



# Localization of Membrane Permeabilization and Receptor Binding Sites on the VP4 Hemagglutinin of Rotavirus: Implications for Cell Entry

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<sup>4</sup>Scripps Clinic, Division of Cardiovascular Diseases, 10666 North Torrey Pines Road La Jolla, CA 92037, USA The surface of rotavirus is decorated with 60 spike-like projections, each composed of a dimer of VP4, the viral hemagglutinin. Trypsin cleavage of VP4 generates two fragments, VP8<sup>\*</sup>, which binds sialic acid (SA), and VP5\*, containing an integrin binding motif and a hydrophobic region that permeabilizes membranes and is homologous to fusion domains. Although the mechanism for cell entry by this non-enveloped virus is unclear, it is known that trypsin cleavage enhances viral infectivity and facilitates viral entry. We used electron cryo-microscopy and difference map analysis to localize the binding sites for two neutralizing monoclonal antibodies, 7A12 and 2G4, which are directed against the SA-binding site within VP8\* and the membrane permeabilization domain within VP5\*, respectively. Fab 7A12 binds at the tips of the dimeric heads of VP4, and 2G4 binds in the cleft between the two heads of the spike. When these binding results are combined with secondary structure analysis, we predict that the VP4 heads are composed primarily of  $\beta$ -sheets in VP8<sup>\*</sup> and that VP5<sup>\*</sup> forms the body and base primarily in  $\beta$ -structure and  $\alpha$ -helical conformations, respectively. Based on these results and those of others, a model is proposed for cell entry in which VP8\* and VP5\* mediate receptor binding and membrane permeabilization, and uncoating occurs during transfer across the lipid bilayer, thereby generating the transcriptionally active particle.

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# Introduction

Rotavirus causes severe gastroenteritis in infants and young animals and is responsible for the death of ~700,000 children per year in developing countries.<sup>1</sup> Rotavirus is a non-enveloped icosahedral virus (diameter ~1000 Å) with three protein layers that encapsidate 11 segments of doublestranded (ds) RNA genome (reviewed by Estes<sup>2</sup>). VP1, VP2 and VP3 are protein components associated with the innermost core layer. The middle

E-mail address of the corresponding author: yeager@scripps.edu and outer capsid protein layers are formed by 260 trimers of VP6 and 780 monomers of the VP7 gly-coprotein, respectively.<sup>3,4</sup> Sixty dimers of the VP4 hemagglutinin extend ~110 Å from the viral surface.<sup>5,6</sup> Each multi-domained VP4 spike has two globular heads attached to a square-shaped body that is connected to a rod-like domain, which merges with a globular base. The VP4 spike penetrates ~90 Å beneath the outer surface and interacts with both the VP7 and VP6 layers.

Infection requires attachment of the virus to membrane receptors of the epithelial cells of the small intestine (reviewed by Estes<sup>2</sup>). There is evidence that rotaviruses have multiple plasma membrane receptors, including sialic acid (SA),<sup>7</sup> integrins such as  $\alpha_2\beta_1$ ,  $\alpha_4\beta_1$  and  $\alpha_V\beta_3^{8-10}$  or other membrane proteins.<sup>11</sup> The attachment of the virus to cell surface receptors is mediated by VP4 (88 kDa).<sup>12</sup> Trypsin cleavage of VP4 generates two

M.T. and K.A.D. contributed equally to this work. Abbreviations used: SA, sialic acid; ds, doublestranded; Mab, monoclonal antibodies; cryo-EM, electron cryo-microscopy; Fab, antigen-binding fragment; DLP, double-layered particle; ER, endoplasmic reticulum; IgG, immunoglobulin G.

virion-associated polypeptides: VP5\* (60 kDa) and VP8\* (28 kDa). This event greatly enhances viral infectivity, induces membrane permeability, and is necessary for maximal viral growth.<sup>13–15</sup> Some animal rotaviruses can bind to the cell either through interactions mediated by VP8\* or VP5\* *via* SA-containing and SA-independent cell surface receptors, respectively.<sup>16</sup> Human strains appear to use an SA-independent route,<sup>17</sup> and an  $\alpha_2\beta_1$  integrin binding motif (DGE) at residues 308-310 may function as the receptor-binding site.<sup>18</sup>

Rotavirus serotypes are designated as G or P depending on whether the antibody response is directed against VP7 or VP4, respectively.<sup>2</sup> VP4 has been directly implicated as a target for sero-type-specific neutralization *in vitro* and protection *in vivo*.<sup>19,20</sup> Homotypic and heterotypic monoclonal antibodies (Mabs) directed against VP4 neutralize both trypsin-activated and non-trypsin treated virus, as well as viral hemagglutination. An effective vaccine for preventing rotavirus infection in infants and neonates <sup>21</sup> was unfortunately associated with significant side effects.<sup>22</sup> Understanding the antigenic properties of the virus is essential to the design of rational preventive or therapeutic strategies.

Libraries of neutralizing Mabs have been analyzed either by competitive inhibition studies or by serologic analyses of Mab mutants.<sup>23,24</sup> Monoclonal antibody 7A12 is homotypic and reacts only with P serotype 5, which includes rhesus rotavirus. The epitope for antibody-binding fragment (Fab) 7A12 includes residue 188 and lies within the SA-binding site of rhesus rotavirus.<sup>25,26</sup> Monoclonal antibody 2G4 is heterotypic and reacts with P serotypes 5, 6 and 7, which includes bovine, rhesus and SA11 rotaviruses. The epitope for Fab 2G4 includes residue 393 and is within a membrane permeabilization domain that is homologous to fusion domains (residues 384-401).<sup>19,26</sup>

Here, we used electron cryo-microscopy (cryo-EM), 3D image reconstruction and difference map analysis to localize the neutralizing Fab molecules 7A12 and 2G4, and thereby the SA-binding site and membrane permeabilization domain on polypeptides VP8\* and VP5\*, respectively.

# **Results and Discussion**

## Localization of Fab fragments

Surface-shaded 3D maps of the Fab-decorated particles show striking similarity to native rotavirus in icosahedral symmetry, the number and distribution of surface holes and the rippled outer capsid surface (Figure 1). In addition, the position and the left-handed helical twist of the square-shaped body of VP4 and the rod-like domain have a similar appearance in native SA11,<sup>5,6</sup> bovine (Figure 1(a)) and rhesus<sup>4</sup> rotavirus. However, there is obvious additional density attached to the VP4 spikes, which we attribute to the bound Fab molecules. For 7A12, each head of the VP4 dimer inter-

acts with one Fab fragment (Figure 1(b)). The 2G4 Fab fragments bind on both sides of the dimeric spike in the cleft between the heads, associated with a slight flattening of the head domains (Figure 1(c); arrows), consistent with an induced fit mechanism for antigen-antibody recognition.<sup>27</sup> Hence, neutralization by these antibodies is due to direct steric interference as well as possible conformational changes that alter cell receptor interactions.

In agreement with neutralization assays, the heterotypic antibody 2G4 was able to bind to rhesus, bovine and SA11 rotavirus, whereas 7A12 only bound to rhesus rotavirus (Figure 1). In particular, the 3D reconstruction of 2G4-decorated bovine rotavirus (Figure 1(c)) was indistinguishable from reconstructions of 2G4-rhesus rotavirus (data not shown) and 2G4-SA11 rotavirus (data not shown) at ~25 Å resolution. Three-dimensional reconstructions of bovine and SA11 rotavirus incubated with 7A12 were indistinguishable from native viruses (data not shown), whereas the additional Fab density on 7A12-rhesus rotavirus was clearly visible (Figure 1(b)).

The density level of the maps was selected according to the expected volume of the capsid shells, and the same level was used for contouring the Fab density. The constant and variable domains of each Fab were especially well defined in the particles decorated with 2G4 (Figure 2(c)), consistent with full occupancy of binding sites. The density of the 7A12 Fab was somewhat less defined at maximal radii (Figure 2(b)), suggesting that this Fab may have had less than full occupancy or more conformational flexibility compared with 2G4. Since the Fab molecules were so well defined, atomic models for canonical Fab structures could be docked within the cryo-EM density maps (Figure 2). Different Fab molecules display elbow angles ranging from a tight 125° to an almost straight 178° angle.<sup>28</sup> The Fab we used for docking has a comparatively tight elbow angle of  $125^{\circ,29}$  which fit quite well into our 3D maps. A top, angled view of the structures with the red (7A12) and green (2G4) Fab densities superimposed on a single VP4 spike revealed that the two Fab molecules bind at  $\sim 90^{\circ}$  with respect to each other (Figure 2(a)). Side views of the individual Fab densities showed that 7A12 Fab molecules bind to the head of the spike angled about  $\sim 10^{\circ}$ from the plane of the viral surface (Figure 2(b)) and 2G4 Fab molecules bind lower in the cleft between the heads angled  $\sim 60^{\circ}$  from the viral surface (Figure 2(c)). These results confirm an earlier analysis, which showed that 2G4 binds to the VP4 spikes.<sup>30</sup> However, in contrast to that study, our maps at higher resolution do not reveal an appreciable difference in the binding orientation on the two sides of the VP4 spike for either 2G4 or 7A12. The side views also demonstrate that the 7A12 and 2G4 Fab orientations are rotated by  $\sim 90^{\circ}$  along their long axes with respect to each other.



**Figure 1.** Surface-shaded quadrant views of the 3D reconstructions of (a) native, undecorated bovine rotavirus, (b) rhesus rotavirus decorated with the Fab 7A12 (in red), which binds to VP8\*, and (c) bovine rotavirus decorated with the Fab 2G4 (in green), which binds to VP5\*. The left and right pairs are wall-eyed and cross-eyed stereo views, respectively. There is close correspondence in the rippled outer capsid surface and the distribution of surface holes in the native and decorated viruses. Although the overall position and shape of the spikes is not changed, binding of 2G4 is associated with slight flattening of the head domains (arrows), while they remain unaltered with 7A12 binding. The spikes in (a) are  $\sim$ 110 Å long.

The separation between the binding sites for 7A12 and 2G4 demonstrates why they do not interfere with each other in competition and inhibition experiments.<sup>25</sup> Both 7A12 and 2G4 decrease infectivity. However, only 7A12 blocks binding, whereas 2G4 does not.<sup>31</sup> These results are understandable in terms of the physical locations for the epitopes in the 3D maps (Figures 2 and 3).

#### Modeling of the secondary structure

Secondary structure analyses suggest the existence of at least two general VP4 domains: the N-terminal two-thirds has a preponderance of  $\beta$ -structure (Figure 3(a), blue) separated by loops, turns and coils, and the C-terminal third has substantial  $\alpha$ -helical content (Figure 3(a), yellow), including one region (residues 490-505) predicted to fold as a coiled-coil domain.<sup>7,32,33</sup> The 7A12 and 2G4 antibodies bind on predicted loops within  $\beta$ -rich regions. The 7A12 neutralization site, including residue 188, resides in the SA-binding site on the head domain. Tyrosine residues 155 and 188, and serine 190, may play an essential role in the SA-binding activity.<sup>7</sup> Surface proteins of many enveloped viruses have well defined sialic acidbinding domains, and most, including the influenza hemagglutinin,<sup>34</sup> are within a  $\beta$ -barrel motif. The 2G4 neutralization binding site, including residue 393, is on a loop located in the membrane permeabilization region <sup>26</sup> that must extend into the cleft of the head domains. Based on this





Figure 3. (a) A schematic representation of VP4, showing the SA-binding region, the conserved trypsin cleavage sites, the  $\alpha_2\beta_1$  integrin binding sequence and the putative membrane fusion region. The two polypeptides, VP8\* and VP5\*, generated by trypsin cleavage are colored red and green, respectively. Yellow represents predicted *a*-helical regions, and blue represents predicted  $\beta$ -structures. The asterisk (\*) identifies a predicted coiled-coil region. The secondary structure predictions are based on the program Jpred, which provides a consensus of six different algorithms.<sup>32</sup> (b) A schematic model for the general topology of VP8\* (red) and VP5\* (green) for one monomer of VP4. The head domain and upper body of the spike contain epitopes for both VP8\* and VP5\* as indicated by the ovals. We propose that the head domain is primarily VP8\* in a β-sheet conformation. VP5\* is proposed to contribute additional  $\beta$ -structure to the body and primarily forms the base comprised mainly of a-helices. Both the SA-binding domain and the putative fusion domain are at the distal end of the spikes.

**Figure 2.** Montage of magnified views of the VP4 hemagglutinin of bovine rotavirus (yellow) complexed with the Fabs 7A12 (red mesh) and 2G4 (green mesh). The blue density corresponds to the outer capsid layer formed by VP7. In (a), the 264 and 7A12 difference maps were both superimposed on the spike of the native, undecorated map (shown in Figure 1(a)), and individually on perpendicular side views in (b) and (c).





Figure 4. Schematic models for budding by rotavirus through the ER membrane during particle assembly (left) and through the plasma membrane during cell entry (right). In the assembly of rotavirus (reviewed by Estes<sup>2</sup>) the immature DLP binds to a virally encoded receptor, NSP4, which is a resident integral membrane protein in the ER. Upon budding through the ER membrane, the particle becomes transiently enveloped and acquires VP7, which is also an integral membrane protein of the ER. In this process, the particle loses NSP4 and the ER membrane but retains VP7 and VP4. The mechanism by which the particle loses its transient envelope and NSP4 but selectively retains VP7 and VP4 is poorly understood. In the model for cell entry by direct membrane penetration, domains within VP8\* and VP5\* bind to receptors on the cell surface. These receptor interactions may elicit a conformational change to facilitate interaction of the VP5\* fusion domain with the lipid bilayer. Such events could bring VP7 into proximity with the lipid bilayer. The mechanism for membrane permeabilization and transfer across the bilayer is unclear. Possibly, calcium chelation by the lipid headgroups could facilitate uncoating of VP7, so that the transcriptionally competent DLP is formed during cell entry.

secondary structure analysis and our maps, we predict that the head of VP4 is formed primarily by  $\beta$ -sheets of VP8\*. This interpretation is supported by a high resolution NMR structure of a fragment of VP8\* (P. Dormitzer & S.C. Harrison, personal communication). The calculated volume of the head density only accounts for about three-

quarters of VP8\*, so a portion of the polypeptide may also contribute to the body of the spike. Since 2G4 binds to a site on VP5\* predicted to be rich in  $\beta$ -structure, we propose that VP5\* forms the body and base primarily in  $\beta$ -structure and  $\alpha$ -helical conformations, respectively (Figure 3(b)). Defining the locations of VP5\* and VP8\* within the spike, as well as epitopes within the polypeptides, provides important fiducial landmarks for docking high resolution structures that will be important for understanding the molecular mechanism of viral entry.

#### **Functional implications**

Several aspects of the rotavirus replication cycle are understood. A remarkable feature of rotavirus assembly is that immature inner capsid particles, also referred to as double-layered particle (DLPs), assemble in the viroplasm adjacent to the endoplasmic reticulum (ER), bind to the carboxy-tail of a virally-encoded ER receptor, NSP4, and then bud into the ER lumen (reviewed by Estes;<sup>2,35</sup> Figure 4(a)). In this process, the particle acquires the outer capsid protein VP7, which is also an integral membrane glycoprotein of the ER. The assembling particle is transiently enveloped and subsequently loses the membrane and NSP4.36,37 The mechanism by which the particle loses its transient envelope and NSP4 but selectively retains VP7 and VP4 is poorly understood.

As yet, the mechanism for cell entry by this nonenveloped virus is even more of a mystery (Figure 4(b)). It is known that particles with cleaved or uncleaved VP4 can bind cells and enter the cytoplasm (as opposed to cell entry of only the dsRNA segmented genome) since the DLPs possess transcriptase activity.38 Trypsin cleavage appears to stabilize the dimeric spike assembly of VP5\* and VP8\*,<sup>39</sup> which may bring critical sites into a conformation that activates cell entry. Particles can enter by receptor-mediated endocytosis 40-42 and/or direct membrane penetration.43-46 It is unclear whether membrane permeabilization occurs exclusively at the plasma membrane, within endosomes, or both. The SA-binding site on VP8\*, at the tips of the heads of VP4 (Figures 1, 2 and 3), is positioned to facilitate interactions with cell surface receptors. This event would be analogous to receptor binding by the surface proteins of enveloped viruses such as HA1 of the influenza hemagglutinin<sup>34</sup> or GP120 of HIV-1 (reviewed by Turner and Summers<sup>47</sup>). This binding event would bring VP5\* into proximity with the membrane to allow binding to secondary receptors such as integrins. By analogy with fusion of enveloped viruses<sup>48,49</sup> the putative fusion domain in VP5\* could mediate interactions of the particle with the lipid bilayer and disrupt the membrane, similar to HA2 of influenza virus34 or GP41 of HIV-1 (reviewed by Turner and Summers<sup>47</sup>). Since the permeabilization domain in VP5\* resides closer to the particle surface in the cleft of VP4 (Figures 1, 2 and 3), VP7 would be in proximity with the lipid bilayer. Interestingly, VP7 contains integrin binding

motifs and may participate in cell attachment.<sup>8,9</sup> The conformational change of VP4 upon binding the 2G4 Fab (Figure 1(c)) may mimic the changes that occur when the particle interacts with membranes. It is notable that the hydrophobic fusion domain within VP5\* is capable of permeabilizing lipid bilayers.<sup>15,50</sup> Rotavirus can also induce the fusion of cells in culture to form small syncytia.<sup>51</sup> These processes may facilitate transfer of the particle across membranes. It is also notable that VP7 is a calcium-binding protein, and the VP7 layer can be removed from particles with EDTA.<sup>52</sup> During entry, the removal of VP7 may be facilitated by chelation of calcium by the phospholipid headgroups of the lipid bilayer. Since VP4 penetrates and interacts with both VP7 and VP6 in mature particles,<sup>5,6</sup> the VP5\* domain of VP4 could not only mediate receptor binding and membrane penetration but could also serve as a tether for the DLP when VP7 is released. It is not known whether VP5\* and VP8\* are released before or after entry. However, VP7 is localized at the plasma membrane, whereas VP6 and the core proteins are present in the cytoplasm.<sup>53</sup> These observations are summarized in Figure 4. An appealing feature of this model for entry into the cytoplasm is that attachment, fusion, uncoating and entry could occur as a coordinated process, and no additional uncoating steps would be necessary after cell entry, thereby generating a transcriptionally active particle.

## **Materials and Methods**

#### Sample preparation

Rhesus, bovine and SA11 rotaviruses were grown in infected monkey kidney cells (MA104) according to Kaljot *et al.*<sup>45</sup> Monoclonal antibodies were generated in mice immunized with rotavirus.<sup>25</sup> The ascites fluid was  $\sim$ 20 mg/ml protein, and the rotavirus immunoglobulin G (IgG) comprised roughly 10-15% of the total protein.

To generate Fab fragments, 30  $\mu$ l of ascites fluid were mixed with 10  $\mu$ l TNC buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1.0 mM CaCl<sub>2</sub>) and 15  $\mu$ l of papain stock solution (500  $\mu$ l TNC buffer, 130  $\mu$ l 0.1 M EDTA, 27 mg cysteine and ~1 mg papain). The mixture was incubated at 37 °C for approximately three hours and was quenched by addition of 20  $\mu$ l of 0.25 M iodoacetamide (final concentration 60 mM).

Bovine and SA11 rotaviruses were labeled with papain-generated Fab molecules by overnight incubation at  $4 \,^{\circ}$ C with gentle rocking. The reaction mixture contained 40-50 µl of virus sample (1.0-1.3 mg/ml), 30-40 µl of quenched papain Fab solution (~8 mg protein/ml) adjusted to a total volume of 100 µl with TNC buffer.

We found that rhesus rotavirus was better decorated with 7A12 IgG compared with the Fab. (Bovine and SA11 rotaviruses tended to disassemble in the presence of papain.) Therefore, virus samples were first labeled with IgG by overnight incubation of 30-60  $\mu$ l of virus sample at 1-3 mg/ml with ~30  $\mu$ l of ascites fluid by gentle rocking at 4°C. Fab-labeled rotavirus was generated by treating the IgG-virus sample with 50  $\mu$ g/ml papain for three hours at room temperature in the presence of 10 mM cysteine and 1 mM EDTA. The reaction was then quenched with 1.5 mM iodoacetamide.

A centrifugal filtration unit (Microcon-100, Amicon, Inc.) was used to concentrate the Fab-decorated virus samples, exchange the buffer and remove excess antibodies. The ~100  $\mu$ l samples were diluted to ~500  $\mu$ l with TNC buffer and then concentrated to 20-40  $\mu$ l. The samples were again diluted with TNC and reconcentrated. The TNC wash was repeated again, and the final virus concentration was ~1 mg/ml.

#### Electron cryo-microscopy and image analysis

Aliquots of  $\sim 4 \mu l$  of virus-antibody complexes were applied to holey carbon films and frozen in ethane as described.<sup>6</sup> Low-dose electron micrographs were recorded at a nominal magnification of  $35,000 \times$  on a Philips CM120 transmission electron microscope operated at 100 kV. The grids were maintained at  $-185 \degree$ C using a Gatan 626 cryo-stage. Micrographs with minimal astigmatism and drift, as assessed by visual inspection and optical diffraction, were digitized at 21 µm intervals using a Zeiss microdensitometer, corresponding to 6.0 Å on the specimen. Particle images were extracted with the program X3D<sup>54</sup> and were processed on a DEC Alpha by common-lines and polar Fourier transform methods.55 The reconstructions of native bovine, 2G4-bovine, 2G4-SA11, 7A12-SA11 and 2G4-rhesus rotaviruses were respectively performed using 120, 132, 151, 206 and 108 particles from several micrographs in each case. The reconstructions of native bovine rotavirus, 7A12-rhesus rotavirus and 2G4-bovine rotavirus shown in Figure 1 were each recomputed with 120 particle images. Particles were selected that showed the strongest density on the perimeter of the particles, consistent with maximal antibody decoration. A restricted range of particle radii, encompassing the capsid shell only (r = 340-650 Å), was selected to optimize the search procedure. To assess the resolution of the maps, each data set was divided in half to compute two independent reconstructions. Cross correlation analysis indicated that the effective resolution limit was 26 Å, using a cut-off of 0.5. This was a conservative estimate since the final map was computed from twice as many particles. All surface-shaded representations were visualized using AVS software.56 Contour levels were chosen to include the volume occupied by the capsid shell calculated from the number of the copies and the molecular mass of VP7. Difference maps were computed by (a) radially scaling the maps based on the peak position of the VP7 shell in spherically averaged radial density profiles, (b) setting the solvent density at radii beyond the Fab position to zero, (c) multiplying the maps by a scale factor that yielded the same density for the VP7 peak, and (d) computing the arithmetic difference between the decorated and undecorated maps. Since there is no crystal structure available for Mabs against rotavirus, the atomic structure of an immunoglobulin Fab fragment (resolution 2.9 Å) of a neutralizing antibody directed against an epitope of GP41 from HIV-1 (PDB code 1NLD<sup>29</sup>) was used for visually docking into the cryo-EM density map using AVS.

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