Supporting Online Material for

Cryo-EM Model of the Bullet-Shaped Vesicular Stomatitis Virus

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CryoEM Model of the Bullet-Shaped Vesicular Stomatitis Virus

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**Material and Methods**

**Isolation and CryoEM of VSV virions**

VSV virion samples were produced as previously described, except that the inoculum was passaged multiple times in HeLa cells with a very low multiplicity of infection (MOI), 0.001, in order to suppress truncated defective-interference (DI) particles (1). We isolated full VSV particles (B-particles) in a sucrose gradient and plaque purified the final inoculum in HeLa cells. We pelleted the then cultured virions at 30,000g for 2 hours and resuspended them in phosphate buffered saline (PBS). We subjected the stock to another low speed centrifugation at 12,000 rpm (12,000g) for 5 minutes in a desktop centrifuge (Spectrafuge 16M, Labnet, Woodbridge NJ) to remove large aggregates. The resulting suspension was banded on a 10ml density gradient containing 0-50% potassium tartrate and 30-0% glycerol. The VSV-containing band was extracted using a syringe, diluted in PBS, pelleted at 30,000g for 2 hours and resuspended in PBS for cryoEM. Each aliquot of 2.5 µl of the purified sample was applied to a quantifoil 3.5/1 µm grid (Quantifoil Micro Tools mbH, Germany), blotted by filter paper, and plunged into liquid nitrogen-cooled liquid ethane to make a vitreous-ice embedded VSV sample. We recorded cryoEM images with a TVIPS 4Kx4K CCD camera, first at a magnification of 59,000x, subsequently at 98,000x, using an FEI Tecnai Polara electron microscope (Hillsborough, Oregon) operated at 300kV.

**Selection of data processing strategies and generation of the initial model**

For image processing and 3D reconstruction, we worked out an integrative approach involving combined use of B-Soft package (2), EMAN (3), IHRSR (4) and Spider (5) packages.
We manually selected a total of 1183 virion trunk segments from the 59,000x images. Each segment was sub-divided into 3 pieces based on the “90% overlap” scheme (4). For the conical tip and trunk base, 2877 particles were chosen for two-dimensional classification with the refine2d.py program of the EMAN package. We corrected each image for phase and Wiener filtered each with the B-Soft package (2) in accordance with parameters determined manually with the ctfit program in the EMAN package (3).

To determine the helical parameters, we first tried the SUPRIM(6) package to index the layer lines in the Fourier transforms of individual VSV trunk particles. However, since the particles were too short – the length of the helical trunk is less than twice its diameter – layer-line data alone was not sufficient to determine helical parameters. Therefore, we turned to the IHRSR package (4), which does not require initial knowledge of helical parameters to do helical reconstruction. However, the typical IHRSR approach that used cylindrical objects as starting models did not work.

As an alternative, we introduced our first model for IHRSR reconstruction by doing the following: We classified the “90% overlap” (4) pieces by mutual similarity with the “refine2d.py” program in the EMAN package (3). A grand-class-average was generated by iteratively aligning and averaging the class-averages using the “classalign2” program in EMAN. This grand-class-average was used to center particles and align them for the in-plane rotation (phi angle in EMAN). We then assigned a random choice from discrete azimuths (in 4° intervals from 0° to 360°) and defined 90° altitude to every particle. We classified the particles based on the assigned orientation, averaged the classes, and back-projected the averages to generate a crude 3D model. We then imposed the initial helical parameters (see below) on the crude model. We subsequently used this helically symmetrized model as the initial model in the second iteration of IHRSR refinement. We iterated for 50 rounds or until convergence.
Determination of helical parameters

We determined the helical parameters based on the following strategy: First, we
determined the pitch of the helix from the calculated layer lines (Fig. 1A, inset). Second,
we did a reference-free 2D classification of the virion-trunk images. The resulting classes
clearly showed that one N subunit sat between the shoulders of two N subunits in a
lower turn (Fig. 4A, left panels). Therefore, the helical symmetry was \((2n+1)/2\)
subunits/turn. The layer lines in the incoherently averaged Fourier transforms of the
trunk images (Fig. 1A, inset) also agreed with such helical symmetry.

Since previous literature suggested that the number of subunits/turn was around
38 (7), we scanned the parameter space by \((2n+1)/2\) subunits/turn, \(32.5 \leq n \leq 42.5\). For
each of the helical parameter sets, we ran an IHRSR refinement and checked whether
the refinement converged at its initial helical parameter. Only the cases for 32.5
subunits/turn and 37.5 units/turn did so. We also tried to count two subunits as one,
considering the possibility that two N proteins shared one P protein (8). If this had been
the case, the helical symmetry would have been reduced. For example, a 37.5
subunits/turn or \(75_2\) helix would have been reduced to 18.75 subunits/turn or a \(75_4\) helix.
When we started the refinement with 38.5/2 (19.25) subunits/turn or a \(77_4\) helix, the
refinement converged at 37.5/2 (18.75) subunits/turn or a \(75_4\) helix; when we used
32.5/2 (16.25) subunits/turn or a \(65_4\) helix as the starting parameter, the refinement
converged at 37.5/3 (12.5) subunits/turn or a \(75_6\) helix (fig. S4).

Since both \(75_4\) and \(75_6\) helixes were multiples of \(75_2\) or 37.5 subunits/turn helix,
these findings proved that 37.5 subunits/turn was the major helicity among the
population and suggested that 32.5 subunits/turn was not a stable solution. In addition,
when we tried to dock the crystal structures of M_{CTD} and N into the final reconstructions,
the structure from the 37.5 subunits/turn helicity fit well with the crystal structure (Fig. 3A), but that from the 32.5 subunits/turn helicity did not. These results demonstrate that the helicity of 37.5 subunits/turn is the true solution.

**Final structural refinement**

Further structure refinement of the trunk was done by using a modified IHRSR procedure with EMAN in place of Spider. We imposed no symmetry during the EMAN refinement by setting the symmetry option to C1 (i.e., no symmetry). At the end of each iteration, we used the `hsearch_lorentz` program from the IHRSR package to re-estimate the helical parameters and impose them on the C1 density map generated by EMAN using program `himpose`. We used only the best 76% of the particles within each class by setting the “classkeep” parameter in EMAN to 0.7.

The structure generated from the 59,000x images was found to be of 15Å resolution. We then manually selected a fresh 644 virion trunk particles from higher-resolution, 98,000x images and segmented them into 1443 pieces (~ 434,700 asymmetric units) based on the “90% overlap” scheme(4). These 1443 virion trunk segments were used to reconstruct a higher-resolution structure by following the above refinement scheme with the final reconstruction from the 59,000x data as the starting model.

The final structure was found to be of 10.6Å resolution, as determined by the 0.5 Fourier shell correlation (FSC) criterion between two reconstructions obtained by splitting the full dataset into two subsets to generate separate 3D reconstructions (fig. S2). Because the glycoprotein (G) layer was smeared out in our reconstruction, we masked off this smeared density outside the membrane with a cylindrical mask in the IHRSR package to improve the clarity of the map. We confirmed the handedness of the
helix and the identities of the components by favorable docking of the crystal structures
of N and M proteins. Docking of the N and M\textsubscript{CTD} structures to the density map was first
done interactively and then optimized using the “fit-to-model” module in Chimera (9). The
cross-correlation coefficients between these crystal structures and cryoEM density maps
were 0.70 and 0.63. As controls, we docked the M crystal structure to the N density map,
the N crystal structure to the M density map, an upside-down version of the N and M
crystal structures to the N and M density maps, producing cross-correlation coefficients
of 0.42, 0.33, 0.44, and 0.41, which indicate significantly poorer fits.

Supporting Discussions

Structure-based mutagenesis suggests roles of N-N interactions

We carried out a structure-based mutagenesis study of the N-RNA complex. We
knocked out two of the seven key interactions along the side-by-side interface of the C-
terminal lobe of N protein, one interaction in each of the two N protein mutants, R309A
and T324A. Mutating either Arg309 or Tyr324 to Ala resulted in a preference for rings
larger than the decamer observed in the wild type: In the wildtype N-RNA complex,
decamers were dominant (78\%) (Fig. 3D), but in the mutants, larger rings were more
common, decamers making up only 58\% and 11\% of the total (Fig. 3D, Supplementary
Table 1). This data suggest that removing interactions along the side-by-side interface of
the C-terminal lobe of N protein disturbs size control of N rings. Therefore, we
hypothesize that these seven interactions serve as determinants for different energy
modes for the formation of N rings of different sizes.
**Location of phosphoprotein P in the rhabdovirus**

Our cryoEM structure of the nucleocapsid N may also provide some hint about the location of the VSV phosphoprotein P. In a recent study, Green et al successfully co-crystallized the C-terminal domain of P ($P_{CTD}$) and the decamer ring of N (10). The interface between N and $P_{CTD}$ is located at the distal end of the C-terminal lobe of N. This interface faces the interior of the helical cylinder in our structure, suggesting that the P binding site of the N protein is in the central cavity. However, since the number of P in a virion is only about one-third of the number of N, and because P is an oligomer (11, 12), only a portion of all available N subunits would be occupied by P (7). As a result, P would not be expected to be resolved in our density map.

**A Rigid and Precise Helix**

It is not easy to maintain long range periodicity in anything but a crystal (4). In this regard, the VSV structure is remarkable. The virus has employed a scheme similar to that of a crystal lattice. In VSV, the M layer lattice contributes to the rigidity of the helix by restricting all degrees of freedom (stretching, twisting and bending) of a flexible helical structure. Simultaneous binding of M to two N subunits requires that the distance and the aspect angle between substrate N subunits hold constant; similarly, the two different types of distances (lateral and vertical) between neighboring M subunits are constrained in a tightly packed, helically symmetric, 2D mesh. Thus, the VSV virion seems to have established a rigid periodicity without formation of a crystal.

**Comparison between the M conformation in the virion trunk and the crystal structure (2W2R)**

Recently, the full length M has been crystallized (13). This crystal structure can be docked into our cryoEM density of M in the virion trunk (Fig. 2A). The newly resolved
loop 41-52 (in the M-hub) and its binding partner are found at the interface between M-hub contact point 4 and the tip of an M from the lower turn. Although part of this loop is outside the cryoEM density of the virion M, the residues that are critical for interaction (45-50) are encompassed by the density, demonstrating good agreement between these two independent investigations.

However, the conformation of M in its linear polymer in the crystal structure is different from that in its linear polymer in the virion in several respects (fig. S5). In the virion, the M subunits present the same face to the membrane. In the crystal structure, the linear polymer folds like an accordion, with subunits alternating in their orientation (fig S5). The longest dimension of every subunit in the virion is more nearly parallel to the axis of the polymer, whereas alternating subunits in the crystal structure tilt 55° and 180° from the axis of the polymer. The polymer in the crystal structure can be seen as a compressed or fanfolded version of its linear arrangement in the virion (fig. S5). Thus, the distance between neighboring subunits in the crystal structure (35Å) is shorter than that in the virion (58Å). The loop consisting of residues 52 to 58 in the crystal structure (11Å) is also shorter than that in the virion (17Å), all within the permissible range for a 7-residue loop (27Å).

The G trimers simultaneously bind to three neighboring M

G has been crystallized as a trimer (14, 15). We suppose that the tails of three G protomers bind three underlying M subunits simultaneously (fig. S6). In fact, the trimerization of solublized G monomers is promoted by the M-nucleocapsid complex or the M protein oligomers (half as well) but not by naked nucleocapsid (16), suggesting that this triple interaction does exist and depends on the triangular packing of M subunits in the M helix. In this way, the G trimers tack the intervening envelope membrane to the
M helix and by extension to the N helix, playing a role in maintaining virion stability and promoting membrane envelopment while budding.

**Implications for pseudotyping**

VSV is known to have a wide range of pseudotyping ability. The glycoproteins that pseudotype VSV can be divided into two groups. Some glycoproteins, like those from influenza virus, can be incorporated into VSV without modification ("direct pseudotyping"), while other glycoproteins, such as the HIV \textit{env}, cannot be incorporated without a G tail from VSV (17) ("montage pseudotyping"). We suggest above that each glycoprotein binds an M protein through its transmembrane tail, a feature that must be considered for the design of VSV-derived vaccine pseudotypes and therapeutic VSV-variants. In addition, selectivity of glycoprotein incorporation is likely the result of motif recognition between the glycoprotein tail and the M protein of VSV. For example, a sequence motif in the cytoplasmic tail has been discovered to be necessary for the Nipha virus F protein to pseudotype into VSV (18). Not surprisingly, most of the direct pseudotype glycoproteins are from NSRVs, which are genetically related to VSV (19-22).

**Implications for glycoprotein arrangement**

Local clustering of G trimers is necessary for viral entry (15). The water soluble pre-fusion form of the ectodomain of the G protein crystallizes with hexagonal packing (15, 23), so G might be supposed to concentrate on the surface of the virus in a similar manner (15). However, the lattice in the virion is more widely spaced than in the crystal, perhaps related to interactions among the extended, membrane proximal regions of G (24) that are absent in the crystal structure. In addition, we suggest that G spikes have a regular arrangement on the surface of the virion envelope in part because their membrane proximal regions have a tendency to trimerize and in part because their
transmembrane domain interacts with the underlying, triangularly-packed M lattice. The array of interactions between the G layer and the underlying M protein lattice may be used to selectively recruit and organize G and facilitate virion budding. Indeed, in synergy with other “quality control” measures, this array may exclude numbers of nucleocapsid complexes per helical turn that fail to produce binding sites in an equilateral triangular arrangement, as in the 752 helix of VSV. However, these remarks are speculative, since as a result of incompatible symmetry and/or incomplete occupancy (25), the glycoprotein (G) layer was not resolved in our reconstruction with helical symmetry.

**Online Movie Legends:**

**Movie S1**
Architectural representation of the virion trunk, rendered in 3D animation. 4 turns of M, part of the membrane bilayer, incomplete subunits, and a 30° wedge have been computationally removed. The virion trunk was first rotated around its helical axis and then around the horizontal axis to show the interior.

**Movie S2**
3D animation of the virion trunk, as seen from the central cavity. The nucleocapsid (red) and the matrix (pink) are rotating around their helical axis. The background music is from Mozart’s piano sonata in C-Major (K545).

**Movie S3**
3D animation showing the fit of the crystal structure of N (yellow ribbon) with vRNA (dark ribbon) into the cryoEM density map (semi-transparent shaded surface). The movie then
zooms into the C-lobes of N’s. The conformation of N in the virion trunk versus that in
the decamer ring is contrasted by morphing between the two structures. We notice that
the hydrogen bonds and hydrophobic interactions observed in the side-by-side interface
of N as in the decamer ring are pulled apart by ~9Å in the virion trunk.

**Movie S4**

Four adjacent M (cyan) with two N (green) subunits in the neighborhood of one M
with its M-hub in yellow. The volume is contoured at 1.0σ.

**Movie S5**

Matching between high density regions (gray blobs, contoured at 3.0σ above the
mean) in the M density map and α helices in the docked crystal structure of M\textsubscript{CTD}
(PDB:2W2R) (ribbons).

**Movie S6**

Matching between high density regions (magenta wireframe, contoured at 3.5σ
above the mean) in the N density map and α helices in the docked crystal
structure of N (PDB:2GIC) (yellow and blue ribbons).

**References and Notes:**

Supplementary Figure S1  Representative class-averages from the 2D classifications of VSV head regions, with annotations.
Top to bottom: Four selected class-average images from the 2D classification of VSV head region images. (left column) four original class averages; the number at the bottom-left corner of each class average gives the number of raw images used for the average. (middle column) the first turn of the ribonucleoprotein (RNP) is overlaid with a side projection image of the decameric RNP ring, the latter shown by itself at the top of each image; (right column) color enhanced images illustrating the protein layers of VSV: yellow: G layer; pink: outer leaflet of envelope; violet: inner leaflet; cyan: M; red: the first turn of the RNP overlaid with a side projection image of the decameric ring; green: N
Supplementary Figure S2  Fourier shell correlation plot.

The Fourier shell correlations (FSC) between even-odd divided reconstructions at all spatial frequencies are plotted against their spatial frequency values. The lowest spatial frequency that has a FSC of 0.5 is 1/10.6 Å/Angstrom, suggesting the resolution of the reconstruction is 10.6 Å.
Supplementary Figure S3

Packing of nucleoprotein (N) and RNA in the VSV virion.
The cryoEM structure of the nucleocapsid (left panel, green) extracted from the virion trunk is displayed at a threshold of 1.0 σ above the mean. The pseudo-atomic model (right panel, straw coloured) of the nucleocapsid is generated by flexibly docking the crystal structure of N (PDB:2GIC) to the cryoEM density.
Supplementary Figure S4  IHRSR convergence plots

(A) IHRSR refinement starting with 37.5 subunits/turn and measured pitch, converging at 37.5 subunits/turn. Three separate refinements were done, with the helical parameter searching applied on N layer only (N), M layer only (M) and both N and M layers (N+M). The difference in final rise per subunits among these three runs is within 0.3%. (B) IHRSR refinement starting with 38.5/2 (19.25) subunits per turn, converging at 37.5/2 (18.75) subunits per turn. (C) IHRSR refinement starting with 32.5/2 (16.25) subunits per turn, converging at 37.5/3 (12.5) subunits per turn.

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Note: The values for rise per subunit are approximate and may vary slightly due to the iterative refinement process.
Supplementary Figure S5  
Comparison of the linear polymer of M in the virion trunk and in the crystal structure of M (PDB:2W2R)

A linear polymer of six M subunits is extracted from the 3D density map of the virion trunk and shown as cyan contour. A linear polymer of six M subunits from the crystal structure of M is shown in ribbon diagram with alternating cyan and magenta colors. The orientation of these M subunits are marked by their neighboring trapezoids. As seen from the figure, the distance covered by the six M subunits in the linear polymer as in the crystal structure is shorter than that covered by their counterparts in the virion.
Supplementary Figure S6 Cartoon illustrating the attachment slot provided by three M subunits for the simultaneous binding of three cytoplasmic tails of a G-spike trimer (yellow).
Supplementary Table S1: Statistics of different ring sizes in N-RNA complexes formed by wildtype N protein and N protein mutants R309A and Y324A, observed in negatively stained TEM experiments.

<table>
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<th>Wildtype</th>
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<th>Y324A</th>
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<td>78%</td>
<td>58%</td>
<td>11%</td>
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<td>Ring Size: 11-13</td>
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Each percentage displayed in the table is the ratio of N proteins in rings of a certain ring size over total N proteins observed.