Atomic model of a nonenveloped virus reveals pH sensors for a coordinated process of cell entry

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Viruses sense environmental cues such as pH to engage in membrane interactions for cell entry during infection, but how nonenveloped viruses sense pH is largely undefined. Here, we report both high- and low-pH structures of bluetongue virus (BTV), which enters cells via a two-stage endosomal process. The receptor-binding protein VP2 possesses a zinc finger that may function to maintain VP2 in a metastable state and a conserved His866, which senses early-endosomal pH. The membrane-penetration protein VP5 has three domains: dagger, unfurling and anchoring. Notably, the β-meander motif of the anchoring domain contains a histidine cluster that can sense late-endosomal pH and also possesses four putative membrane-interaction elements. Exposing BTV to low pH detaches VP2 and dramatically refolds the dagger and unfurling domains of VP5. Our biochemical and structure-guided-mutagenesis studies support these coordinated pH-sensing mechanisms.

Entry of viruses into host cells to establish infection is a highly coordinated process. The molecular and chemical details of this process are relatively clear for enveloped viruses, such as influenza viruses, human immunodeficiency viruses, herpesviruses and flaviviruses1,2. However, the molecular details of this dynamic process and the coordination of the two outer-layer proteins are unclear in part because of the lack of atomic structures of the outer-layer proteins of BTV. To reveal how the two outer-layer proteins sense endosomal pH, how they coordinate during cell entry and how VP5 acquires membrane-penetration activity, we determined the structures of BTV under both physiological and low-pH conditions by using cryo-electron microscopy (cryoEM), revealing dramatic conformational changes induced by low pH. Atomic modeling, biochemical analyses and structure-guided mutagenesis elucidated the mechanism of the coordinated cell-entry process of BTV. This mechanism bears certain similarities to that used by some enveloped viruses and provides general insights into cell entry of large nonenveloped viruses.

RESULTS

Overall structure
We recorded cryoEM images (Supplementary Fig. 1a) of intact BTV virions with a direct electron detector operated at super-resolution counting mode and obtained a 3.5-Å-resolution structure (Fig. 1, Supplementary Fig. 1b and Supplementary Video 1) by single-particle analysis. The BTV virion contains an outer layer of 60 VP2 trimers and 120 VP5 trimers; a middle layer with 260 VP7 trimers; and an inner layer formed by 120 VP3 monomers (Fig. 1a). Each VP2 trimer binds atop four VP7 trimers (described below). Situated at a six-coordinated position of the icosahedral lattice, each VP5 trimer bridges the channel formed by six surrounding VP7 trimers (Fig. 1a and Supplementary Video 1). At 3.5-Å resolution, our cryoEM map resolves most amino acid side chains, permitting atomic modeling for both the VP2 and VP5 proteins as detailed below (Fig. 1b,c, Supplementary Videos 2–6 and Table 1). The structural features of the middle and inner layers, including amino acid side chains (Supplementary Fig. 1c,d), match the atomic structures of core proteins VP3 and VP7 solved previously by X-ray crystallography7.

Structure of the receptor-binding protein VP2
VP2 exists as triskelion-like trimers on the outermost layer of the particle. Each VP2 monomer can be divided into four domains:

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Figure 1 CryoEM reconstruction of the BTV virion. (a) CryoEM density map of the BTV virion, shown as radially colored surface representation, and a close-up view of the boxed area containing an asymmetric unit. (b,c) Close-up views of the α17 helix (b) and a β-strand of VP5 (c), showing side chain density of amino acid residues in a helix and a loop, respectively. The atomic model is shown as ribbons or sticks superimposed with the density (mesh).

hub (M1–Y49, G121–C162 and K839–V961), hairpin (D50–V120), body (L163–K190 and Y408–T838) and external tip (described below) (Fig. 2a,b and Supplementary Video 2). The hub domain contains a ten-stranded lectin-like β-barrel flanked by three helices outside, as well as both N and C termini (Fig. 2a and Supplementary Fig. 2a). Each VP2 trimer is formed through trimerization of the hub domains of three monomers and sits atop the jelly-roll domain of four VP7 trimers, one via its hub domain and three via the body domains of the three monomers (Fig. 1c and Supplementary Video 1). The interaction interface of the VP2 hub domain is highly conserved (Fig. 2b), including H866 (91.6% identity in 25 serotypes), thus suggesting a possible role of the VP2-VP7 interface in VP2 detachment in the early endosome. The hairpin domain consists of a loop, two short α-helices and a two-stranded β-sheet (Fig. 2a). The pyramid-shaped body domain has a β-sheet–rich apex and an α-helix–rich base (Fig. 2a). The apex of the pyramid connects to and interacts with the overlying tip domain.

The external tip domain contains at least four helices and a β-sheet (Fig. 2a), but its density is weaker and at a lower resolution (5–10 Å), thus suggesting flexibility. Consequently, this domain was not modeled, and its sequence was deduced to be P191–I407 only by the process of elimination. The exposed nature of this domain is consistent with its role as a key determinant of the host antibody response 8–11.

Table 1 Statistics of atomic-model refinement

|        | VP2       | VP5       |
|--------|-----------|-----------|
| Resolution | 3.5 Å     | 3.5 Å     |
| Rwork (overall) | 0.18 (40–3.5 Å) | 0.16 (40–3.5 Å) |
| Rfree (overall) | 0.14 (40–3.5 Å) | 0.14 (40–3.5 Å) |
| Rwork (highest-resolution zone) | 0.28 (4.0–3.5 Å) | 0.24 (4.0–3.5 Å) |
| Rfree (highest-resolution zone) | 0.30 (4.0–3.5 Å) | 0.21 (4.0–3.5 Å) |

Ramachandran-plot values

|                | VP2       | VP5       |
|----------------|-----------|-----------|
| Most favored   | 89.54%    | 88.66%    |
| Generously allowed | 8.42%    | 9.49%    |
| Disallowed     | 2.04%     | 1.85%     |

Because zinc fingers in some proteins are known to control conformations, and BTV VP2 does not bind nucleic acids, we speculated that the zinc-finger motif of VP2 might have a role in controlling conformational changes during cell entry. To measure the effect of Zn\(^{2+}\) binding on the conformation of VP2, we treated VP2 with Chelex-100 to remove bound divalent ions. This apo-VP2 was destabilized relative to untreated VP2, as indicated by the decrease in melting temperatures (Fig. 2c), suggesting that bound Zn\(^{2+}\) has a stabilizing role. To prevent the formation of a disulfide bond between cysteine of the CCCH motif after removal of the bound metal ion, we repeated this measurement in the presence of the reducing agent DTT. Indeed, we observed additional reduction of melting temperature (Fig. 2c), indicating further destabilization of VP2 in a reducing environment. These observations suggest that Zn\(^{2+}\) binding affects the conformation of VP2.

Our observations above offer clues as to how VP2 detaches from viral cores in the early endosome. VP2 detachment could result from disruption of the weak interaction involving the conserved H866 at the VP2-VP7 interface by high salt and low pH, and from perturbation to the zinc finger at low pH16,17. To verify that VP2 alone is pH sensitive, we subjected purified VP2 to different pH conditions. The melting temperature of VP2 changed when the pH was shifted from neutral (7.5) to early-endosomal pH (6.5–6.0), thus suggesting that VP2 has different physical properties under neutral and endosomal pH conditions (Fig. 2d). Together, our structural and biochemical data suggest a pH-sensing mechanism that involves the CCCH zinc finger, H866 and possibly other events such as receptor binding, and that enables VP2 detachment within the early endosome.

Structure of the penetration protein VP5 at high and low pH

At pH 8.8, 521 of the total 526 residues of VP5 were resolved in the 3.5-Å structure, and our atomic model of VP5 contains 19 α-helices and only two β-sheets (Fig. 3a,b, Supplementary Fig. 2b and Supplementary Video 4). This atomic model confirms the α-helix-rich nature of VP5, although the previous provisional sequence mapping18 to the observed helices was incorrect, owing to insufficient resolution to trace backbone and the differences of the secondary-structure predictions in the previous report18. Unlike any known viral fusion and penetration proteins, VP5 has a near-rectangular shape and consists of three domains: dagger (M1–S68), unfurling (K69–F354) and anchoring (I355–A526) (Fig. 3a). The dagger domain is located at the N terminus and is sequestered in the canyons between two adjacent
**Figure 2** Structure of VP2. (a) Ribbon model of the VP2 monomer with its three resolved domains: body (red), hairpin (green) and hub (blue). The tip domain is shown as density (yellow). The zinc-finger motif in VP2, a putative Zn\(^{2+}\)-binding site, is highlighted by the black box, and its density view is shown in the upper inset. The hairpin loop region interacting with VP7 and VP5 is shown in the lower inset. N and C termini are denoted by magenta and cyan balls, respectively. (b) Conservation surface of the VP2 trimer. The ribbon model (red) shows a VP7 trimer interacting with the hub domains of a VP2 trimer. The inset is a close-up view of the interface on VP2, showing two histidines (H866 and H947). Color-scale bar indicates the level of conservation from complete (blue) to partial (cyan) to none (white). (c) Effects of divalent metal on the stability of purified VP2. Fluorescence intensities as a function of temperature were measured by differential scanning fluorimetry. Inset, comparison of melting temperatures (midpoints between the upper and lower levels). Results at pH 7.5 for metal-bound VP2, apo-VP2 and apo-VP2 in the presence of DTT are shown. Error bars, s.d. (n = 3 technical replicates). A.u., arbitrary units. (d) Similar to (c), but showing the effects of pH on the stability of purified VP2. Error bars, s.d. (n = 3 technical replicates). The VP2 sequence annotated with secondary-structure elements resolved in the cryoEM map is shown in Supplementary Figure 2a.

**Figure 3** Structure of VP5. (a) Ribbon model of the VP5 monomer colored by anchoring (green), unfolding (red) and dagger (blue) domains. The stem helix (cyan ribbon) of the unfolding domain is shown with the N and C termini indicated as magenta and cyan balls, respectively. (b) Ribbon model of a VP5 trimer as viewed from outside of the BTY capsid. Color coding is as in (a). The arrow indicates the view direction in (c). (c) View of the two helices (α1 and α2) of the dagger domain from the direction indicated by the arrow in (b), showing their interactions with two adjacent VP7 trimers. The VP5 sequence annotated with secondary-structure elements resolved in the cryoEM map is shown in Supplementary Figure 2b.
helices (Fig. 3a) but also augmented by the fifth, N-terminal, β-strand (β1 in Fig. 3a) (K3–R6), thus tethering the dagger domain (Fig. 3a). Interestingly, the previously identified WHXL motif (W411–L414), responsible for membrane interactions20, is located on the extension of the β7 strand in the β-meander motif (described below).

Membrane-permeability activity of VP5 was inhibited by VP2 in an experiment using recombinant proteins of VP2 and VP5 engineered with membrane-anchoring tags6. Our atomic structure of the BTV virion reveals the interactions between VP2 and VP5 that might account for such inhibition. First, the loop of the VP2 hairpin domain contains H95 and interacts with the WHXL motif, a loop (352–357) and helix α7 of VP5 (Fig. 2a). Second, the hub domain of VP2 interacts with helices α6, α7 and α12 of the VP5 unfurling domain (Fig. 3a). Therefore, we reason that the activation of VP5’s membrane permeability activity requires the initial removal of VP2. Indeed, cryoEM of BTV under low-pH conditions (pH 3.4 and 5.5) revealed that VP2 dissociates while VP5 remains attached to the VP7 layer but refolds substantially to form a new filamentous barb-like structure that protrudes from the remaining VP5 (Fig. 4a and Supplementary Fig. 1e,f). This barb-like structure is long (~150 Å) and flexible and thus cannot be resolved in the three-dimensional (3D) density map at 9-Å resolution (Fig. 4b). When the map was filtered to lower resolution (i.e., 15-Å resolution; Fig. 4b), the base of the barb-like structure appeared at the center of the remaining VP5 trimer, thus suggesting that it is probably a filamentous trimer. Rigid-body docking of the VP5 atomic model into the remaining VP5 density indicated that the anchoring domain remains attached to the VP7 layer while both the dagger and unfurling domains refold to form the filamentous structure (Fig. 4c,d). Comparison of the orientations of the anchoring domains of the two structures indicated that low pH triggers a flower-like opening of the VP5 trimer, which is probably required to relieve restraints and create space to accommodate the simultaneous refolding of the dagger and unfurling domains (Fig. 4e,f).

Identification of low-pH sensor in VP5

After detachment of the protective VP2, VP5 must sense the low-pH environment of the late endosome with a hitherto-unknown mechanism to trigger the dramatic refolding described above. We expected the pH sensor to be composed of histidines, because each VP5 monomer contains a total of 19 histidines. Remarkably, 13 of them localize...
to the anchoring domain, and five (H272, H319, H384, H385 and H386) are clustered closely at the interface between the β-meander motif of the anchoring domain and the beam helices of the unfurling domain (Fig. 5a and Supplementary Video 6). Three of these (H272, H319 and H386) are completely conserved among all 25 known BTV serotypes, thus indicating that they may have important roles. In addition, fully conserved E316 is also located at the interface, where it interacts with the histidine cluster (Fig. 5a and Supplementary Video 6). Protonation of these histidine residues at late-endosomal pH would change their charge properties, thereby altering the interactions with each other as well as with E316. Therefore, we expect that some or all of these conserved residues could function to sense pH changes in the late endosome during cell entry.

To confirm the role of the histidines at the interface, we mutated four histidines to phenyalanines (because the serotype-specific phenyalanine substitutions seen in this region suggest that this residue is tolerated) in two domains, including (i) the buried H272 in the unfurling domain (single mutation H272F) and (ii) single (H384F), double (H384F H385F) and triple (H384F H385F H386F) histidines in the anchoring domain. Initially we introduced a single H272F mutation in the unfurling domain and a single H384F mutation in the anchoring domain into the recombinant protein. The H272F mutation substantially reduced the expression level of VP5, thus suggesting that this residue has a critical role in maintaining properly folded VP5. In contrast, the H384F mutation was well tolerated. We tested wild-type (WT) protein and the single H384F mutation in a pore-formation assay with synthetic liposomes, which mimic the composition of anionic lipids of the endosomal membrane. WT VP5 exhibited membrane-disruption activity with maximum pore formation at pH 5.5, and the effect in the single (H384F) mutation was only marginal (Fig. 5b). Alignment across serotypes of this histidine cluster displayed single substitutions of histidine to phenyalanine in the 384 and 385 positions, thus indicating that a minimum of two histidines in this region are required for sensing of low pH by VP5. Further, mutation of the conserved H386 in the replicating genome did not hinder the virus recovery by reverse genetics. These results led us to hypothesize that the net charge of this cluster may be a critical factor in VP5 function. Indeed, the pore-formation activities for the double mutant (H384F H385F), which still preserved the conserved H386) and the triple mutant (H384F H385F H386F) were reduced to only ~30% and ~17% of that of the WT VP5 at pH 5.5 (Fig. 5b).

Together, these observations suggest that the H384–H386 cluster functions as a pH sensor of VP5 and that the histidine residues in the cluster probably act cooperatively. However, further analysis is required to pinpoint the specific role of each residue in the cluster.

The VP5 structure indicates that residues H384–H386 interact with conserved residues H272, H319 and E316 in the unfurling domain (Fig. 5a and Supplementary Video 6). The proximity of their locations implies that disruption of these interactions by protonation would lead to unfolding of VP5 to expose the dagger domain for membrane interaction. Our result of the single mutation H272F also indicates that the change of interactions in this region, either by protonation or by mutagenesis, would greatly affect the stability or conformation of the VP5 protein.

Membrane-interaction elements in VP5

Our atomic structure of VP5 resolves the membrane-interaction elements previously identified by bioinformatics and biochemical analyses.
as an N-terminal peptide (M1–S41) and a WHXL lipid-interaction motif (W411–L414) usually found in SNARE proteins. M1–S41 is part of the dagger domain, which is inaccessible to the membrane at physiological pH but refolds dramatically to project outward at low pH conditions (Fig. 4a,b and Supplementary Fig. 1c,f). The WHXL motif interacts with cholesterol-rich membrane rafts and is an extension of the β7 strand of the highly conserved β-meander motif (Fig. 5c and Supplementary Fig. 3a). Moreover, the β-meander motif possesses three additional membrane-interaction elements, which are usually found in bacterial outer-membrane β-barrel proteins: a hydrophobic surface (Supplementary Fig. 3b,c) and two aromatic clusters (Fig. 5c). Indeed, prediction with BOCTOPUS identified six transmembrane β-barrel strands in the anchoring domain, two of which lie in the β-meander motif (Supplementary Fig. 3d). Conceivably, the transmembrane β-strands from multiple copies of VP5 could join together to form a transmembrane β-barrel. Interestingly, rotavirus also contains more than one membrane-interacting element. Nevertheless, the exact role of the anchoring domain and especially its β-meander motif in membrane interaction remains to be determined. One plausible role for the β-meander motif is to promote oligomerization of the unfurled VP5 in the late endosome and subsequent pore formation, as observed in the self-assembly of other β-rich structures containing aromatic clusters.

Interactions between VP2, VP5 and VP7 proteins

VP2 interacts with both VP7 and VP5 on the virion. A VP2 trimer attaches to four VP7 trimers through the bases of its hub, body and hairpin domains. The bases of three hub domains of a VP2 trimer sitting on the jelly-roll domain of a VP7 trimer and two of the histidines (H866 and H947) of VP2 are located at the interacting interface. In addition, the loop of the VP2 hairpin domain also interacts with the jelly-roll domain of the VP7 trimer (Fig. 2a).

VP2 interacts with VP5 at two regions. First, the VP2 hub domain interacts with helices α6, α7 and α12 of the VP5 trimer (Fig. 3a). Second, the loop (A89–H95) of the VP2 hairpin domain interacts with the WHXL motif, a loop (352–357) and the α7 helix of an adjacent VP5 trimer. These relatively weak interactions are consistent with VP2’s disengagement during cell entry when VP2 must dissociate to permit the conformational change of VP5.

At physiological pH, VP5 interacts with VP7 extensively through all three of its domains, whereas at endosomal pH, VP5 interacts with VP7 only through the anchoring domain, and its dagger and unfurling domains protrude out.

DISCUSSION

BTV responds to pH changes inside early and late endosomes in a coordinated manner to gain entry into the cytoplasm. The structures of BTV under high-pH (pH 8.8) and low-pH conditions (pH 3.4 and 5.5) suggest that the highly conserved zinc finger and H866 in VP2, as well as the H384–H386 cluster in VP5, might act as the pH sensors that coordinate detachment of VP2 and subsequent unfurling of VP5. Our functional analysis confirmed the essential role of the H384–H386 cluster of VP5 for its pH-dependent membrane-penetration activity, whereas H272 of VP5 is required for protein folding and/or structural stability. These data, together with previous biochemical observations, support a model of cell entry (Fig. 6). Upon entrance into the early endosome (Fig. 6, steps 1,2), VP2 senses the local pH (6.5–6.0) and detaches from the virion (Fig. 6, step 3). In the late endosome (~pH 5.5) and without VP2’s restraints, VP5 senses the lower pH and extends its unfurling and dagger domains (Fig. 6, step 5). This rearrangement allows VP5 to interact with the membrane while it remains tethered to the viral core by its anchoring domain (Fig. 6, steps 5,6), thus resulting in a membrane-enclosed virus intermediate (Fig. 6, step 6). We hypothesize that the membrane-bound VP5 undergoes further conformational changes to detach from the viral core, disrupt the membrane and consequently release the transcriptionally active core into the cytoplasm (Fig. 6, steps 7,8). Though our structural and functional data are consistent with this hypothetical model, the mechanisms of viral entry proposed in this model still need to be established or refuted. For example, what factors promote the final detachment of the refolded VP5 from the viral core? What are the functions, if any, of the membrane-interaction elements in the anchoring domain of VP5? Does the membrane penetration form a large pore across the membrane or simply disrupt it into fragments (Fig. 6, step 7) in order to translocate the viral core? Answers to these questions should further advance understanding of the mechanism of membrane penetration used by nonenveloped viruses.

Many other dsRNA viruses, notably mammalian reovirus and rotavirus, use one protein for receptor binding and another for membrane penetration; their membrane-interaction proteins are protected and must be exposed to cellular factors for membrane penetration. Though the structure of the ‘exposed’ mammalian reovirus penetration protein is unknown, the exposed penetration proteins of BTV and rotavirus both are filamentous, and their final detachment from the viral cores probably involves additional triggers. Despite these similarities, the atomic structures of the receptor-binding and membrane-penetration proteins of BTV at high pH reported here bear no similarities to those in other dsRNA viruses and thus represent an extraordinary example of mechanistic diversity among members of this family of viruses, perhaps as a result of the unique route of transmission of arbovirus through arthropod vectors. Other nonenveloped viruses use a variety of cell-entry strategies with no similarity to the BTV mechanism. For example, acidification within endosomes triggers small-RNA viruses, such as picornaviruses, to undergo ‘molecular plate-tectonic’ movement, thereby exposing myristylated internal proteins. Entry by nonenveloped DNA viruses may involve partial removal of the penton unit, as in the case of adenoviruses, or interactions with Golgi and endoplasmic reticulum components within the cell, as in the case of papillomaviruses.

Low pH–triggered cell-entry processes are well understood for enveloped viruses, as exemplified by the influenza and dengue viruses. Among nonenveloped viruses, the mechanism by which BTV enters cells (Fig. 6) bears the greatest level of similarities to that used by enveloped viruses. In a sense, VP5 is similar to some classical viral fusion proteins, such as the influenza HA2 protein, in that these proteins use histidine residues as pH sensors and undergo a dramatic conformational change at the late-endosomal pH. Along the landscape of cell-entry strategies used by nonenveloped and enveloped viruses, BTV, along with rotavirus, is therefore situated near enveloped viruses; as such, the results presented here should be of general importance to understanding of cell entry by both nonenveloped and enveloped viruses.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The atomic models and cryoEM density map have been deposited in the Protein Data Bank and Electron Microscopy Data Bank under accession numbers PDB 39D (VP2), PDB 39E (VP5), EMD-6444 (whole map, pH 8.8), EMD-6445 (whole map, pH 3.4), EMD-6239 (VP2 averaged map, pH 8.8) and EMD-6240 (VP5 averaged map, pH 8.8).
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AUTHOR CONTRIBUTIONS

Z.H.Z., P.R. and X.Z. designed the experiments. A.P. expressed proteins and performed the mutagenesis and biochemical experiments. C.C.C. and X.Y. grew Z.H.Z., P.R. and X.Z. designed the experiments. A.P. expressed proteins and performed the mutagenesis and biochemical experiments. C.C.C. and X.Y. grew Z.H.Z., P.R. and X.Z. interpreted the structures and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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CryoEM imaging. Virions were purified as previously described. To prepare cryoM grids, we applied 2 µl of purified BTV sample (pH 8.8) to thin continuous carbon films on lacy grids (Ted Pella) for 3 min, blotted for 9 s in an FEI Vitrobot with 100% humidity and then plunged into liquid ethane. CryoEM images were collected at liquid-nitrogen temperature in an FEI Titan Krios cryo electron microscope operated at 300 kV with parallel illumination. Before data collection, the microscope was carefully aligned, and beam tilt was minimized by coma-free alignment. Images were recorded on a Gatan K2 camera with super-resolution mode at a magnification of 14,000x. The magnification was calibrated as 24,140x with a catalase crystal sample, yielding a pixel size of 1.036 Å/pixel on the specimen. The dose rate of the electron beam was set to ~9 counts/physical pixel x s on the camera, and the corresponding dose reading on the electron microscope was ~2.6 e⁻/Å² x s on the specimen, owing to the miscounted electrons by the K2 camera. The image stacks were recorded at 2 frames/s for 15 s. After drift correction with the UCSF software, the first 15 frames of each image stack were merged to generate a final image with a total dose of ~20e⁻/Å² on specimen.

Data processing for BTV structure at physiological pH. From a total of 3,309 recorded micrographs, 1,630 were selected, and 12,513 images of individual particles were boxed for image processing. The underfocus values of these micrographs were determined to range between 0.8 µm and 4.5 µm with CTFFIND3. The 2x-binned data set was used in the global search, and the original data set was used in the local refinement. The orientation and center parameters of each of the 12,513 particle images were first determined by a global averaging the three VP2 and six VP5 monomers in each asymmetric unit.

The effective resolution of the whole virion was estimated to be ~3.5 Å on the basis of FSC (20,143), and the resolution for the averaged VP2 and VP5 densities was estimated to be better than 3.5 Å, on the basis of the R-factor criterion (the R factor given by Phenix reached 0.5 at 3.5 Å for both VP2 and VP5 densities) (Table 1). These estimated resolutions are consistent with the observed structural features of the density maps (Fig. 2 and Supplementary Videos 2-6). The capsid and averaged maps were filtered to the spatial frequency of 1/(3.4 Å) and sharpened with a reverse B factor of ~150 Å². This B factor was chosen with a trial-and-error method on the basis of optimization of noise level, backbone density continuity, and emergence of side chain densities. Visualization and segmentation of density maps were carried out with UCSF Chimera.

CryoEM and data processing of BTV at low pH. Virions were purified as previously described. To prepare cryoEM grids, 2 µl purified BTV sample (pH 8.8) was applied to thin continuous carbon films. After 3 min, the sample was blotted, and 2 µl citrate buffer (pH 3.4 or 5.5) was immediately applied to the grid for 30 s. The grid was blotted for 9 s in an FEI Vitrobot with 100% humidity and then plunged into liquid ethane. CryoEM images were collected in an FEI Titan Krios cryo electron microscope operated at 300 kV. For the pH 3.4 condition, a total of 350 images were recorded on a Gatan 4k × 4k CCD at a final concentration of 0.1 mg/ml VP5 and 0.1 mg/ml total lipid for liposomes. Virions were purified as described above, and the final 3D map was reconstructed from 4,503 particles at a resolution of 9.0 Å (FSC = 0.143). For the pH 5.5 condition, 159 particle images recorded on a Gatan K2 camera were used for a reconstruction at 25 Å-resolution (FSC = 0.143), which appeared identical to that of the above pH 3.4 structure filtered to the same resolution.

Atomic modeling and model refinement. On the basis of the averaged density maps of VP2 and VP5, we first built initial Cα and full-atom models for VP2 and VP5 with Coot without referring to any existing models of other proteins. The initial full-atom models were regularized by constraining Ramachandran geometry and secondary structures in Coot.

These initial full-atom models were iteratively refined with structural information of both amplitude and phase (from Fourier transformation of the cryoEM maps) in the following three steps: First, a trial-and-error method was performed in Phenix with Ramachandran restraint. The second step (automatic) was also performed in Phenix to regularize the new model. To regularize the model, hydrogen atoms were added to all atoms of the model from the last refinement, and this was followed by regularization and removal of hydrogens. The latest models were refined iteratively until no further improvement was apparent on the basis of both Ramachandran geometry and R factors. Then in the third (manual) step, amino acid residues with invalid Ramachandran backbone geometries were identified and manually corrected in Coot. This process of automatic and manual model refinement steps was iterated until no further improvement on both Ramachandran geometry and R factors was evident.

Protein expression. Both VP2 and VP5 proteins were expressed with a recombinant baculovirus system in S9 cells, which were purchased from Invitrogen (cat. no. 11496-01S; lot no. 1296885) and had been checked for mycoplasma. For both proteins, a 200-ml culture of 2 × 10⁵ cells/ml was infected at an MOI of 10. Cells were harvested by centrifugation at 3,000g 48 h (VP5) and 65 h after infection (VP2). Recombinant VP2 was expressed as a N-terminally His₆- GST–tagged fusion protein with a glycine-serine linker and a TEV cleavage site. Recombinant VP5 was expressed similarly except with an N-terminal His₆-MBP fusion. Cells were lysed by Dounce homogenization (20 strokes) in a lysis buffer (20 mM Tris, 150 mM NaCl, 20 mM imidazole, and 1% Triton X-100, pH 8.5). Lysate was subsequently clarified by centrifugation at 15,000g for 30 min. Soluble lysate was purified with an Äktara explorer FPLC column (GE Healthcare), first by immobilized metal affinity chromatography (IMAC) with a 5-mL HisTrap HP column (GE Healthcare) and second by affinity chromatography with a 1-mL GSTrap HP column (GE Healthcare). VP5 and mutants were purified in an analogous manner with a 1-mL MBPTrap HP column (GE Healthcare) used in the second affinity step. Eluted proteins were concentrated to 200 µl and cleaved with 20 µg of TEV protease (Sigma-Aldrich) overnight at 4°C. Cleaved tags and proteases were removed with IMAC by passing the cleaved protein suspension through a 1-mL HisTrap HP column (GE Healthcare) three times. Mutant proteins were produced by site-directed mutagenesis of WT expression constructs.

Differential scanning fluorimetry (DSF). DSF was performed with an MX300P Q-PCR system (Agilent Technologies) in a manner similar to that described by Neisen et al. Briefly, purified VP2 was reconstituted in DSF buffer (20 mM Tris 150 mM NaCl, pH 7.5) and used at a final concentration of 0.2 mg/ml with 5× SYPRO orange and additional additives. If stated, temperature was ramped from 25 to 95°C at 45 s/°C. For metal-binding experiments, VP2 was left untreated (metal bound) or was incubated with Chelex-100 resin (Sigma-Aldrich) for 1 h (apo). For pH experiments, protein was acidified to the stated pH values with a predetermined titration of 0.1 N HCl. Raw data were fitted to a Boltzmann distribution with Prism (GraphPad), and the derived melting midpoints (Tₘ) were plotted.

Production of calcein-loaded liposomes. 5 mg total lipid with a ratio of 4:2:1 PC/PE/PS (Avanti Polar Lipids) in chloroform was dried under vacuum to yield a solvent-free film. This was hydrated in 1 ml of calcein buffer (50 mM calcin, 100 mM NaCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄) and freeze-thawed three times. The lipid suspension was then extruded ten times through a 1-µm polycarbonate membrane filter (Whatman) with a Mini Extruder (Avanti Polar Lipids). Unencapsulated calcine was removed from the extruded suspension by size-exclusion chromatography with Sephadex G-50 resin (Sigma-Aldrich), and PBS, pH 7.5 (liposome buffer), was used as the aqueous-phase buffer. Liposomes were stored at 4°C and used within 1 week of preparation.

Pore formation assay. Purified WT and H384-6F VP5 were reconstituted in PBS, pH 7.5, containing 1 µg/ml of anti-gp64 monoclonal antibody B12DS (ref. 6) (liposome buffer). Calcein-loaded liposomes and VP5 protein were mixed in a flat black 96-well plate (Greiner) and incubated at room temperature for 10 min at a final concentration of 0.1 mg/ml VP5 and 0.1 mg/ml total lipid for liposomes.
Mixtures were then acidified to the stated pH values with a predetermined titration of 0.1 N HCl and incubated at 37 °C for 20 min. Fluorescence was then measured with a SpectraMax M5 plate reader (Molecular Devices) with top-read mode and an Ex/Em of 485/535 nm. The percentage calcein release was calculated with the formula \( R_\% = \frac{R - R_0}{R_{100} - R_0} \), where \( R_\% \) is percentage release, \( R \) is the measured fluorescence, \( R_0 \) is the measured fluorescence of liposomes acidified with buffer alone, and \( R_{100} \) is the fluorescence of liposomes with buffer containing 1% Triton X-100.

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