified VP6f was a trimer.

DXMS. We performed comparative deuterium exchange mass spectrometry (DXMS) studies with VP6f construct alone or with VP6f in complex with RV6-25 Fab. Quench conditions optimal for maximum peptide fragmentation were determined prior to the deuteration experiments, as previously described (24, 25). Complexes of VP6f and Fab were prepared by mixing VP6f and RV6-25 Fab at a 1:1.4 stoichiometric ratio and incubating the mixture at 0°C for 30 min.

Functional hydrogen-deuterium exchange reaction of free VP6f was initiated by diluting 0.7 μ l of stock solution into 1.3 μ l of H₂O buffer (8.3 mM Tris, 150 mM NaCl, in H₂O, pH 7.15) and then mixed with 6 μ l of D₂O buffer (8.3 mM Tris, 150 mM NaCl, in D₂O, pD_{READ} 7.15) at 0°C. At 10 s, 100 s, or 1,000 s, 12 μ l of optimized cold quench reagent (0.8 M GuHCl in 0.8% formic acid) was added to the respective samples and then samples were frozen at -80°C . The functionally deuterated antibody-

FIG 1 Structure of RV6-25 Fab bound to RV-DLP. Density map of the full virion structure of RV6-25-DLP complex reconstructed to 21.5-Å resolution, based on an FSC 0.5 criterion, viewed along the icosahedral 5-fold axis. The VP2, VP6, or RV6-25 Fab density is represented by cyan, gray, or salmon, respectively. The yellow circle indicates the density that represents the VP6-binding components of the third Fab. The enlarged gray box indicates a type I channel (in mesh) showing Fabs fitted into density of the third Fab. The blue ribbons represent the heavy chains, and the red ribbons represent the light chains.

bound VP6f samples were prepared by diluting 1.5 μ l of complex solution (9.2 mg/ml) into 0.5 μ l of nondeuterated buffer and then mixed with 6 μ l of D₂O buffer (8.3 mM Tris, 150 mM NaCl, in D₂O, pD_{READ} 7.15) at 0°C. At 10 s, 100 s, or 1,000 s, 12 μ l of optimized cold quench reagent was added to the respective samples and then samples were frozen at -80°C. In addition, nondeuterated samples (incubated in H₂O buffer mentioned above) and equilibrium-deuterated back-exchange control samples (incubated in D₂O buffer containing 0.5% formic acid overnight at 25°C) were prepared as previously described (24–26). Later, the samples were thawed automatically on ice and then immediately passed over an AL-20-pepsin column (16- μ l bed volume, 30 mg/ml porcine pepsin [Sigma]), which was run at a flow rate of 20 μ l/min with 0.05% trifluoroacetic acid. The resulting peptides were collected on a C₁₈ trap and separated using a C₁₈ reversed-phase column (Michrom Bioresources) running a linear gradient of 0.046% (vol/vol) trifluoroacetic acid, 6.4% (vol/vol) acetonitrile to 0.03% (vol/vol) trifluoroacetic acid, 38.4% (vol/vol) acetonitrile over 30 min with column effluent directed into an LCQ mass spectrometer (Thermo-Finnigan LCQ Classic) for epitope mapping. Data were acquired in both data-dependent MS:MS mode and MS1 profile mode. SEQUEST software (Thermo-Finnigan Inc.) was used to identify the sequence of the peptide ions. The centroids of the isotopic envelopes of nondeuterated, functionally deuterated, or fully deuterated peptides were measured using DXMS Explorer (Sierra Analytics Inc., Modesto, CA) and then converted to corresponding deuteration levels with corrections for back-exchange (27).

RESULTS

RV6-25 bound to the apical surface of VP6 molecules on DLPs.

RV6-25 and another anti-VP6 human antibody previously isolated from our laboratory, designated RV6-26, are closely related genetically as they are encoded by the same heavy chain variable gene segment (15). However, RV6-26 has been shown to inhibit virus replication following transfection into cells, whereas the RV6-25 antibody that is the subject of the current studies did not inhibit virus replication after transfection (18). It was of interest to determine the pattern of binding of RV6-25 to DLPs, in order to begin to determine the structural basis for the difference in the

ability of these genetically related antibodies to inhibit RV. We determined the structure of the complex of RV6-25 Fab with RV DLPs using cryo-electron microscopy (cryo-EM) reconstruction; data are deposited in the Electron Microscopy Data Bank (EMDB ID pending). The reconstruction of the Fab-DLP complex was computed from 1,062 complex particle images extracted from corresponding cryo-EM micrographs. The estimated final resolution of the RV6-25-DLP complex was 21.5 Å, using the 0.5 FSC criterion. Like most cryo-EM structures that have been determined for icosahedral viral particles in complex with antibodies and their target antigens (28), RV6-25 Fab protrudes from the VP6 trimers and extends away from the viral surface in a regular pattern depending on the position of the VP6 trimer in the VP6 layer (Fig. 1).

RV6-25 Fabs bound to all available VP6 epitopes around the type I channels. All of the VP6 trimers around the 5-fold axis of symmetry, which form the type I channel, were bound by three Fab molecules each, in a ratio of one Fab per VP6 protomer. The density for two of the Fabs on each trimer was obvious; however, the densities of the Fabs bound to protomers oriented toward the type I channel were not visible at higher thresholds (isosurface contour levels), presumably because of lower occupancy (Figs. 1 and 2A and B). At a lower threshold, an averaged density of bound Fabs, which formed a ring-like density that projected above the transcription pore, was clear (data not shown). We fitted a ribbon tracing of Fab into the density of the Fabs oriented toward the type I channel, and this revealed that these Fab densities are oriented such that they project laterally with respect to the pore, toward the adjacent Fab densities, and thus they do not occlude the pore (Fig. 1, inset). The connected nature of the Fab density above and around the lip of the transcription pore indicates that steric clashes occur among bound Fabs that lie laterally to the pore, consistent with lower occupancy at this site. A model of the RV6-25 Fab was fit into the cryo-EM density using the UCSF

FIG 2 Binding of RV6-25 Fab to VP6 trimers at the type I, II, or III channel. (A) Surface representation of RV6-25–DLP complex particle, highlighting the RV6-25 Fabs bound to the VP6 trimers (green) around the type I channel in the context of the whole virion particle (gray). (B) Surface representation of the type I pore showing two visible Fab densities bound to each VP6 trimer around the channel. At this threshold, density for the third Fab bound to each trimer is not

FIG 3 Demonstration that VP6f is a trimer in solution. Purified VP6f was run on an analytical Superdex 200 10/300 GL column (GE Healthcare) at 0.5 ml/min, in 10 mM Tris, 150 mM NaCl, pH 7.5, and compared to Bio-Rad gel filtration standards. The figure shows an overlay of these standards and VP6f. VP6f eluted between the 158,000- and 44,000-Da standards. A least-squares regression line based on these standards (excluding the 1.35-kDa standard) predicted a molecular mass of 70,254 Da for VP6f. The expected molecular mass of a VP6f trimer is 68,316 Da. These data indicate that VP6f is a trimer under dilute buffer conditions.

Chimera program (29), Fit-In-Map function, which found a best-orientation fit with a cross coefficient correlation score of 0.9217 (Fig. 2B). The P trimers surround the 5-fold axis of symmetry and form the type I channel. The RV6-25 Fab densities bound at an orientation to the P trimers such that they projected out to the adjacent channel and over adjacent (P') trimers.

RV6-25 Fabs bound to only one of the available Fab epitopes on the P' trimers around the type II channels. Two of the P trimers form part of the type II channels that surround each of the type I channels on the surface of the DLP. The binding angle of the Fabs to the P trimers of VP6 in the type I channel (Fig. 2A and B) appeared to preclude full occupancy of the available epitopes on the adjacent P' trimers. The obstructed availability of the Fab epitopes on the P' trimers allowed for binding only to the epitopes where the Fab densities were oriented toward the 2- or 3-fold icosahedral axes (Fig. 2C and D). Interestingly, under these conditions Fabs did not bind to the D or T' trimers adjacent to the 2-fold or 3-fold axes of symmetry, respectively. The D and T' trimers, together with two P and P' trimers each, form the type II channels. Fitting of an RV6-25 Fab model into one of the Fab densities bound to a P' trimer yielded a cross correlation coefficient of 0.8707 (Fig. 2D).

RV6-25 Fabs bound to all Fab epitopes on the triad trimers. The icosahedral symmetry of the DLP places a trimer, design-

nated T, directly at the 3-fold axis of symmetry (4, 13). The T trimer is one of the six trimers that form the type III channel. The structure of DLP complexed with Fab showed that all of the available Fab epitopes on the T trimer were occupied by Fabs (Fig. 2E and F). Fitting of a Fab model into one of the Fab densities bound to a T trimer showed a cross correlation coefficient of 0.8769 (Fig. 2F). The P' trimer that is also part of the type III channel showed a bound Fab density oriented toward the 2-fold symmetry, similar to the type II channel. Furthermore, as in the type II channel, Fabs did not bind to the D or T' trimers (Fig. 2F). In summary, Fab binding to the epitopes on the distinct P, P', T, T', or D VP6 trimers under saturating conditions exhibited a striking and reproducible pattern that showed that steric clashes result in altered occupancy at chemically identical sites (Fig. 2G and H).

RV6-25 bound to an epitope on the apical loops of VP6. The cryo-EM complex structure suggested regions that are contact surfaces on Fabs and VP6 trimers. We next sought to determine the precise residues of the VP6 head domain that formed the epitope recognized by Fab RV6-25. We employed enhanced amide hydrogen-deuterium exchange mass spectrometry (DXMS) to identify the residues that comprise the Fab epitope.

We prepared a recombinant fragment of VP6 head domain

visible. One of the Fab densities is depicted in a transparent mesh with a fitted Fab. The trimers around the type I channel are designated P trimers. (C) Surface representation of RV6-25-DLP complex particle, highlighting the RV6-25 Fabs bound to the VP6 trimers around the type II channel in the context of the whole virion particle (gray). The green color represents two of the 5-fold VP6 trimers (P) with bound Fabs, pink represents the trimers directly adjacent to the 5-fold VP6 trimers (P') with bound Fabs, yellow represents one of the VP6 trimers at the 2-fold axis (D), and cyan represents one of the trimers directly adjacent to the VP6 trimer at the 3-fold axis (T'). (D) Surface representation of the type II pore showing the two visible Fab densities bound to the two P VP6 trimers in the adjacent type I pore and single Fab densities each bound to P' VP6 trimers, directly adjacent to the P trimers. One of the Fab densities is depicted in a transparent mesh with a fitted Fab. Fab densities are not visible on D and T' trimers. The trimers around the type II channel are designated P, P', D, or T'. (E) Surface representation of RV6-25-DLP complex particle, highlighting the RV6-25 Fabs bound to the VP6 trimers around the type III channel in the context of the whole virion particle (gray). The pink color represents the P' trimer with one bound Fab density, yellow represents the two trimers at the 2-fold axis (D), light blue represents the trimer at the 3-fold axis (T), and cyan represents two of the trimers directly adjacent to the 3-fold trimer (T'). (F) Surface representation of the type III pore showing one visible Fab density bound to the P' VP6 trimer that is also part of the adjacent type II channel and three Fab densities bound to the T trimer. One of the Fab densities bound to the T trimer is depicted in a transparent mesh with a fitted Fab. Fab densities are not visible on D or T' trimers. The trimers around the type III channel are designated P', D, T, or T'. (G and H) Surface representation of the Fab-DLP complex highlighting all three channels (types I, II, and III) in the context of the whole particle (gray) (G) and showing the trimer designations (H). In all images, the Fab heavy and light chains are shown in red and blue, respectively. The black line, triangle, and star represent the icosahedral 2-, 3-, or 5-fold symmetry, respectively.

FIG 4 Determination of VP6 epitope for RV6-25 by deuterium exchange mass spectroscopy. (A and B) Ribbon map showing percent deuterated (% D) of VP6 alone (A) or VP6 bound to RV6-25 Fab (B). The top row shows the residue position number, the second row shows the residue, and the rest of the rows show protein dynamic features at different on-exchange time points (10, 100, or 1,000 s). As indicated in the colored bar, cold colors suggest relatively stable regions and warm colors suggest relatively flexible regions. All prolines are shown in white, because prolines do not have amide hydrogens. Residues uncovered by surface deuteration are also shown in white. (C) Difference map showing the influence of RV6-25 Fab binding to VP6 indicated by changes in % D. Blue suggests the regions that exchange slower upon Fab binding; red suggests the regions that exchange faster upon Fab binding. (D) Side view of the predicted epitope regions of RV6-25 Fab on the head domain of the VP6 structure (PDB: 1QHD). The different shades of gray represent the three protomers that make up the VP6 trimer, and the different shades of blue represent the predicted epitope regions mapped on each protomer. (E) The side view of the VP6 trimer highlighting, in red, the loops that are most likely involved in binding to RV6-25. (F) The top view of the VP6 trimer with all the predicted epitope regions visible on the structure. (G) The top view of the VP6 trimer highlighting, in red, the loops that are most likely involved in binding to RV6-25.

termed VP6f and showed that it is a trimer in solution. Purified VP6f was run on an analytical Superdex 200 column and compared to gel filtration standards. **Figure 3** shows an overlay of the elution signal from these standards and VP6f. VP6f eluted between the 158,000- and 44,000-Da standards. A least-squares regression line based on these standards (excluding the 1.35-kDa standard) predicted a molecular mass of 70,254 Da for VP6f. The expected molecular mass of a VP6f trimer is 68,316 Da. These data indicate that VP6f is a trimer under dilute buffer conditions.

Using DXMS, we identified peptides derived from regions including residues 197 to 214 (region 1) and 306 to 324 (region 2) to contain residues forming the epitope of RV6-25 (**Fig. 4A** to **C**). The DXMS approach identifies peptides that contain residues of the epitope, but the technique does not provide single-residue resolution for epitope mapping. Therefore, we highlighted these peptides onto the three-dimensional structure of VP6 (PDB: 1QHD) (13) to visualize the position of peptides identified by the DXMS data. It was clear from inspection of this projection and comparison with the cryo-EM structure that the epitope is within two apical loops of the VP6 structure. Using the conventional nomenclature for jelly-roll motifs (30), region 1 spans residues within the loop emanating from β B to β C (**Fig. 4D** and **F**). Region 2 extends from the loop emerging from the short α A helix down to β I (**Fig. 4D** and **F**). Taken into context with the orientation of the docked Fab in the cryo-EM density map, residues 197 to 214 and 308 to 316 in the loops between β B- β C and the short α A helix and β I are involved directly in the binding of RV6-25 (**Fig. 4E** and **G**).

DISCUSSION

The cryo-EM structure of the RV6-25-DLP complex revealed an interesting binding pattern of the RV6-25 Fab that suggests differ-

ential accessibility of a VP6 epitope in different trimers on the surface of RV DLPs, depending on their position in the particle. A consequence of icosahedral packaging used by many viruses is that the protein subunits inevitably exhibit quasiequivalent (31) or nonequivalent (32, 33) variations among the conformations and interactions of their arrayed subunits. RV exhibits quasiequivalence and a symmetry mismatch between the VP2 and VP6 layers (4, 13). Variability in occupancy among quasiequivalent forms of the same epitope appears to be common among viral antigens, as observed in the configuration of major capsid proteins of herpes simplex virus (34) and papillomavirus (35). This property of differential presentation of antigens that are genetically identical but vary due to position in the particle may offer an additional mechanism for generating antigenic diversity on a viral capsid. The antibody investigated in the cytomegalovirus (CMV) study (36) could discriminate between A, B, and C subunits of the capsid protein, by binding only to the A subunit closest to the 5-fold axis of symmetry. It is possible that the selectivity of the CMV Fab could reflect structural differences in the epitopes of different capsid subunits, but this seems unlikely since the majority of the β H- β I loops containing the epitopes adopt essentially identical structures. Therefore, Bowman et al. (36) reached the conclusion that the antibody recognized differences in subunit arrangements. In other words, Fabs acquired specificity by virtue of quaternary differences in the subunit arrangements within pentons and hexons. The RV6-25 epitope described here lies in similar loops on the apical surface of the VP6 protein. It is therefore plausible that the binding pattern of RV6-25 Fab is driven by constraints imposed by the geometry of the type I, II, or III channels on the DLP surface. While the type I channels form symmetrical pentamers,

the hexamers formed in the type II and III channels are slightly distorted as a consequence of the structural arrangement of the VP6 layer. These distortions are reflected in the arrangement of the VP6 trimers within the channels and hence could affect the availability for binding of some of the RV6-25 epitopes in the particle.

The RV6-25 Fabs bound to the epitope at the corner of the VP6 trimer project off the particle surface at an angle of about 45°. This orientation allows the Fabs to extend out sideways over a relatively long distance, enlarging the effective footprint of the antibody and blocking access to binding sites on neighboring trimers. Furthermore, analysis of the VP6 interactions in the viral capsid has shown a pattern of lateral interaction between VP6 trimers in the viral capsid that reveals that quasiequivalence is only partially obeyed in the icosahedral T=13 layer formed by VP6, since both quasiequivalent and nonequivalent intertrimer contacts were observed. Hence, the accessibility of the antibody epitopes may be affected by differences in intertrimer contacts around the head of the VP6 molecule (13). The binding pattern of RV6-25 is likely a function of the combination of distinct environments around the DLP VP6 trimers and accessibility of epitopes influenced by steric clashes of the antibody.

We reported previously that the related human Fab RV6-26 fully occupies the type I channel by binding deep in the channel and blocks transcription of RNA by the DLP (19). In contrast, RV6-25 binds an epitope on the apical edge of the transcriptional pore rather than an epitope within the pore, and this Fab is not inhibitory. The comparison suggests that the efficiency of occlusion of the type I channel by VP6 Fabs is determined in part by the depth of location of the antibody epitope in the channel. Another contributing factor to the difference in binding is likely the fact that the affinity of binding of RV6-26 for VP6 is higher than that of RV6-25 (18). Subtle differences exist in the display of capsid protein on viral surfaces. These differences should be considered in the rational design of vaccines based on virus-like particles, as capsid arrangements can influence epitope presentation and accessibility and thus can affect the host immune response.

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