pH-Induced Conformational Change of the Rotavirus VP4 Spike: Implications for Cell Entry and Antibody Neutralization

Joseph B. Pesavento,^{1,2,†‡} Sue E. Crawford,^{3,‡} Ed Roberts,¹ Mary K. Estes,³ and B. V. Venkataram Prasad^{1,2,*}

Verna and Marrs McLean Department of Biochemistry and Molecular Biology,¹ W. M. Keck Center for Computational Biology,² and Department of Molecular Virology and Microbiology,³ Baylor College of Medicine, Houston, Texas 77030

Received 15 December 2004/Accepted 9 March 2005

The rotavirus spike protein, VP4, is a major determinant of infectivity and neutralization. Previously, we have shown that trypsin-enhanced infectivity of rotavirus involves a transformation of the VP4 spike from a flexible to a rigid bilobed structure. Here we show that at elevated pH the spike undergoes a drastic, irreversible conformational change and becomes stunted, with a pronounced trilobed appearance. These particles with altered spikes, at a normal pH of 7.5, despite the loss of infectivity and the ability to hemagglutinate, surprisingly exhibit sialic acid (SA)-independent cell binding in contrast to the SA-dependent cell binding exhibited by native virions. Remarkably, a neutralizing monoclonal antibody that remains bound to spikes throughout the pH changes (pH 7 to 11 and back to pH 7) completely prevents this conformational change, preserving the SA-dependent cell binding and hemagglutinating functions of the virion. A hypothesis that emerges from the present study is that high-pH treatment triggers a conformational change that mimics a post-SA-attachment step to expose an epitope recognized by a downstream receptor in the rotavirus cell entry process. This process involves sequential interactions with multiple receptors, and the mechanism by which the antibody neutralizes is by preventing this conformational change.

A critical step in the productive infection of a virus is its successful entry into a host cell. For many viruses, a single protein is implicated in this complex process that often involves multiple steps and multiple receptors. Such is the case with rotavirus, the major pathogen of infantile gastroenteritis accounting for nearly half a million deaths annually worldwide (8). Rotavirus, a member of the *Reoviridae* family, is a large icosahedral virus with a complex organization consisting of three concentric capsid layers that encapsidate 11 genomic double-stranded RNA segments (16, 41). The innermost capsid layer is composed of 120 copies of VP2 on a T=1 lattice (30), and 12 copies each of the RNA-dependent RNA polymerase VP1 (44) and the guanylyltransferase VP3 (6, 31) attach as heterodimeric complexes to the inner surface of this layer at the fivefold axial positions (41). The intermediate capsid layer is composed of 780 copies of VP6 arranged as 260 trimers on a T=13 lattice. The outermost capsid layer contains 780 copies of VP7, with the same icosahedral organization as the intermediate layer, and 120 copies of VP4, which interacts with VP6 and emanates as distinct bilobed spikes through the VP7 capsid layer. Antibody labeling and cryo-electron microscopy (cryo-EM) showed that the spikes on the surface of the triple-layered particle (TLP) are present as 60 dimers of VP4 located near the type II channels surrounding each fivefold vertex (40, 43).

Although earlier studies implicated VP7 in the cell entry process (19, 42), subsequent studies have increasingly indicated that VP4 is the major player in this process. VP4 is implicated not only in cell attachment and penetration but also in hemagglutination, neutralization, virulence, and protease-enhanced infectivity of rotavirus (25, 32, 34). The latter phenomenon is particularly relevant considering that rotavirus replicates in the mature enterocytes of the small intestine, an environment rich in proteases. Proteolytic cleavage of VP4 primes the virus for efficient entry into cells (1, 17, 26). During proteolysis, VP4 (88 kDa) is cleaved into VP8* (28 kDa) and VP5* (60 kDa), and the cleavage products remain associated in the virion (18). Our recent structural and biochemical studies have shown that VP4 undergoes a conformational transition from a disordered to an ordered state upon trypsinization, and this transition appears to be responsible for trypsin-enhanced infectivity observed with rotavirus (10). The X-ray crystallographic structures of VP8* and VP5* have provided strong evidence that the distal globular domain of the VP4 spike is composed of VP8*, with the remaining body of the spike consisting of VP5* (12, 13, 43).

A consensus opinion that has emerged from recent biochemical studies is that rotavirus entry into cells is a multistep process involving sialic acid (SA)-containing receptors in the initial cell attachment step and integrins such as $\alpha_v\beta_3$, $\alpha_4\beta_1$, and $\alpha_2\beta_1$ during the subsequent postattachment steps (9, 22, 23, 45). In this process, the VP8* domain, which has a galectin fold, is involved in the interactions with SA, whereas VP5* is implicated in the interactions with integrins. Involvement of SA during rotavirus infection is not an essential step for all rotavirus strains. For the majority of rotavirus strains, including human rotaviruses, cell entry is SA independent (7). In

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-5686. Fax: (713) 798-1625. E-mail: vprasad@bcm.tmc.edu.

† Present address: University of California, Lawrence Livermore National Laboratory, P.O. Box 808, L-448, Livermore, CA 94551-0808.

‡ J.B.P. and S.E.C. contributed equally to this study.

these viruses, the majority of neutralizing monoclonal antibodies (MAbs) that recognize VP4 select mutations in VP5* (27, 28, 38), suggesting that cell entry is mediated mainly by VP5*. It also is thought that cell penetration of rotavirus may require a hydrophobic, fusion domain, which resides on the VP5* cleavage product. These hydrophobic regions could aid in membrane penetration after cell attachment (11, 14).

As has been observed with other viruses such as influenza virus (4), flavivirus (36, 37), alphavirus (20), and picornaviruses (3), it is possible that VP4 undergoes distinct conformational changes at various stages during cell entry to mask certain epitopes and reveal others to optimally interact with different receptors and the cellular membrane. We have already seen that VP4 undergoes a drastic conformational change upon trypsinization. Recent X-ray crystallographic studies of VP5* also suggest the possibility of significant structural changes in the spike structure during rotavirus cell entry (12). Tracking these conformational changes and identifying the epitopes to understand the molecular basis of how VP4 interacts with various receptors during entry is indeed difficult, particularly considering that there is no established reverse genetic system for any member of *Reoviridae*, including rotavirus. In lieu of reverse genetics, we have devised a strategy to further our understanding of structure-function relationships in rotavirus. In this strategy, we perturb the particle structure by varying the chemical conditions such as pH, ionic strength, and temperature and evaluate how these perturbations affect virion function. Such a strategy has been useful for gaining insights into the structural organization of the genome in rotavirus (39). Using a combination of cryo-EM and biochemical techniques, we show here that at elevated pH the VP4 spike undergoes an irreversible conformational change from a bilobed structure to a distinctly stunted trilobed structure to alter the cell binding characteristics of the virus. This structural change is completely abrogated by a neutralizing MAb, allowing us to propose a mechanism of neutralization by this antibody.

MATERIALS AND METHODS

Cells, virus, and antibodies. Rotavirus TLPs (RRV and SA11-4F), grown in the presence of trypsin in MA-104 cells, were purified as described previously and suspended in TNC buffer (10 mM Tris, 150 mM NaCl, 10 mM CaCl₂ [pH 7.5]) (39). MAbs were purified from ascites by using the MAb-Trap Kit (Amersham Pharmacia Biotech, Piscataway, NJ), and Fab fragments were created by using the ImmunoPure Fab Preparation kit (Pierce, Rockford, IL). Purified Fabs were eluted into 0.1 M phosphate-buffered saline (PBS) at a concentration of 0.7 mg/ml. Prior to addition to rotavirus for structural study, Fabs were dialyzed against 10 mM Tris (pH 7.5) to inhibit clumping of the virus particles. To study the effect of various chemical and pH conditions on the virus, the specimen was dialyzed by using a microdialysis button (Hampton Research, Laguna Niguel, CA) for 30 min in either 250 mM ammonium hydroxide (pH 11.5) or CAPS buffer (pH 11.5). In all biological assays, the specimen was brought back to a normal pH of 7.5 by dialysis in TNC. The term "pH-treated," unless otherwise stated, refers to particles taken to high pH, to induce conformational changes, and then brought back to normal pH.

Virus radiolabeling. To quantitate the relative amounts of VP5* and VP8* before and after ammonium high-pH treatment, SA11-4F rotavirus was radiolabeled during infection and viral replication. MA104 cells were starved of methionine and cysteine for 3 h by using Dulbecco's modified Eagle medium ((1×) lacking L-glutamine, sodium pyruvate, L-methionine, and L-cysteine (Life Technologies, Rockville, MD)). The cells were then infected and, after 1 h, L-methionine and L-cysteine (³⁵S-Met/Cys; Amersham Pharmacia Biotech) were added, and the virus was allowed to grow overnight. Virus was purified as described above, and incorporation of ³⁵S-Met/Cys following purification was measured by scintillation counting. Virus concentration was calculated based on

the absorbance at 260 nm, and the specific activity of radiolabeled virus was calculated to be ~7,000 cpm/μg. Both untreated and pH-treated radiolabeled virus particles were repurified again by cesium chloride ultracentrifugation to separate the intact capsid from any potentially soluble protein fragment removed by the pH treatment. Treated virus banded at the same density as untreated virus, and both types of sample were pelleted for analysis. The purified virus samples were then analyzed again to measure protein and radioactivity and found to have no significant decrease in specific activity.

SDS-PAGE. To determine protein stoichiometry and apparent molecular weights of the protein components of untreated and pH-treated virions, both samples of repurified radiolabeled particles were denatured by boiling and then separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-12% PAGE). Gels containing radiolabeled proteins were dried and exposed to PhosphorImager screens, and analyzed in a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The data were quantitated by using ImageQuant software, and protein bands were boxed out for stoichiometric analysis.

Western blots. Verification of protein identity and antigenicity was confirmed by Western blotting. A SDS-12% polyacrylamide gel of untreated and pH-treated virus was transferred to a Hybond nitrocellulose membrane (Amersham Pharmacia Biotech) and exposed overnight with primary antibodies to SA11-4F, VP5*, and VP8*. Western blots were developed by using chemiluminescence.

Determination of virus infectivity. The infectivity of the untreated and pH-treated viruses was determined either by sequential passage of virus in MA104 cells or by plaque assay (5). Samples were passaged three times in six-well plates to determine whether any infectious virus survived the pH treatment. Initially, 10 μl of 1 mg of untreated and pH-treated virus/ml was incubated with 10 μg of Worthington trypsin/ml. After each passage, the plates were frozen and thawed three times, the samples were treated with Worthington trypsin for 30 min at 37°C, and new cells were inoculated. After each passage, the wells were observed to determine whether the cells exhibited cytopathic effect. The control, untreated sample induced cytopathic effect, whereas the pH-treated virus did not. Samples from both conditions also were subjected to plaque assay after each passage.

Hemagglutination. To determine whether virus binding to sialic acids was affected by pH treatment or the binding of 2G4 Fabs, hemagglutination assays (HAS) were performed. Untreated RRV, RRV taken to either pH 9.75 or 11.5 and then returned to pH 7.5, RRV pretreated with 2G4 Fabs and then subjected to high pH treatment, or RRV taken to either pH 9.75 or 11.5, returned to pH 7.5 and then treated with 2G4 Fabs were serially twofold diluted in a 96-well plate. The first well-contained a 1:100 dilution of the initial virus concentration of 0.34 mg/ml. Type O human red blood cells were washed three times by diluting with 10 ml of PBS and centrifuged at 1,000 rpm for 10 min each. Then, 50 μl of 0.5% red blood cells in PBS was added to each 50 μl of virus per well. After incubation for 1 h at room temperature, the plate was read for hemagglutination activity. The hemagglutination titer was determined as the reciprocal of the final dilution resulting in hemagglutination.

Cell-binding assays. Cell-binding assays were performed essentially as previously described (10). Untreated and pH-treated methionine-labeled TLPs were serially diluted in TNC containing 1% bovine serum albumin, and added to monolayers of MA104 cells in a 96-well plate. The specific activity of the virus was calculated to yield an initial value of 4 μg of virus. Both radiolabeled virus and radiolabeled virus plus a 10-fold concentration of unlabeled competitive virus were added to the plates in duplicate. The cells were incubated on ice for 1 h, washed twice with TNC buffer, and then lysed with 50 μl of 1% SDS. The lysate was counted by using a Beckman LS 3801 scintillation counter (Beckman Instruments, Fullerton, CA). Each assay with the TLPs was performed in duplicate and repeated twice.

Cryo-EM. Specimen preparation for cryo-EM was carried out by using standard procedures (10, 15). Each specimen, at a concentration of ~1.5 mg/ml (5-μl aliquot) was pipetted to a holey carbon-coated copper grid (Quantifoil MicroTools, Jena, Germany). The grid was blotted and flash-frozen in liquid ethane (–190°C) and loaded on a Gatan (Gatan Instruments, Pleasanton, CA) cold-stage cryoholder. Electron micrographs were recorded on a JEOL 1200 transmission electron microscope operating at 100 kV with a magnification of ×30,000 by using an electron dose of 5 e[–]/Å². From each specimen area in the grid, a focal pair with intended defocus values of 1 and 2 μm was recorded. The images were taken with 1-s exposure to Kodak S0-163 EM film (Eastman Kodak Co., Rochester, NY) and developed for 12 min in Kodak D-19 developer, followed by fixation for 10 min in Kodak fixer.

Antibody labeling of particles. Decoration of rotavirus TLPs with the Fab fragment of MAb 2G4 was carried out to observe whether the epitopes were conserved. Purified TLPs were pretreated overnight at a stoichiometry of 5 Fabs per VP4 monomer. After Fab addition, some of the samples were dialyzed

FIG. 1. Rotavirus structures with or without high-pH treatment and Fabs. Cryo-EM reconstructions of RRV rotavirus that was at pH 7.5 (A), ammonium high pH treated (B), 2G4 Fab bound and then high pH treated (C), and 2G4 Fab bound after high-pH treatment (D). All structures are shown along the icosahedral threefold axis. The capsids are radially color coded with the VP4 protein in red, VP7 in yellow, and the Fab fragments in cyan.

against ammonium hydroxide (pH 11.5), redialyzed with TNC (pH 7.5), and then flash frozen. In other cases, virus that had been pH treated was then treated overnight with Fabs. Samples were then imaged by cryo-EM as before.

Three-dimensional reconstructions. Cryo-electron micrographs were selected for correct defocus, ice quality, contrast, and particle concentration and were scanned on a Zeiss SCAI microdensitometer (Carl Zeiss, Inc., Englewood, CO), with a scanning interval of 14 μm corresponding to 4.67 $\text{\AA}$ in the object. Reconstructions were carried out by using the data from closer-to-focus images to a resolution within the first zero of the contrast transfer function with appropriate corrections. The defocus values were $-1.34 \mu\text{m}$ (using 136 particles to a resolution of 22.3 $\text{\AA}$) for pH 7.5, $-1.34 \mu\text{m}$ (using 175 particles to a resolution of 22.3 $\text{\AA}$) for pH 11.5, and $-1.46 \mu\text{m}$ (using 118 particles to a resolution of 22.8 $\text{\AA}$) for Fab pretreatment, followed by high-pH treatment, and $-1.46 \mu\text{m}$ (using 75 particles to a resolution of 22.8 $\text{\AA}$) for Fab labeling after high-pH treatment. Determination of the defocus values, orientation determination, refinement, and three-dimensional reconstructions were carried out by using the ICOS toolkit software (29), and resolution assessment and choice of the appropriate contour levels were conducted as described previously (39). For each reconstruction, the number of particles with unique orientations was found to be adequate by examining the spread of inverse Eigen values, which was <0.1 for 99% of the data. After the final refinement, in each case, the average phase residual between the images and their corresponding projections was $<45^\circ$. Threshold values for

the reconstructions were chosen to account for 780 molecules of VP6 between radii of 250 and 350 $\text{\AA}$ in all reconstructions.

Difference maps and antibody fitting. The maps of rotavirus before and after addition of Fab and/or high-pH treatment were scaled, and differences were computationally calculated. Maps without Fabs were subtracted from maps containing Fabs, and differences (representing Fab densities) were color coded to indicate regions of interaction with VP4.

RESULTS

VP4 is altered by high pH. Prior to determining structural alterations of rotavirus by various chemical treatments, all virus preparations were observed under normal pH and ionic strength conditions to verify the purification and the structure of the VP4 spikes. When either purified rhesus rotavirus (RRV) or simian rotavirus (SA11-4F) strains were imaged under normal pH conditions (TNC [pH 7.5]), the standard conformation of the trypsinized spike was seen in both cryo-electron micrographs and image reconstructions (Fig. 1A and

FIG. 2. Close-up of VP4 with or without high-pH treatment and/or Fabs. Close-up view of RRV VP4 spike structures under various conditions: normal pH (7.5) (A), high pH (pH 11.5) (B), 2G4 Fab bound and then high pH treated (C), and Fab-bound after high-pH treatment (D). Row I shows the spikes as they appear from the side, and row II shows them from the top, rotated 90° downward and 180° clockwise to illustrate the movement relative to the type II channel. Blue arrows in panel A indicate normal VP4 connections with VP7. The red arrows in panel B indicate altered spike connections to VP7. The dashed arrows in row II, panels A and B, indicate the shift that the altered spike makes from the edge of the type II channel to directly cover the channel. The violet arrows indicate Fab densities in row II, panel D, with the stronger Fab density indicated by the solid arrow and the weaker Fab densities indicated by the dashed arrows.

2A). This spike, which is ~ 110 Å long, has a dimeric shape with two large globular domains at the tip of the spike.

To determine the effects of various pH and ionic strengths on the rotavirus capsid, cryo-EM reconstructions were performed after altering the conditions by dialysis (see Materials and Methods). When the virus was treated with either NH_4OH (pH 11.5) or CAPS buffer (pH 11.6), the spike in the reconstructions is stunted to a trilobed shape (Fig. 1B and 2B). The spike on the virus at high pH is 66 Å long and 70 Å wide and has also shifted from its location at the edge of the type II channel toward the fivefold vertex by ~ 20 Å (Fig. 2A and B, arrows), directly over the channel (Fig. 2B). The spike, which originally made two connections to the VP7 layer (arrow, Fig. 2A), has made a new connection to VP7 and lost the smaller connection (arrows, Fig. 2B). It appears from the image reconstructions that a loss of mass, notably in the distal lobes, has occurred in the altered spike structure.

The conformational change in VP4 spikes is irreversible. After observing the conformational change in VP4, we sought

to determine whether the change was reversible when particles were brought back to neutral pH. When returned to pH 7.5 after high-pH treatment, the cryo-EM reconstructions showed that the spike remained stunted on both RRV and SA11-4F viruses (data not shown). This is in contrast to the structural alterations induced by high-pH treatment in the rotavirus genome organization, which are reversible (39). This reversible condensation and expansion in the genome structure was found to be a synergistic effect of NH_4^+ and pH. However, the alterations in the spike structure are the consequence of high pH alone, since identical changes are observed with or without NH_4^+ .

The conformational change occurs only at high pH. The virus was subjected to both extremely low and moderate pH levels to determine the range of the response of VP4 to various pH conditions. When virus was dialyzed against high pH, TNC (pH 9.75) and observed by cryo-EM, image reconstructions showed no conformational change in the VP4 spike (data not shown). This suggests that the change occurs only at very high

FIG. 3. Protein composition of native and pH-treated rotavirus. Untreated and pH-treated radiolabeled rotavirus (SA11-4F) were analyzed on a 12% SDS gel. Western blot analysis with the VP8* specific antibody detected the two bands indicated by VP8* and * (10). Molecular weights are indicated on the left, and the corresponding viral proteins are labeled along the right.

pH, as evidenced by reconstructions of rotavirus treated with pH 11.6 CAPS or pH 11.5 NH_4OH . In addition, when virus was dialyzed at low pH against HCl (pH 3.5), the spike also did not exhibit any structural changes (data not shown). Finally, virus treated with pH 2.2 HCl remained morphologically intact, but the particles tended to aggregate and were unsuitable for image processing (data not shown). This indicates the overall broad range of rotavirus stability at low pH levels.

Neither VP5* or VP8* are lost by high-pH treatment. To determine whether the altered VP4 structure was due to a loss of protein, either VP5* or VP8*, from the spike, untreated, and pH-treated radiolabeled particles were repurified by cesium chloride density gradient centrifugation after treatment, and equivalent counts of each preparation were analyzed by SDS-PAGE (Fig. 3). Western blot analysis was performed with antibodies to SA11-4F, VP5*, and VP8* to identify each of the proteins (data not shown). Two forms of VP8* were identified by Western blotting with the VP8* specific antibody as described previously (10). After PhosphorImager analysis, the ratio of counts for VP5* and for VP8* or for both VP5* and VP8* to VP6 or VP2 were unchanged in untreated or pH-treated particles. Since the counts for VP6 for the treated and untreated particles were equivalent, these results indicate that the amount of VP5* and VP8* remained the same after pH treatment.

Mapping the altered VP4 structure with Fabs. To examine whether the antigenic structure of VP4 is maintained and recognizable in the altered structure, we carried out antibody labeling with a VP5* specific neutralizing MAb called 2G4 (Fig. 1C and D). At normal pH, two Fab fragments of 2G4, as shown previously (40, 43), bind toward the distal ends of the spike in an area localized between the globular heads (Fig. 1C and 2C). The binding site of 2G4 has been mapped to amino acid 393 with a 2G4 escape mutant (35). Cryo-EM reconstruction of 2G4-Fabs bound to pH-treated virus shows the presence of the Fabs on short spikes as three mass densities, one on each of the three lobes (Fig. 1D and 2D). One of the densities has the size and shape of an entire Fab, while the other two mass densities, although clearly showing the overall shape of a Fab, were not as strong (Fig. 2D). It is possible that one of the VP4 monomers has a stable conformation, whereas the other monomer exists in two alternate states. Although two of the three Fab densities are weak, the three parts of the altered trilobed spike structure are equally strong. Alternatively, the trilobed appearance of the spike may indicate that the spike in the altered conformation is composed of three monomers of VP4 in accordance with the proposal by Dormitzer et al. (12) and, due to either steric hindrance or antibody-induced conformational changes, only one of the Fabs has full occupancy.

Pretreatment of virus with 2G4 Fabs inhibits the conformational change. We next sought to determine whether different conformational states could be obtained for Fab-bound and pH-treated virus. Rotavirus was pretreated with 2G4 Fabs and then taken to high pH and returned to normal pH. In contrast to results obtained with pH-treated particles without Fabs, the spike in the Fab-bound and then pH-treated particles remained in a fully elongated state (Fig. 1C and 2C). The Fab density and binding location were identical to what was observed with Fab-bound untreated particles used as a control. Thus, not only does 2G4 protect VP4 from undergoing a structural change with pH treatment, it remains bound to the spikes despite drastic changes in the pH (from pH 7 to ~11 and back to pH 7). These results demonstrate the remarkable strength of the Fab-VP5* interaction and its ability to withstand high-pH treatment. In addition, the ability of the 2G4 Fab bound to this specific domain of VP4 to block the high-pH-induced conformational change may indicate a possible mechanism of 2G4 neutralization if a conformational change in VP4 is required for infectivity.

Fab-bound and pH-treated virus hemagglutinates red blood cells, but hemagglutination is lost with virus containing the high-pH-induced altered VP4. Previous studies have shown that 2G4 binding to SA-dependent viruses, such as RRV, inhibits hemagglutination and internalization but not cell attachment (34). However, in our studies we found that 2G4-Fab-bound virus still retained the ability to hemagglutinate red blood cells, a function mapped to amino acids 93 to 208 of the VP8* domain (Fig. 4). This apparent discrepancy is likely due to the use of Fabs in our studies instead of the immunoglobulin G antibodies used in the previous studies. To confirm that the 2G4-Fabs protected the spike from high-pH-induced conformational changes, we first mixed RRV with 2G4-Fabs, took the virus-Fab complex to pH 11.2, and then returned the mixture to pH 7.5. These particles showed no loss of hemagglutinating activity and were able to hemagglutinate red blood cells to the

FIG. 4. HA of type O red blood cells with RRV (RRV), pH-treated RRV (RRV pH), 2G4 Fab-bound RRV (RRV 2G4), or RRV bound with 2G4 Fabs and then treated with high pH (RRV 2G4 pH). HA titers expressed as the reciprocal of the highest dilution resulting in hemagglutination.

same level as the Fab-bound virions at pH 7.5 (Fig. 4). This result clearly emphasizes that 2G4 not only protects the structural integrity of the spike but also preserves the functional properties of the spike through pH changes. In contrast, pH-treated RRV from the same preparation did not display any hemagglutinating activity, indicating that the apparent disorder of VP8* due to high-pH treatment results in the loss of this functional property.

Rotavirus with altered VP4 shows a loss of infectivity. Next we wanted to examine whether the pH-treated particles with altered spikes retain their ability to infect cells. Three rounds of blind passage were conducted by infecting MA104 cells in six-well plates in duplicate with untreated and pH-treated virus. After each round, plaque assays were performed, and they revealed no infectious particles for the pH-treated virus, whereas the average titer for untreated virus was found to be 2×10^8 PFU/ml.

Rotavirus with an altered VP4 maintains the ability to bind to MA104 cells in a SA-dependent manner. After the observation of loss of infectivity of the pH-treated virus, we performed cell-binding assays to determine whether the conformational change in VP4 spikes had any effect on the ability of the pH-treated virus to bind to cells. Both untreated and pH-treated TLPs specifically bound to MA104 cells (Fig. 5A). Both RRV and SA11-4F strains that we used in our studies are SA-dependent strains, i.e., they require the presence of *N*-acetylneuraminic (sialic) acid for efficient infectivity. Since the pH treatment of the virus particle resulted in the loss of hemagglutination but still retained the ability to bind to cells, we wanted to examine whether these particles bound to cells in an SA-dependent manner as do native particles. We determined the ability of native and pH-treated virus to bind to cells that were treated with neuraminidase to remove sialic acid from the cell surface. As expected, cell binding with native virus was abrogated on neuraminidase-treated cells; however, surprisingly, the pH-treated particles showed an increase in cell binding with cells that were treated with neuraminidase compared to nontreated cells (Fig. 5A). The increase seen for pH-treated virus in binding to neuraminidase-treated cells compared to

FIG. 5. Cell-binding assay. Specific cell binding of untreated or pH-treated radiolabeled SA11-4F rotavirus on MA104 cells. (A) Specific cell binding of ^{35}S -labeled SA11-4F (4F) and pH-treated SA11-4F (4F pH) to MA104 cells treated with (+) or without (–) neuraminidase. (B) Specific cell binding of SA11-4F (4F), pH-treated SA11-4F (4F pH), 2G4-Fab-bound, and pH-treated SA11-4F (4F 2G4 pH), and pH-treated SA11 4F then bound with 2G4 Fab (4F pH 2G4). Error bars represent the standard deviation.

nontreated cells is similar to that observed for SA-independent rotavirus strains. This result indicates that VP8* on the SA-dependent rotavirus strain is no longer required for the initial cell binding on neuraminidase-treated cells and that a downstream receptor is likely used by the altered VP4 spike to bind to cells.

VP5* has been suggested to be the domain responsible for downstream receptor interactions (33). To determine whether the cell binding of pH-treated particles could be blocked, 2G4 Fabs were bound to pH-treated virus and specific cell-binding assays performed (Fig. 5B). As described above, untreated virus decorated with 2G4 Fab showed specific binding to MA104 cells. However, cell binding was blocked when 2G4 Fabs were bound to pH-treated virus. The 2G4 binding site is mapped to a putative fusion domain in VP5* (positions 384 to 401) (35). The contrasting properties of the native and the pH-treated particles with respect to the effect of 2G4-Fab on particle cell-binding properties suggest the following. First, the region that 2G4 binds in VP4 has a minimal role in differentiating between SA-dependent and SA-independent cell binding of rotavirus per se because this region is accessible in both native and pH-treated particles. Second, the pH treatment exposes a new region, located in the immediate vicinity of the 2G4 binding site, that may be important for the SA-independent cell-binding activity of pH-treated particles but is masked in the native particle. Finally, the binding of 2G4-Fab to pH-treated particles makes this new region inaccessible for SA-independent cell binding.

DISCUSSION

The study presented here, together with other structural studies on rotavirus, clearly shows a conformational flexibility inherent in VP4 spikes. Our previous studies showed trypsinization transforms flexible spikes into rigid 120 Å bilobed structures that are clearly visible in cryo-EM reconstructions of trypsinized virions at physiological conditions (10). Cryo-EM reconstructions with VP4 specific antibodies have shown that these spikes are dimers of VP4 (40, 43). However,

recent crystallographic studies have shown that the tryptic fragment VP5*, which constitutes the central body of the spike, forms a trimer (12). These crystallographic studies have further suggested that VP4 may undergo a transformation from a dimeric to a trimeric structure during the cell entry process. Considering that several biochemical studies have increasingly suggested that cell entry of rotavirus may involve sequential interactions with multiple receptors (33), such a conformational flexibility is perhaps not surprising.

In our studies presented here, we have shown that VP4 spikes at high pH undergo a drastic irreversible conformational change resulting in a stunted structure with a pronounced trilobed appearance. In contrast, the overall structure of the virion, including the spikes, is unaffected by low or moderate pH conditions consistent with its ability to survive in the low-pH environment of the gastrointestinal tract. The mass density calculations of the spikes altered by high pH clearly indicated significant mass loss. Our initial conjecture was that the tryptic fragment VP8*, which is localized to the distal globular domains of the native spike (13), was dislodged from the spike. However, Western blot and stoichiometric analyses of radiolabeled particles unambiguously indicated that both VP5* and VP8* are present on particles in the same stoichiometric proportions before and after high pH treatment. This indicates that the interactions, possibly hydrophobic, between these two cleavage products in the spike structure are strong enough to withstand high-pH treatment and remain associated with the capsid. These observations further suggest that a significant portion of the spike structure becomes disordered or flexible at high pH and therefore is not visible in the reconstructions.

What is the chemical composition of the visible portion of the altered spike? The distal portion of the altered spike is recognized by a VP5*-specific antibody 2G4, suggesting that the altered spike consists of VP5*. Because this antibody recognizes only a conformational epitope (35), this observation further indicates that, despite high-pH treatment, the structural integrity of the antigenic epitope in VP5* is intact. The observations that pH-treated particles have lost the ability to hemagglutinate and interact with SA indicate that VP8*, which is responsible for these two properties, is likely the portion that is disordered and/or flexible and not visible in the reconstruction. Three Fab densities are seen attached to each of the trilobed spikes. There are two possible interpretations to this observation. One possibility is that the trilobed spike represents a trimerized VP5*. Recent crystallographic studies clearly indicate that VP5* forms trimers with strong interactions between the monomeric subunits (12). The authors of that study proposed a model in which each spike is a trimer of VP4 and, upon trypsinization, two of the monomers form the visible spike in cryo-EM reconstruction rotavirus particles, while the other monomer being floppy is not visible in the reconstruction. During cell entry, by yet unknown entry associated events, the floppy VP4 monomer, together with the other two molecules, trimerize as seen in the VP5* crystal structure. It is plausible that high-pH treatment renders VP8* disordered and triggers dimer-to-trimer transition of VP5*, perhaps mimicking a post-SA-attachment step during cell entry. Such a drastic conformational rearrangement, although first proposed for nonenveloped viruses, has been visualized

for enveloped viruses such as flavivirus (36, 37) and alphavirus (20). Another possible interpretation, which cannot be ruled out, is that the trilobed spike represents one stable monomer and another monomer in two alternate conformations. Higher-resolution cryo-EM structural analysis of the pH-treated particles may be required to resolve this question unambiguously.

Although the pH-treated particles lose the ability to hemagglutinate, cell-binding assays indicate that they retain the ability to specifically bind to cells in a SA-independent manner. This is in contrast to the untreated virions, which exhibit SA-dependent cell binding. For SA-dependent rotavirus strains, the initial step in cell entry constitutes interactions with SA-containing receptors. However, cell binding for most rotavirus strains, including human rotavirus, is SA independent (7). In these strains, the majority of the neutralizing MAbs that recognize VP4 select mutations in VP5* (27, 28, 38), suggesting that cell entry is mediated mainly by VP5*. Thus, a likely interpretation is that the altered spike represents a transitional state geared to interact with one of the downstream receptors after SA attachment in the multistep cell entry process of rotavirus. Possible involvement of VP7 in the cell binding of pH-treated particles is ruled out because the cell binding is inhibited by the VP5*-specific 2G4-Fab. In its cell attachment, the pH-treated particles appear to resemble the nar3 mutant of RRV (21, 45). This mutant, which exhibits SA-independent cell binding in contrast to its parental strain, has been shown to attach to the cell surface by interacting with integrin $\alpha_2\beta_1$ through the DGE motif in VP5*. In this mutant also, just as in our pH-treated particles, the 2G4 antibody inhibits cell binding (46). A distinct possibility is that the DGE motif (residues 308 to 310) becomes exposed in the pH-treated particles, and the 2G4-Fab inhibits cell binding of the pH-treated particles by sterically hindering the accessibility of this motif. Although pH treatment exposes a domain on the spike that can interact with a cellular factor, it appears to have destroyed the other determinants required for successful internalization, since pH-treated particles are not infectious.

A remarkable observation made during the course of these experiments is that 2G4, a VP5*-specific neutralizing antibody, can completely protect the spike from pH-induced conformational changes. Equally remarkable is its ability to stay attached to the spikes despite drastic changes in pH (from ca. pH 7 to 11 and back to pH 7). To our knowledge, no other studies have provided a structural demonstration of such a strong antibody-antigen interaction. In general, an antibody-antigen interface is less hydrophobic with relatively more intermolecular hydrogen bonds than found at the protein-protein interface of a homodimer (24). Because 2G4-VP5* interactions are not sensitive to variations in pH, we can argue that they are predominantly hydrophobic. In this respect, 2G4-VP5* interactions perhaps are an exception to the general trend found in other antibody-antigen interactions. Studies with 2G4 escape mutants indeed show involvement of a hydrophobic region between residues 384 and 401 of VP5* (35).

The observation that 2G4 protects the structural and functional integrity of the VP4 spike allows us to propose a mechanism of neutralization by this antibody. It is known that 2G4 does not interfere with cell binding but inhibits internalization (34). We propose that the mechanism by which 2G4 neutralizes rotavirus is by inhibiting a postattachment conformational

change that is required for interacting with downstream receptors. This is also consistent with our observation that 2G4 binding to pH-treated particles inhibits cell binding. The ability of a certain antibody to prevent conformational changes necessary for effective internalization of a virus has indeed been demonstrated in yet another case. An antibody that binds to membrane-distal domains of the influenza virus hemagglutinin can prevent the low-pH transition required for fusion activity (2).

Despite the lack of a reverse genetics system for rotavirus, which would have been ideal to dissect the role of VP4 in cell entry process, our "structural mutagenesis" strategy has provided useful insights into conformational properties of VP4 in relation to the rotavirus cell entry process. A hypothesis that emerges from the various observations we have made is that high-pH treatment induces a conformational change in the VP4 spikes that mimics a post-SA-attachment step in the rotavirus cell entry process, which involves sequential interactions with multiple receptors. This conformational change is inhibited by 2G4 MAb to neutralize the virus.

ACKNOWLEDGMENTS

This study was supported by NIH grants AI-36040 (B.V.V.P.) and DK-30144 (M.K.E.). J.B.P. acknowledges the support of NSF training grant BIR-9256580.

We acknowledge the use of cryo-EM facilities at the NIH-funded National Center for Macromolecular Imaging at the Baylor College of Medicine.

REFERENCES

- Arias, C. F., P. Romero, V. Alvarez, and S. Lopez. 1996. Trypsin activation pathway of rotavirus infectivity. *J. Virol.* **70**:5832–5839.
- Barbey-Martin, C., B. Gigant, T. Bizebard, L. J. Calder, S. A. Wharton, J. J. Skehel, and M. Knossow. 2002. An antibody that prevents the hemagglutinin low pH fusogenic transition. *Virology* **294**:70–74.
- Belnap, D. M., D. J. Filman, B. L. Trus, N. Cheng, F. P. Booy, J. F. Conway, S. Curry, C. N. Hiremath, S. K. Tsang, A. C. Steven, and J. M. Hogle. 2000. Molecular tectonic model of virus structural transitions: the putative cell entry states of poliovirus. *J. Virol.* **74**:1342–1354.
- Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* **371**:37–43.
- Burns, J. W., D. Chen, M. K. Estes, and R. F. Ramig. 1989. Biological and immunological characterization of a simian rotavirus SA11 variant with an altered genome segment 4. *Virology* **169**:427–435.
- Chen, D., C. L. Luongo, M. L. Nibert, and J. T. Patton. 1999. Rotavirus open cores catalyze 5'-capping and methylation of exogenous RNA: evidence that VP3 is a methyltransferase. *Virology* **265**:120–130.
- Ciarlet, M., and M. K. Estes. 1999. Human and most animal rotavirus strains do not require the presence of sialic acid on the cell surface for efficient infectivity. *J. Gen. Virol.* **80**:943–948.
- Cohen, J. 2001. Medicine: rethinking a vaccine's risk. *Science* **293**:1576–1577.
- Coulson, B. S., S. L. Londrigan, and D. J. Lee. 1997. Rotavirus contains integrin ligand sequences and a disintegrin-like domain that are implicated in virus entry into cells. *Proc. Natl. Acad. Sci. USA* **94**:5389–5394.
- Crawford, S. E., S. K. Mukherjee, M. K. Estes, J. A. Lawton, A. L. Shaw, R. F. Ramig, and B. V. Prasad. 2001. Trypsin cleavage stabilizes the rotavirus VP4 spike. *J. Virol.* **75**:6052–6061.
- Denisova, E., W. Dowling, R. LaMonica, R. Shaw, S. Scarlata, F. Ruggeri, and E. R. Mackow. 1999. Rotavirus capsid protein VP5* permeabilizes membranes. *J. Virol.* **73**:3147–3153.
- Dormitzer, P. R., E. B. Nason, B. V. Prasad, and S. C. Harrison. 2004. Structural rearrangements in the membrane penetration protein of a non-enveloped virus. *Nature* **430**:1053–1058.
- Dormitzer, P. R., Z. Y. Sun, G. Wagner, and S. C. Harrison. 2002. The rhesus rotavirus VP4 sialic acid binding domain has a galectin fold with a novel carbohydrate binding site. *EMBO J.* **21**:885–897.
- Dowling, W., E. Denisova, R. LaMonica, and E. R. Mackow. 2000. Selective membrane permeabilization by the rotavirus VP5* protein is abrogated by mutations in an internal hydrophobic domain. *J. Virol.* **74**:6368–6376.
- Dubochet, J., M. Adrian, J. J. Chang, J. C. Homo, J. Lepault, A. W. McDowell, and P. Schultz. 1988. Cryo-electron microscopy of vitrified specimens. *Q. Rev. Biophys.* **21**:129–228.
- Estes, M. K. 2001. Rotaviruses and their replication, p. 1747–1785. *In* D. M. Knipe and P. M. Howley (ed.), *Fields virology*, 4th ed. Lippincott-Raven Publishers, Philadelphia, Pa.
- Estes, M. K., D. Y. Graham, and B. B. Mason. 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. *J. Virol.* **39**:879–888.
- Fiore, L., H. B. Greenberg, and E. R. Mackow. 1991. The VP8 fragment of VP4 is the rhesus rotavirus hemagglutinin. *Virology* **181**:553–563.
- Fukuhara, N., O. Yoshie, S. Kitaoka, and T. Konno. 1988. Role of VP3 in human rotavirus internalization after target cell attachment via VP7. *J. Virol.* **62**:2209–2218.
- Gibbons, D. L., M. C. Vaney, A. Roussel, A. Vigouroux, B. Reilly, J. Lepault, M. Kiellian, and F. A. Rey. 2004. Conformational change and protein-protein interactions of the fusion protein of Semliki Forest virus. *Nature* **427**:320–325.
- Graham, K. L., P. Halasz, Y. Tan, M. J. Hewish, Y. Takada, E. R. Mackow, M. K. Robinson, and B. S. Coulson. 2003. Integrin-using rotaviruses bind alpha2beta1 integrin alpha2 domain via VP4 DGE sequence and recognize alphaXbeta2 and alphaVbeta3 by using VP7 during cell entry. *J. Virol.* **77**:9969–9978.
- Guerrero, C. A., E. Mendez, S. Zarate, P. Isa, S. Lopez, and C. F. Arias. 2000. Integrin $\alpha_3\beta_3$ mediates rotavirus cell entry. *Proc. Natl. Acad. Sci. USA* **97**:14644–14649.
- Hewish, M. J., Y. Takada, and B. S. Coulson. 2000. Integrins $\alpha_3\beta_1$ and $\alpha_4\beta_1$ can mediate SA11 rotavirus attachment and entry into cells. *J. Virol.* **74**:228–236.
- Jones, S., and J. M. Thornton. 1996. Principles of protein-protein interactions. *Proc. Natl. Acad. Sci. USA* **93**:13–20.
- Kalica, A. R., J. Flores, and H. B. Greenberg. 1983. Identification of the rotaviral gene that codes for hemagglutination and protease-enhanced plaque formation. *Virology* **125**:194–205.
- Kaljit, K. T., R. D. Shaw, D. H. Rubin, and H. B. Greenberg. 1988. Infectious rotavirus enters cells by direct cell membrane penetration, not by endocytosis. *J. Virol.* **62**:1136–1144.
- Kirkwood, C. D., R. F. Bishop, and B. S. Coulson. 1996. Human rotavirus VP4 contains strain-specific, serotype-specific and cross-reactive neutralization sites. *Arch. Virol.* **141**:587–600.
- Kobayashi, N., K. Taniguchi, and S. Urasawa. 1990. Identification of operationally overlapping and independent cross-reactive neutralization regions on human rotavirus VP4. *J. Gen. Virol.* **71**:2615–2623.
- Lawton, J. A., and B. V. Prasad. 1996. Automated software package for icosahedral virus reconstruction. *J. Struct. Biol.* **116**:209–215.
- Lawton, J. A., C. Q. Zeng, S. K. Mukherjee, J. Cohen, M. K. Estes, and B. V. Prasad. 1997. Three-dimensional structural analysis of recombinant rotavirus-like particles with intact and amino-terminal-deleted VP2: implications for the architecture of the VP2 capsid layer. *J. Virol.* **71**:7353–7360.
- Liu, M., N. M. Mattion, and M. K. Estes. 1992. Rotavirus VP3 expressed in insect cells possesses guanylyltransferase activity. *Virology* **188**:77–84.
- Lizano, M., S. Lopez, and C. F. Arias. 1991. The amino-terminal half of rotavirus SA114fM VP4 protein contains a hemagglutination domain and primes for neutralizing antibodies to the virus. *J. Virol.* **65**:1383–1391.
- Lopez, S., and C. F. Arias. 2004. Multistep entry of rotavirus into cells: a Versaillesque dance. *Trends Microbiol.* **12**:271–278.
- Mackow, E. R., J. W. Barnett, H. Chan, and H. B. Greenberg. 1989. The rhesus rotavirus outer capsid protein VP4 functions as a hemagglutinin and is antigenically conserved when expressed by a baculovirus recombinant. *J. Virol.* **63**:1661–1668.
- Mackow, E. R., R. D. Shaw, S. M. Matsui, P. T. Vo, M. N. Dang, and H. B. Greenberg. 1988. The rhesus rotavirus gene encoding protein VP3: location of amino acids involved in homologous and heterologous rotavirus neutralization and identification of a putative fusion region. *Proc. Natl. Acad. Sci. USA* **85**:645–649.
- Modis, Y., S. Ogata, D. Clements, and S. C. Harrison. 2004. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**:313–319.
- Mukhopadhyay, S., R. J. Kuhn, and M. G. Rossmann. 2005. A structural perspective of flavivirus life cycle. *Nat. Rev. Microbiol.* **3**:13–22.
- Padilla-Noriega, L., S. J. Dunn, S. Lopez, H. B. Greenberg, and C. F. Arias. 1995. Identification of two independent neutralization domains on the VP4 trypsin cleavage products VP5* and VP8* of human rotavirus ST3. *Virology* **206**:148–154.
- Pesavento, J. B., J. A. Lawton, M. E. Estes, and B. V. Prasad. 2001. The reversible condensation and expansion of the rotavirus genome. *Proc. Natl. Acad. Sci. USA* **98**:1381–1386.
- Prasad, B. V., J. W. Burns, E. Marietta, M. K. Estes, and W. Chiu. 1990. Localization of VP4 neutralization sites in rotavirus by three-dimensional cryo-electron microscopy. *Nature* **343**:476–479.
- Prasad, B. V., R. Rothnagel, C. Q. Zeng, J. Jakana, J. A. Lawton, W. Chiu, and M. K. Estes. 1996. Visualization of ordered genomic RNA and localization of transcriptional complexes in rotavirus. *Nature* **382**:471–473.
- Sabara, M., J. E. Gilchrist, G. R. Hudson, and L. A. Babiuk. 1985. Preliminary characterization of an epitope involved in neutralization and cell at-

- tachment that is located on the major bovine rotavirus glycoprotein. *J. Virol.* **53**:58–66.
43. **Tihova, M., K. A. Dryden, A. R. Bellamy, H. B. Greenberg, and M. Yeager.** 2001. Localization of membrane permeabilization and receptor binding sites on the VP4 hemagglutinin of rotavirus: implications for cell entry. *J. Mol. Biol.* **314**:985–992.
44. **Valenzuela, S., J. Pizarro, A. M. Sandino, M. Vasquez, J. Fernandez, O. Hernandez, J. Patton, and E. Spencer.** 1991. Photoaffinity labeling of rotavirus VP1 with 8-azido-ATP: identification of the viral RNA polymerase. *J. Virol.* **65**:3964–3967.
45. **Zarate, S., R. Espinosa, P. Romero, C. A. Guerrero, C. F. Arias, and S. Lopez.** 2000. Integrin alpha2beta1 mediates the cell attachment of the rotavirus neuraminidase-resistant variant nar3. *Virology* **278**:50–54.
46. **Zarate, S., R. Espinosa, P. Romero, E. Mendez, C. F. Arias, and S. Lopez.** 2000. The VP5 domain of VP4 can mediate attachment of rotaviruses to cells. *J. Virol.* **74**:593–599.