pH-Induced Conformational Changes of Human Bocavirus Capsids

Mengxiao Luo,^{a,*} Mario Mietzsch,^a Paul Chipman,^a Kangkang Song,^{b,c} Chen Xu,^{b,c} John Spear,^d Duncan Sousa,^d Robert McKenna,^a Maria Söderlund-Venermo,^e Mavis Agbandje-McKenna^a

^aDepartment of Biochemistry and Molecular Biology, Center for Structural Biology, McKnight Brain Institute, College of Medicine, University of Florida, Gainesville, Florida, USA

^bDepartment of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts, USA

^cCryo-EM Core Facility, University of Massachusetts Medical School, Worcester, Massachusetts, USA

^dBiological Science Imaging Resource, Department of Biological Sciences, Florida State University, Tallahassee, Florida, USA

^eDepartment of Virology, University of Helsinki, Helsinki, Finland

Mengxiao Luo and Mario Mietzsch contributed equally to this work. The author order was determined alphabetically.

ABSTRACT Human bocavirus 1 (HBoV1) and HBoV2 to -4 infect children and immunocompromised individuals, resulting in respiratory and gastrointestinal infections, respectively. Using cryo-electron microscopy and image reconstruction, the HBoV2 capsid structure was determined to 2.7-Å resolution at pH 7.4 and compared to the previously determined HBoV1, HBoV3, and HBoV4 structures. Consistent with previous findings, surface variable region III (VR-III) of the capsid protein VP3, proposed as a host tissue tropism determinant, was structurally similar among the gastrointestinal strains HBoV2 to -4, but differed from that of HBoV1 with its tropism for the respiratory tract. Toward understanding the entry and trafficking properties of these viruses, HBoV1 and HBoV2 were further analyzed as species representatives of the two HBoV tropisms. Their cell surface glycan-binding characteristics were analyzed, and capsid structures determined to 2.5- to 2.7-Å resolution at pHs 5.5 and 2.6, conditions normally encountered during infection. The data showed that glycans with terminal sialic acid, galactose, GlcNAc, or heparan sulfate moieties do not facilitate HBoV1 or HBoV2 cellular attachment. With respect to trafficking, conformational changes common to both viruses were observed under low-pH conditions localized to the VP N terminus under the 5-fold channel, in the surface loops VR-I and VR-V and specific side chain residues such as cysteines and histidines. The 5-fold conformational movements provide insight into the potential mechanism of VP N-terminal dynamics during HBoV infection, and side chain modifications highlight pH-sensitive regions of the capsid.

IMPORTANCE Human bocaviruses (HBoVs) are associated with disease in humans. However, the lack of an animal model and a versatile cell culture system to study their life cycle limits the ability to develop specific treatments or vaccines. This study presents the structure of HBoV2, at 2.7-Å resolution, determined for comparison to the existing HBoV1, HBoV3, and HBoV4 structures, to enable the molecular characterization of strain and genus-specific capsid features contributing to tissue tropism and antigenicity. Furthermore, HBoV1 and HBoV2 structures determined under acidic conditions provide insight into capsid changes associated with endosomal and gastrointestinal acidification. Structural rearrangements of the capsid VP N terminus, at the base of the 5-fold channel, demonstrate a disordering of a “basket” motif as pH decreases. These observations begin to unravel the molecular mechanism of HBoV infection and provide information for control strategies.

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Address correspondence to Mavis Agbandje-McKenna, mckenna@ufl.edu.

* Present address: Mengxiao Luo, Room 302, Dajinzhong Road, Baiyun District, Guangzhou, Guangdong, China.

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The genus *Bocaparvovirus*, belonging to the *Parvovirinae* subfamily of the *Parvoviridae* family (1), was named after the first two virus members, bovine parvovirus (BPV), reported in 1961 (2, 3), and canine minute virus (CnMV), isolated in 1967 (4). In 2005, a third member, human bocavirus 1 (HBoV1), was discovered in children (<2 years of age) suffering from acute respiratory tract infections (5). Three more human bocaviruses, HBoV2, HBoV3, and HBoV4, were subsequently found in pediatric patients with acute gastroenteritis (6–8). HBoV infections have been described worldwide in young children (7, 9–13), with high seroprevalences, up to 80% for HBoV1 and 50% for HBoV2 already at the age of 6 years (14, 15).

The HBoV genomes contain three open reading frames (ORFs) (16, 17). ORF1 encodes nonstructural protein 1 (NS1), involved in genomic DNA replication (18). ORF2 encodes a phosphorylated nucleoprotein (NP) also important for DNA replication, capsid protein expression, and interferon signaling (19–21). ORF3 encodes three overlapping viral proteins, VP1 (~74 kDa), VP2 (~64 kDa), and VP3 (~60 kDa) (16, 17), which assemble the capsid, with $T=1$ icosahedral symmetry, from 60 VPs in an approximate 1:1:10 ratio (22). The VPs are expressed from different start codons within the same transcript and therefore share a C terminus (23–25). However, 60 copies of the common VP3 are capable of assembling capsids (26). Importantly, VP1 contains a unique N-terminal region (VP1u) containing a phospholipase A2 (PLA2) domain conserved in most parvoviruses (27). Its enzymatic activity is required for the capsid to escape the endo/lysosomal pathway after entry into host cells. The structure of the VP3 common region contains two α -helices (α A and α B) and an eight-stranded anti-parallel- β -barrel (β B to β I) that forms the conserved core of the capsid. Large loops inserted between the β -strands form the exterior capsid surface (28, 29). These loops display the highest amino acid sequence variability and structural diversity among the HBoV1, HBoV3, and HBoV4 capsids and contain regions defined as variable regions (VRs) (28, 29). Characteristic features of the capsids include depressions at the icosahedral 2-fold axes and surrounding a channel at the 5-fold axes, three separate protrusions that surround the 3-fold axes, and raised capsid regions between the 2- and 5-fold axes, termed the 2-/5-fold wall (28, 29).

The HBoV capsid, as with other parvoviruses, serves as a protective coat for the packaged genome during the infection process that initiates with receptor attachment and cellular entry and involves trafficking in the endo/lysosomal pathway, nuclear entry, genome release and replication, transcription and translation, and ultimately capsid/virion assembly (30–32). For host cell recognition the capsid attaches to specific receptor(s) on the cell surface. With the exception of BPV, reported to bind glycoprotein A (GPA) with terminal α -2,3 O-linked sialic acid on erythrocytes, nothing is known about cellular entry receptor usage by other bocaviruses (33). As shown for many other parvoviruses, the capsid traffics through the endo/lysosomal pathway, to the nucleus for genome replication after cellular entry, and requires a pH gradient from the early endosome (pH 6.0) to late endosome (pH 5.5) and lysosome (pH 4.0) conditions for successful infection (34, 35). This acidification is reported to serve as a trigger for virus escape from the transport vesicles, utilizing the VP1u PLA2 enzymatic activity (31). Similar to the case with other parvoviruses, the bocavirus VP1u is postulated to become externalized through the 5-fold channel of the capsid, from the interior of the capsid, to enable its PLA2 function upon acidification of the endosomes (34, 36, 37).

In this study, the capsid structure of HBoV2 was determined to 2.7-Å resolution by cryo-electron microscopy (cryo-EM) and image reconstruction and compared to the available capsid structures of HBoV1, HBoV3, and HBoV4, determined with the same method to 2.9-, 2.8-, and 3.0-Å resolution, respectively (28). The HBoV2 structure is similar to those of HBoV3 and -4, the other enteric viruses. All four structures, determined at neutral pH, exhibit surface loop topology differences in the VRs associated with

TABLE 1 Summary of data collection, image processing parameters, and refinement statistics

Parameter	Value(s) for:				
	HBoV1		HBoV2		
	pH 2.6	pH 5.5	pH 2.6	pH 5.5	pH 7.4
Total no. of micrographs	785	711	691	927	2,921
Defocus range (μm)	0.84–4.03	0.82–3.74	0.83–4.02	0.82–4.03	0.78–4.39
Electron dose ($\text{e}^-/\text{\AA}^2$)	32	32	32	32	64
Frames/micrograph	50	50	50	50	36
Pixel size ($\text{\AA}/\text{pixel}$)	0.90	1.04	0.90	0.91	0.96
Particles used for final map	50,342	66,471	19,648	30,308	188,857
Resolution of final map ($\text{\AA}$)	2.54	2.74	2.51	2.74	2.71
PHENIX model refinement statistics					
Map CC	0.81	0.80	0.84	0.87	0.83
RMSD (bonds) ($\text{\AA}$)	0.01	0.01	0.01	0.01	0.01
RMSD (angles) ($\text{\AA}$)	0.85	0.84	0.88	0.84	0.81
All-atom clash score	11.88	10.55	9.66	8.17	10.17
Ramachandran plot					
Favored (%)	96.7	95.9	96.9	97.2	96.8
Allowed (%)	3.3	4.1	3.1	2.6	3.2
Outliers (%)	0	0	0	0.2	0
Rotamer outliers (%)	0.5	0.2	0.2	0.2	0.2
C- β deviations	0	0	0	0	0

tropism disparities (among parvoviruses), and all display a “basket” motif formed by the N termini of VP3, extending the 5-fold channel into the capsid interior. The cell surface glycan-binding properties of HBoV1 and HBoV2 were also investigated and their structures determined at pHs 5.5 and 2.6, to characterize the effect of pH acidification encountered during trafficking on the capsid structure. Column- and cell binding-based assays failed to identify potential interacting glycans either because the cell attachment receptor does not contain a glycan, the glycan recognized was not present in the array tested, or the attachment site is not present in VP3. Comparison of the structures at pHs 7.4, 5.5, and 2.6 showed analogous disordering of the basket motif under the 5-fold channel, in addition to conformational changes at several surface loops and modifications of cysteine and histidine amino acid side chains as pH drops. These changes suggest potential rearrangements required for successful virus trafficking. Significantly, capsid stability measurements showed the highest thermal stability (T_m) at pH 5.5, suggesting that concurrent stabilization and rearrangements drive the capsid transitions required for infection, including the disordering of the 5-fold basket motif, predicted to be necessary for externalization of the PLA2 function at low pH. This study provides insight into the mechanism(s) of HBoV infection and potential means to control HBoV spread.

RESULTS AND DISCUSSION

The HBoV2 structure conserves features of other bocavirus members. HBoV2 VP3 virus-like particles (VLPs) were generated using the baculovirus/Sf9 expression system and were produced and purified as previously reported (28) (see Materials and Methods) in quantities and quality suitable for structural studies (data not shown). The structure of HBoV2 at pH 7.4 was determined from a total of 188,857 capsid images, extracted from 2,921 micrographs. The individual capsid images were combined to reconstruct the capsid structure to 2.7- $\text{\AA}$ resolution (Fourier shell correlation [FSC]=0.143 [Table 1]). The HBoV2 capsid density map exhibits surface capsid topology features shared by other known *Parvovirinae* subfamily members, including a

FIG 1 The HBoV2 capsid structure. (A) The reconstructed capsid surface map colored according to radial distance from the capsid center (blue) to the outermost regions (red) as indicated by the scale bar. The positions of the 5-fold channel (5f), 3-fold (3f) and 2-fold (2f) axes, and 2-/5-fold wall are indicated. (B) Cross-sectional view of the capsid. Locations of the 5-fold channel are indicated by arrowheads. Density coloring is as in panel A. (C) Example of fitted amino acid residues, W94 to F99, in the β C strand. The density map is depicted as a black mesh. The VP model is shown in stick representation, and the atoms are colored as follows: C, yellow; O, red, and N, blue. (D) Closeup of a cross-sectional view of the “basket” under the 5-fold channel (refer to panel B). The ribbon diagram of VP3 is shown within the semitransparent density. The first ordered residue, G33, is labeled. This figure was generated using UCSF-Chimera (63).

depressed region at the 2-fold axes, three protrusions surrounding the 3-fold axes, a cylindrical channel at the 5-fold axes, surrounded by a wide depressed region, and a “wall” between the 2- and 5-fold axes (22, 31) (Fig. 1A). A cross-sectional view of the HBoV2 capsid showed a “basket” beneath the 5-fold axes, a structural feature observed for BPV (29), HBoV1, HBoV3, and HBoV4 (28) (Fig. 1B). The HBoV2 amino acid side chains were well ordered in the core secondary structure (Fig. 1C) and most surface loop regions, enabling most of VP3 to be built. Exceptions were missing side chain densities for some residues at the apexes of some surface loops and acidic residues known to be most susceptible to radiation damage in cryo-EM (38). Furthermore, the first 32 residues of VP3 could not be built into the density map due to lack of ordering of the N terminus. Similar to the case with the other HBoV structures (28), the first ordered N-terminal residue of VP3, residue 33, is located under the 5-fold channel projecting into the basket density extending into the interior of the capsid (Fig. 1D). The refined model, from residue 33 to the C-terminal residue 542, has a coefficient of correlation (CC) of 0.83 to the reconstructed density map. The refinement statistics for the model are summarized in Table 1. The VP3 structure of HBoV2 displays the conserved core features of other parvoviruses (29), including an eight-stranded anti-parallel β -barrel (β B to β I) that is organized in two β -sheets, BIDG and CHEF, with an additional β A strand that is antiparallel to β B and an α -helix (α A) between β B and β C (Fig. 2A). In addition, a second α -helix (α B) exists in the HBoV2 VP3 structure located in one of the surface loops between the β E and β F (Fig. 2A), similar to other known bocavirus structures (28, 29).

Comparison of HBoV VP3 structures at pH 7.4 supports a role for VR-III as a tropism determinant. The HBoV VPs have an amino acid sequence identity that ranges from 77% to 90% (7). Structural comparison of the different HBoV capsid structures showed that their core regions are nearly perfectly superposable (Fig. 2B). Furthermore, several VRs exhibit similar conformations, including VR-II, -VI, -VII, -VIII, and -IX (Fig. 2B). The remaining VRs, VR-I, VR-III, VR-IV, VR-V, and VR-VIII, showed differences, with VR-III and -VIII differing in HBoV1 due to a 4-amino-acid (aa) insertion and

FIG 2 HBoV2 (pH 7.4) VP structure. (A) Ribbon diagram of HBoV2 VP structure. The conserved β -barrel core motif (βB to βI), βA strand, and αA and αB helices are indicated. The N and C termini are labeled. The loops inserted between these secondary-structure elements also contain β -strand regions, as indicated. (B) Structural superposition of HBoV1 (blue), HBoV2 (orange), HBoV3 (green), and HBoV4 (red), with the locations of variable loops VR-I to -IX labeled. The approximate icosahedral 2-, 3-, and 5-fold axes are indicated as a filled oval, triangle, and pentagon, respectively. The images were generated with PyMOL (73).

an alternative conformation, respectively, and VR-IV differing in HBoV4 due to a 2-aa insertion as previously reported (28). VR-I and VR-V differed among all HBoV strains despite the lack of insertions or deletions. Overall, the HBoV2 VRs resemble those of HBoV3 with a minor difference in VR-V (Fig. 2B), with an overall $C\alpha$ root mean square deviation (RMSD) of 0.66 Å for their superposed VPs. HBoV4 displays structural differences from HBoV2 in VR-I, -IV, and -V (Fig. 2B) and is the next most structurally similar, with an overall VP $C\alpha$ RMSD of 0.78 Å. HBoV1 has the lowest sequence similarity and a different tissue tropism from the gastrointestinal HBoVs and also exhibits the greatest VP $C\alpha$ RMSD, 1.13 Å, compared to HBoV2. Previously, it was postulated that VR-III, displaying the highest structural difference between the respiratory and the gastrointestinal HBoVs, might be a determinant for tissue tropism (28). HBoV2 conserves the structure of this loop in the gastrointestinal HBoVs. In contrast to the other surface loops, the amino acid sequence of VR-III, residues 196-IHELAEMED(A/S)NAVEKAI-212, is highly conserved among HBoV2, HBoV3, and HBoV4. This further highlights the potential importance of VR-III for tissue tropism. VR-III is located at the 2-/5-fold wall of the capsid, where other parvoviruses are also known to bind to glycan receptors, e.g., adeno-associated virus 1 (AAV1) to sialic acid (39) or AAV9 to terminal galactose (40). This capsid region and VR-I, VR-IV, VR-V, and VR-VIII also serve as dominant antigenic regions (41). HBoV-based vectors are currently being developed as vectors for gene therapy applications (42). The structure information for HBoV2, along with that already available for the other HBoV strains (28), will assist future engineering of vectors to reduce the detrimental effects of preexisting immunity and to improve or alter the tropism as recently shown for HBoV1 (43).

HBoV1 and HBoV2 VP3 VLPs do not bind common cell surface glycans. To date, no viral entry receptors have been identified for any of the HBoVs. To characterize whether the HBoVs use glycans as receptors, HBoV1 and HBoV2, the two most widely

FIG 3 Glycan binding analysis of HBoV1 and HBoV2. (A) SDS-PAGE of heparin binding assay fractions for AAV2, AAV5, HBoV1, and HBoV2 VLPs. FT, flowthrough; W1 to W5, wash fractions; E1 to E5, elution fractions. (B) SDS-PAGE of labeled HBoV1, HBoV2, AAV2, and AAV5 VLPs. Gels on the left stained for proteins show the presence of VP3 of HBoV1 and HBoV2 (~60 kDa) as well as VP1 (~87 kDa), VP2 (~72 kDa), and VP3 (~62 kDa) of AAV2 and AAV5 (AAV5 VP1 is at a low concentration). Gels on the right were imaged under UV light to confirm successful fluorescent capsid labeling. (C) Cell binding assay analysis of fluorescently labeled VLPs incubated with the indicated cell lines. Results show the percentage of cells bound by the fluorescently labeled capsids and are displayed as means $\pm$ standard deviations ($n=3$).

distributed HBoVs representing the two HBoV tropisms and disease phenotypes (5, 7), were analyzed. Heparin binding assays, to mimic heparan sulfate proteoglycan (HSPG) interactions, utilizing VLPs were performed (Fig. 3A) in addition to cell-binding assays with fluorescently labeled VLPs (Fig. 3B). AAV2 VLPs, known to bind HSPG (44), and AAV5 VLPs, which do not bind to HSPGs but bind to terminal sialic acid (45), were used as controls. In the heparin binding assays, AAV2 VLPs appeared in the elution fraction, consistent with binding, while AAV5 and the HBoV1 and HBoV2 VLPs were detected in the wash and flowthrough fractions, indicating lack of binding (Fig. 3A). Cell binding assays, to analyze binding to terminal sialic acid, galactose, or *N*-acetylglucosamine, utilized differential glycan-presenting CHO cell lines. The parental CHO-Pro5 cell line displays terminal sialic acid, the mutant Lec2 cell line displays terminal galactose, and the Lec8 cell line displays *N*-acetylglucosamine, resulting from mutations in specific genes required for glycan biosynthesis (46). AAV5, which is known to bind to sialic acid (45), only bound to Pro5 cells, whereas AAV2, which binds to HSPG (44) and does not require any of the glycans affected by the mutations of the CHO cell lines, bound to all three cell lines with approximately equal efficiencies (Fig. 3C). In contrast, both HBoV1 and HBoV2 showed no significant binding to any of the three cell lines, indicating that they do not use terminal sialic acid, galactose, or *N*-acetylglucosamine as receptors (Fig. 3C). In addition, a series of colon cell lines, HT-29, CaCo-2, and CMT-93 and the lung cell line A549, were analyzed (Fig. 3C). While AAV2-VLPs bound efficiently to all these cells, AAV5 showed weak to medium binding phenotypes and both HBoV1 and HBoV2 showed no significant binding (Fig. 3C). These observations indicate that these viruses might require additional cofactors for cellular attachment, such as bile salts or microbial molecules as described recently for noroviruses (47). It could also be that

FIG 4 HBoV capsids under different pH conditions. (A and B) Negative-stain EM analysis of HBoV1 (A) HBoV2 (B) capsids dialyzed into the indicated pH. The sample labeled “pH2.6>7.4” was dialyzed to pH 2.6 and then subsequently redialyzed to pH 7.4. (C) Capsid stability analysis using differential scanning fluorimetry. The melting temperatures (T_m s) of HBoV1, HBoV2, and AAV5 capsids were determined at pHs 2.6, 4.0, 5.5, 6.0, and 7.4. Results are displayed as means $\pm$ standard deviations ($n = 3$).

they do not utilize glycans for cellular entry or that, unlike AAV2 and AAV5 (48, 49), VP3 alone does not contain the glycan binding determinant and VP1 is required, as reported for B19 (50). Future studies analyzing capsids containing VP1, VP2, and VP3 will address this possibility.

HBoV1 and HBoV2 capsids reveal strain-specific as well as common phenotypes in pH environments associated with infection. During infection and trafficking, HBoVs encounter different cellular environments. HBoV1 is exposed to the mild acidic pH conditions in the respiratory tract ranging from pH 5.5 to pH 6.7 (51). In contrast, HBoV2 to HBoV4, associated with gastrointestinal infections, are initially exposed to the acidic extracellular environment of the stomach, ranging from pH 1.5 to pH 3.5 (52). While experiencing these varied extracellular pH conditions, the capsids have to remain structurally intact to infect cells. In addition, the HBoVs all also experience varied pH conditions after cellular internalization while trafficking through the early (pH 6) and late (pH 5.5) endosome, lysosome (pH 4), and then nucleus (pH 7.4). In order to confirm that HBoV1 and HBoV2 capsids are indeed intact under these conditions, the VLPs were dialyzed into different pH environments and analyzed by negative-stain EM. The result showed that the capsids were visually intact at all the pHs tested, even with multiple changes of pH conditions (from pH 7.4 to pH 2.6 and back to pH 7.4) (Fig. 4A and B).

To further analyze the stability of the capsids at the different pH environments, the melting temperatures (T_m s) of the HBoV VLPs were determined by differential scanning fluorimetry (DSF) with previously characterized AAV5 VLPs for direct comparison. The T_m of AAV5 at pH 7.4 has been reported to be $89.0 \pm 0.1^\circ\text{C}$ (in universal buffer; see Materials and Methods for ingredients) (53) and confirmed in this study (T_m of 89.5°C [Fig. 4C]). Under lower-pH conditions the T_m of AAV5 gradually decreases: 89.5°C at pH 7.4, 88.0°C at pH 6.0, 80.5°C at pH 5.5, 78.0°C at pH 4.0, and 71.5°C at pH 2.6. The

thermal stability of AAV5 capsids has also been analyzed in citric phosphate buffer at pHs 7.4, 6.0, 5.5, and 4.0, with comparable results (34). The thermal stability profile of HBoV2 followed a trend similar to that of AAV5, but at lower temperatures, with the T_m decreasing with pH: 73.2°C at pH 7.4, 70.3°C at pH 6.0, 70.0°C at pH 5.5, 67.8°C at pH 4.0, and 43.5°C at pH 2.6 (Fig. 4C). The profile for HBoV1 differed in that its T_m increased as pH dropped and then decreased again: 65.3°C at pH 7.4, 68.7°C at pH 6.0, 69.7°C at pH 5.5, and then 67.0°C at pH 4.0 and 41.3°C at pH 2.6. A similar trend was previously observed for AAV1, AAV2, and AAV8 (34). The capsid stability is reversible, for example, when dialyzed from pH 2.6 to pH 7.4 (Fig. 4C).

The T_m s of the two HBoVs differed most at pH 7.4, by ~8°C, and differed by only ~2°C from pH 6 to pH 2.6, with HBoV2 being the most stable. The difference at pH 7.4 is consistent with the utility of T_m for identifying AAV serotypes (53) and suggests that the HBoVs can be similarly differentiated by T_m . Notably, the VP1u and VP1/2 common regions of VP1 and VP2 do not contribute to AAV capsid stability, and this phenotype is likely the same for the HBoVs. Despite the lack of minor VPs in the HBoV VLPs, the maintained stability of the capsids at pHs 6.0 and 5.5 is consistent with the need to preserve integrity under the conditions at which the PLA₂ domain within the VP1u is proposed to be externalized for endo/lysosomal escape (54). The reversibility of the pH effects on the capsid, from a destabilized low-pH environment to pH 7.4, provides insight into the requirements to escape into the cytoplasm and travel into the nucleus while protecting the genome.

The capsid structures of HBoV1 and HBoV2 at pHs 2.6 and 5.5 reveal capsid dynamics associated with endo/lysosomal escape. To further analyze the observed contrasting capsid stabilities at pHs 2.6 and 5.5, the structures of HBoV1 and HBoV2 were determined at these pHs to identify any associated structural differences. VLPs were dialyzed to the two pH conditions and vitrified on EM grids for data collection. For HBoV1, 50,342 capsids were selected at pH 2.6 and 66,471 capsids at pH 5.5, resulting in three-dimensional (3D) reconstructed capsid structures at 2.54- and 2.74-Å resolution, respectively (Table 1). Similarly, for HBoV2, 19,648 and 30,308 capsids were selected at pH 2.6 and pH 5.5 for 3D reconstructed capsid structures at 2.51- and 2.74-Å resolution, respectively (Table 1).

Comparison of the HBoV1 and HBoV2 maps at pHs 2.6 and 5.5 with the previously published pH 7.4 HBoV1 map (28) and the map of HBoV2 described above showed minor structural differences on the capsid exterior surface but significant movement of the VP3 N terminus (Fig. 5 to 7). The capsid surface differences are located at the 2-/5-fold wall (VR-I) and 3-fold protrusions (VR-V) of the HBoV1 and HBoV2 capsids at pH 2.6 compared to pHs 7.4 and 5.5 (Fig. 6), with HBoV2 showing additional density at the 3-fold symmetry axis at pH 2.6 (Fig. 5). On the capsid interior, the “basket” structure beneath the 5-fold axis at pH 7.4 becomes less pronounced at pH 2.6, suggesting that low pH induces movements of the VP3 N terminus for both HBoV1 and HBoV2 (Fig. 5). Interestingly, low-resolution cryo-EM structures of both capsids returned to pH 7.4 from pH 2.6 showed the reappearance of the basket structure and loss of the 3-fold density in HBoV2 (data not shown), implying that these structural observations are reversible. This would be consistent with the requirement to maintain capsid integrity following VP1u externalization to protect the encapsulated genome en route to the nucleus for replication.

Similar to the case with pH 7.4, the low-pH structures were ordered from residue 33 to the C terminus, residue 542 in HBoV1 and 537 in HBoV2. The CCs for the models fitted to their respective maps ranged from 0.80 to 0.87 (Table 1). The RMSDs for HBoV1 are 0.6 Å (pH 5.5) and 0.7 Å (pH 2.6) for C α atoms compared to the pH 7.4 structure. As reported previously, two regions of the HBoV1 pH 7.4 map, residues 204 to 209 (VR-III) and 333 to 338 (VR-V), were poorly ordered, and only the main chain was interpreted. Similarly, the HBoV1 map at pH 2.6 displayed disordered density in these loops and as well as residues 78 to 84 (VR-I) (Fig. 6A and C). Despite the high level of disorder in these loops, the main chain was interpretable (Fig. 6A and C). In contrast to the pH 2.6 and pH 7.4 HBoV1 maps, the pH 5.5 density was better ordered in these

FIG 5 Capsid density maps of HBoV1 and HBoV2. HBoV1 structures at pHs 7.4, 5.5, and 2.6 are shown on the left-hand side. Similar pH structures for HBoV2 are shown on the right-hand side. The reconstructed maps are colored according to radial distance (blue to red), as indicated by the scale bar at the bottom. Shown are surface and cross-sectional views of HBoV1 and -2 under the different pH conditions indicated. The images were generated with UCSF-Chimera (63).

loops, with most side chain densities observed (Fig. 6A and C). Overall, the HBoV1 VP structure topologies were similar under all three pH conditions analyzed except for conformational shifts in VR-I and VR-V (Fig. 6E). These structural shifts were more pronounced for pH 2.6, with main chain movements of ~ 9 Å in both VR-I and VR-V compared to pH 7.4 and ~ 3 Å in VR-I and ~ 7 Å in VR-V between pHs 5.5 and 7.4. These observations identify the 2-/5-fold wall and 3-fold protrusions of the capsid as being involved in the capsid dynamics associated with low-pH-mediated transitions required for infection.

For the HBoV2 pH 5.5 and 2.6 structures the overall RMSDs for their VPs superposed onto the pH 7.4 coordinates are 0.4 and 0.7 Å, respectively, for $C\alpha$ atoms. The HBoV2 surface loops were generally more ordered at pHs 7.4 and 5.5 than those of HBoV1 (Fig. 6A to D). At pH 2.6, VR-V was slightly disordered and only the main chain was interpreted (Fig. 6D). Similar to the case with HBoV1, the overall capsid structure was maintained with low-pH-dependent shifts of the main chain observed in VR-I and VR-V. These shifts are also more pronounced at pH 2.6, with main-chain movements of ~ 4 Å in VR-I and ~ 6 Å in VR-V compared to pH 7.4 and < 2 Å in VR-I and < 3 Å in VR-V at pH 5.5 compared to pH 7.4. Thus, the HBoV2 capsid is susceptible to reduced pH-induced conformational changes compared to those for HBoV1. This could be an adaptation to the potential fecal-oral transmission route involving the acidic conditions of the stomach. In addition, the pH-sensitive regions of the HBoV capsids, VR-I and VR-V, located at the side of the 2-/5-fold wall and 3-fold protrusions, is known to bind to various receptors in other parvoviruses (39, 40, 55, 56). The pH-induced movement of these loops may enable the capsid to detach from its receptor after cell entry and acidification of the endosomes.

The HBoV VP N terminus experiences pH-induced conformational changes. The basket-like structure of the HBoVs under the 5-fold channel (Fig. 1D) is formed by the

FIG 6 VP structures of HBoV1 and HBoV2 at various pHs. (A) Conformations of VR-I in HBoV1 at pHs 7.4, 5.5, and 2.6, shown as coil diagrams. Amino acid side chains are shown within the corresponding density map for each pH condition. (B) Conformations of VR-I in HBoV2 shown as in panel A. (C and D) Conformations of VR-V in HBoV1 (C) and HBoV2 (D) presented as in (A). (E and F) Superpositions of the HBoV1 (E) and HBoV2 (F) VP structures under the different pH conditions. The positions of VR-I to VR-IX and the N and C termini are labeled. The images were generated with UCSF-Chimera (63).

glycine-rich N terminus of VP3 (28). The high number of glycine residues grouped at the N terminus of the major VP is a common element among the parvoviruses (22). However, among the parvoviral structures determined to date, only the bocaviruses (28, 29), AAV5 in complex with an antibody (57), and *Penaeus monodon* metallothionein (58) display this feature. The clustering of glycines is believed to provide flexibility to the N terminus for the externalization of the VP1u region with its PLA2 domain through the 5-fold channel. The presence of the basket could thus be thought to prevent premature externalization, acting as a “cork” to the 5-fold channel, or as the region positioned to initiate the process. However, the basket is still present at pH 5.5 (Fig. 5 and 7), the condition encountered by capsids during endosomal acidification, and is thought to be associated with the externalization of the VP1u region. At pH 2.6 the basket under the 5-fold channel disappears, with density observed leading away from the 5-fold channel, on the capsid interior surface, interpreted as aa 24 to 29 of the VP3 N terminus (Fig. 5 and 7). This density runs antiparallel to the β G strand in both HBoV1 and HBoV2 (Fig. 7B and D) and possesses several glycines on both ends. It is possible that this movement away from the 5-fold channel (Fig. 7E and F) is a mechanism to prevent premature externalization of VP1u or that externalization is via a different portal, for example, the 2-fold axis. However, these studies were performed on HBoV VP3-only VLPs. The wild-type capsid contains, on average, 50 copies of VP3, 5 of VP1, and 5 of VP2, with extended N termini of 129 and 35 aa, respectively. Due to the incompatibility of the number of VP1 and VP2 with icosahedral symmetry (imposed

FIG 7 The structures of the N termini of HBoV1 and HBoV2 at various pHs. (A) Cross-sectional views of the HBoV1 density maps and fitted models shown as ribbon diagrams for each pH condition (pH 7.4, blue; pH 5.5, yellow-orange; and pH 2.6, red) studied. The 5-fold symmetry axis, glycine 33, and β -strand G are indicated. (B) The amino acids of the HBoV1 N terminus and β -strand G are shown in stick representation inside the pH 2.6 mesh density map. (C and D) Cross-sectional views of HBoV2 as in panel A (C) and the amino acids of the HBoV2 N terminus and β -strand G inside the pH 2.6 mesh density map as in panel B (D). (E and F) Representation of the interior surface of the HBoV1 (E) and HBoV2 (F) capsids from a 9-mer at a pH of 2.6. At this condition, the N terminus is extending away from the 5-fold channel. The N-terminal residues, aa 25 to 32, are colored red and the last N-terminal residue ordered, amino acid 24, is colored blue. The asymmetric unit, with the 2- and 3-fold symmetry axes, is indicated. Panels A to D were generated with UCSF-Chimera (63) and panels E and F with PyMOL (73).

during structure determination), their structures would also be absent in the density map of wild-type capsids (22). Nonetheless, in future studies, it would be interesting to see whether VP1 and VP2 have an effect on the basket structure under the 5-fold channel.

pH-induced residue level changes targets cysteines and histidines. In addition to the structural shifts described above, a number of side chain rotamer changes and unassigned densities proximal to side chains were observed in the HBoV1 and HBoV2 pH 2.6 and 5.5 maps compared to pH 7.4 (Fig. 8). The amino acids affected the most

FIG 8 Modification of cysteines under low-pH conditions. (A) HBoV1 and HBoV2 model and density map (black mesh) are shown at pHs 7.4, 5.5, and 2.6. Additional density relative to pH 7.4 is indicated as a red mesh. Density meshes are contoured at a σ -level threshold of 2.0. The additional cysteine density for Cys159 causes F243/239 to flip at pHs 5.5 and 2.6, indicated by a red arrow. (B) Similar depiction (as in panel A) for cysteines with additional densities exclusively at pH 2.6. In HBoV2 C393 causes extra density at the center of the 3-fold symmetry axis. The view is rotated by 90° without density mesh for the VP3 amino acids. The images were generated with UCSF-Chimera (63). (C and D) Position of cysteines on the exterior and interior surface of the HBoV1 (C) and HBoV2 (D) capsids. Red labeling of cysteines indicates that these possess additional densities, whereas cysteines in blue display no additional densities. The images were generated with PyMOL (73).

are cysteines and histidines. The HBoV1 and HBoV2 VP3 contain a total of 10 and 8 cysteines, respectively. None of these are in close enough proximity to form disulfide bonds. However, in HBoV1, at pHs 5.5 and 2.6, two and eight cysteines, respectively, exhibited modifications or additional density, not consistent with the cysteine side chain alone, as seen at pH 7.4 (Fig. 8A and B; Table 2). The same observation was made for HBoV2, with three cysteines at pH 5.5 and five at pH 2.6 exhibiting additional density (Fig. 8A and B; Table 2). In some cases, the additional density on the cysteines caused steric clashes with neighboring amino acids, resulting in conformational shifts of amino acid side chains. For example, conserved C159's modification flips the orientation of a nearby phenylalanine (F234 in HBoV1 and F239 in HBoV2) at pHs 5.5 and 2.6 (Fig. 8A). Generally, conserved cysteines showed modifications in both HBoVs except for C473 in HBoV2, which did not show any additional densities, unlike structurally equivalent C477 in HBoV1 (Fig. 8B). This density extends to nearby C104, not conserved in HBoV2 (Table 2), and M177 in HBoV1. Conversely, there were also unique cysteine densities in HBoV2, e.g., C393 (valine in HBoV1), modified by additional density. This cysteine is located near the 3-fold symmetry axis (Fig. 8B). The three symmetry-related cysteines created the large extra density seen at the center of the 3-fold axis (Fig. 5 and 8B). Generally, the cysteines with modifications in the HBoV1 and HBoV2 capsids are located on either the exterior or interior surface of the capsid (Fig. 8C and D) and are located in hydrophobic environments. In contrast, unmodified cysteines are either buried or situated in less hydrophobic regions (data not shown).

The cysteine modification phenomenon appears to be independent of the buffer used, because glycine HCl was used at pH 2.6 and universal buffer at pH 5.5 (see

TABLE 2 Summary of observed modification of side chain densities^a

HBoV1 residue	Extra density			Comment	HBoV2 residue	Extra density			Comment
	pH 7.4	pH 5.5	pH 2.6			pH 7.4	pH 5.5	pH 2.6	
C89	No	No	Yes		C89	No	Yes	Yes	Causes flip of K262
C104	No	No	Yes	Density to C477, M177	S104	No	No	No	
C159	No	Yes	Yes	Causes flip of F243	C159	No	Yes	Yes	Causes flip of F239
C247	No	Yes	Yes		C243	No	Yes	Yes	
C322	No	No	Yes		V318	No	No	No	
C347	No	No	Yes		S343	No	No	No	
V397	No	No	No		C393	No	No	Yes	Density at 3-fold
C421	No	No	Yes	Density towards H170	C417	No	Weak	Yes	Density towards H170
C471	No	No	No		C467	No	No	No	
C477	No	No	Yes	Density to C104, M177	C473	No	No	No	
C531	No	No	No		C527	No	No	No	
H48	No	No	No		Y48	No	No	No	
H70	No	No	Yes		H70	No	No	Yes	Flip of K450/Y462 nearby
H105	Yes	Yes	Yes	Coordinated ion?	H105	Yes	Yes	Yes	Coordinated ion?
Q127	No	No	No		H127	No	No	Yes	Shift of K129 nearby
H156	No	No	Yes	Paired with H230	H156	No	Yes	Yes	Paired with H226
H163	No	No	Yes	Paired with H443	H163	Yes	Yes	Yes	Paired with H439
H170	No	Weak	Yes	Density towards C421	H170	No	Weak	Yes	Density towards C417
N197	No	No	No		H197	No	No	No	
H230	No	No	Yes	Paired with H156	H226	Yes	Yes	Yes	Paired with H156
N278	No	No	No		H274	No	No	—	
H329	—	No	—		A325	No	No	No	
H429	No	No	Yes		N425	No	No	No	
H443	No	No	Yes	Paired with H163	H439	Yes	Yes	Yes	Paired with H163
H497	No	No	Yes		H493	No	No	Yes	
H515	No	No	No		H511	No	No	No	

^aEquivalent amino acids that are not cysteines or histidines in the other HBoV are shaded. "Yes" results are in bold. —, local resolution was not sufficient to determine whether extra density is present.

Materials and Methods for ingredients). It is important to note that pH-induced cysteine modifications have not been previously described. Deposited EM maps in the Electron Microscopy Data Bank (EMDB) mostly described protein structures at neutral pH and the few maps at lower pH do not possess the necessary resolution required for this level of comparison, with one exception. EMD-4063 (PDB identifier [ID] [5LK7](#)) is the structure of the slow bee paralysis virus virion at pH 5.5 (in sodium acetate buffer) at a resolution of 3.42 Å. This structure contains five cysteines. While not discussed in the publication (74), two of these cysteines, Cys117 and Cys241 in chain A, show modifications similar to those seen in the HBoV maps. The fact that these additional densities were not observed at pH 7.4 indicates that the low pH may be responsible through the reaction of glycine or acetate ions with the thiol group. In future studies, mass spectrometry analyses may clarify the nature of these cysteine modifications. A low-resolution structure of HBoV2 determined from a sample dialyzed to 2.6 and then back to pH 7.4 did not show the extra density at the center of the 3-fold axis. This indicates that these modifications are reversible. The biological significance of these cysteines and their modifications needs to be evaluated, especially given that Cys159 and Cys247/243, showing these densities at pHs 2.6 and 5.5 (Fig. 8A; Table 2), are conserved in all HBoVs (28).

HBoV1 and HBoV2 both contain 12 histidines within their VP3 sequences (Table 2). Among these, there are two conserved pairs with continuous density between them: H156:H230/226 and H163:H443/439 for HBoV1 and HBoV2, respectively (Fig. 9A; Table 2). At pH 2.6, additional density occurs between the H156:H230/226 pair in HBoV1 and HBoV2, but not between the H163:H443/439 pair (Fig. 9A). These "joined" histidine pairs are connected within the same VP monomer and are conserved in all HBoVs. In addition, a series of "single" histidines also exhibited modifications at pH 2.6 (Table 2). Conserved histidines in both HBoVs behaved identically, with either both having

FIG 9 Modification of histidines under low-pH conditions. (A) HBoV1 and HBoV2 paired histidines and density (black mesh) are shown at pHs 7.4, 5.5, and 2.6. Additional density relative to pH 7.4 is indicated as a red density mesh. Density meshes are shown at a σ -level threshold of 2.0. The movement of H230 is indicated by a red arrow. (B) Similar depiction (as in panel A) for single histidines. The conformational change to Y462 at pH 2.6 is indicated by a red arrow. The images were generated with UCSF-Chimera (63). (C and D) Positions of histidines on the exterior and interior surface of HBoV1 (C) HBoV2 (D) capsids. Red labeling of histidines indicates that these possess extra densities, orange labeling indicates the paired H163 with H443/439, and blue labeling indicates histidines that displayed no additional densities. The images were generated with PyMOL (73).

additional density at pH 2.6, e.g., H497/493 (Fig. 9B), or both not having additional density, e.g., H515/511. In some cases, amino acids near histidines with extra density underwent a conformational change, such as K459 and Y462 near H70 in HBoV2 (Fig. 9B). Interestingly, these residues did not make the same conformational rotation in HBoV1 despite being conserved. In contrast to the cysteines mentioned above, histidines with and without additional density are found on both the capsid interior and exterior surfaces (Fig. 9C). Histidines have been shown to change their orientation at different pHs in AAV8 (35). However, additional density was not reported at low pH in that study, which utilized X-ray crystallography. In this study, it was seen only at pH 2.6 (Fig. 9A and B). To our knowledge, no cryo-EM structure of sufficient resolution has been published at this pH for other parvoviruses, and thus, relevance requires further studies beyond the scope of this work.

In summary, the HBoV2 capsid structure presented here completes the panel for the currently available HBoV strains. As anticipated, HBoV2 is structurally similar to the other gastrointestinal HBoVs, HBoV3 and HBoV4, and differs from HBoV1 at VR-III, supporting a role for this VP region in tissue tropism as originally proposed (28). The capsid dynamics associated with endo/lysosomal trafficking was documented with HBoV1 and HBoV2 as representatives of the respiratory and gastrointestinal strains, respectively. Both show similar N-terminal VP rearrangements and residue-level, cysteine and histidine, modifications at specific positions on the interior and exterior capsid surfaces. The N-terminal changes support progression toward the externalization of the PLA2 enzyme required for phospholipid cleavage and escape to the cytosol or interaction with the nuclear membrane. The modifications are reversible, suggesting that they may be required for interaction with other molecules in vesicle membranes.

Following escape from the endo/lysosomal pathway, the HBoV capsid reverts to protecting the packaged genome and trafficking to the nucleus for genome replication.

MATERIALS AND METHODS

HBoV VLP generation and purification. The baculovirus expression system in Sf9 (*Spodoptera frugiperda*) cells was used to express VLPs of HBoV1 and HBoV2. Briefly, 250 ml of Sf9 cells (1.7×10^6 to $\sim 2.0 \times 10^6$ cells/ml) were infected by a recombinant baculovirus expressing HBoV1 or HBoV2 VP3 at a multiplicity of infection (MOI) of 5 and incubated at 28°C for 3 days. The infected cells were lysed by three rounds of freeze/thaw cycles in TNTM buffer (25 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 mM MgCl₂, 0.2% Triton X-100). After the third cycle, the completely thawed pellet was treated with Benzonase (250 U/ μ l; 1 μ l/ml of sample) to remove nucleic acid. The VLPs were pelleted through a 20% (wt/vol, in TNTM buffer) sucrose cushion centrifugation at 45,000 rpm for 3 h at 4°C. Subsequently, the pelleted VLPs were resuspended in TNTM buffer and further purified by sucrose gradient (5% to 40% sucrose [wt/vol] in TNTM buffer) centrifugation at 35,000 rpm for 3 h at 4°C. Various fractions (20 to 25%, 25 to 30%, and 30 to 35%) of sucrose gradient were collected and analyzed for the presence of VLPs. The fractions were dialyzed three times into 1 $\times$ phosphate-buffered saline (PBS; pH 7.4) at 4°C for 3 h each and concentrated to ~ 1 to 2 mg/ml using an Apollo concentrator column. SDS-PAGE and negative-stain EM using a Tecnai G² Spirit transmission electron micrograph (TEM; FEI Co.) were used to analyze the purity and VLP integrity, respectively, as described previously (57). Alternatively, the purified HBoV1 or HBoV2 samples were dialyzed into glycine HCl (50 mM glycine, adjusted to pH 2.6) or universal buffer (20 mM HEPES, 20 mM morpholineethanesulfonic acid [MES], 20 mM sodium acetate, 150 mM NaCl, 5 mM CaCl₂) adjusted to a pH of 4.0, 5.5, 6.0, or 7.4. All pH-treated VLP samples were analyzed by SDS-PAGE and negative-stain EM prior to 3D structure determination.

Cryo-EM and data collection. HBoV1 VLPs (~ 1 mg/ml) were applied to C-flat holey carbon grids (Protochips, Inc.), while HBoV2 VLPs were applied to thin carbon-coated holey Quantifoil grids to overcome aggregation. The samples were vitrified using a Vitrobot Mark IV (FEI Co.) and screened on a 16-megapixel CCD camera (Gatan, Inc.) in a Tecnai (FEI Co.) G2 F20-TWIN transmission electron microscope (200 kV, ~ 20 e⁻/Å²) prior to data collection. For high-resolution data collection for HBoV2 at pH 7.4, micrographs were collected by using a Titan Krios electron microscope (FEI Co.) operating at 300 kV with a DE20 (Direct Electron) direct electron detector. This data set was collected as part of the NIH Southeastern Center for Microscopy of Macromolecular Machines (SECM4) project. For the HBoV VLPs under low-pH conditions, data were collected using a Titan Krios electron microscope on a K2 Summit (Gatan) direct electron detector at the UMass cryo-EM core. Images were recorded as “movies” consisting of multiple frames, and their parameters are summarized in Table 1. Subsequently, the frames of the micrographs were aligned for motion correction and to enhance the signal-to-noise ratio by using the DE_process_frames software (Direct Electron) with corresponding dark and bright reference images without radiation dose damage compensation for the data collected on the DE20 direct electron detector (59). The frames collected on the K2 Summit direct electron detector were aligned using MotionCor2 (60).

Determination of the structure of HBoV VLPs. For the 3D image reconstruction of HBoV1 and HBoV2 under the different pH conditions, the cisTEM software package was utilized (61). Briefly, the aligned micrographs were imported into the program and their contrast transfer function (CTF) parameters estimated. The CTF information was used to eliminate micrographs of poor quality. This was followed by automatic capsid picking using a radius of 125 Å. The selected capsids for each data set were subjected to 2D classification that eliminated ice particles and debris from the automatic picking process. Following 2D classification, the structures were reconstructed using default settings. This included *ab initio* 3D model generation, autorefinement, and density map sharpening with a precutoff (low resolution amplitudes) B-factor value of -90 Å² and variable postcutoff (high-resolution amplitudes) B-factor values such as 0, 20, and 50 Å². The sharpened density maps were inspected in the Coot and Chimera applications (62, 63). The -90 -Å² (precutoff) and 0-Å² (postcutoff) sharpened maps were used for assignment of the amino acid main and side chains for the majority of the capsid. For the more disordered surface loops, VR-I and VR-V (particularly at pH 7.4 and pH 2.6), the -90 -Å² (precutoff) and 50-Å² (postcutoff) sharpened maps were used, which were less noisy and allowed a better assignment of the amino acid main chain in these VP regions. The resolution of the cryo-reconstructed maps was estimated based on a Fourier shell correlation (FSC) of 0.143 (Table 1).

VP3 model building and structure refinement. A model of the HBoV2 VP3 monomer was generated based on the protein sequence (NCBI accession number [AFW98869.1](https://www.ncbi.nlm.nih.gov/nuclot/AFW98869.1); previously listed as VP2) on the SWISS-MODEL protein structure homology-modeling server (<https://swissmodel.expasy.org/>) (64) using the structure of HBoV3 as the template (28, 64). The resulting monomer was utilized to build an icosahedral 60-mer capsid model in VIPERdb (65). The 60-mer capsid model was docked into the HBoV2 cryo-reconstructed density map using the “fit in map” subroutine in UCSF-Chimera (63). The quality of the fit between the map and the model was evaluated by a correlation coefficient (CC) calculation. The pixel size of the reconstructed map was adjusted to obtain the best fit of the 60-mer model in the reconstructed map. Using the e2proc3d.py subroutine in EMAN2 and the program MAPMAN, a CCP4 format map file with the correct pixel size was generated that also represents a compatible file type for Coot (66–68). In Coot the reference VP3 model was fitted into the density map by adjusting the position of residues through interactive model building and the real-space-refine options (64). For the HBoV capsids under low-pH conditions, the pH 7.4 VP3 model was fitted into the maps as described above and structural rearrangements were refined in Coot using the real-space-refine option. All VP3 models were

further refined using PHENIX real space refinement with the default settings (69). The resulting model was analyzed in Coot with the density map, and side chains were modified if necessary. Finally, a B-factor refinement of the models was conducted in PHENIX.

Structure alignment of HBoVs. For comparative analysis, the VP3 structures of HBoV1 to -4 or of HBoV1 and HBoV2 under the different pH conditions were superimposed with each other by using the secondary-structure matching (SSM) program in Coot (70). The program also calculated root mean square deviations (RMSDs) for the superposed structures and the distances between the aligned C α positions. The cartoon representations of the VP3 structures and side chain density images were generated in Chimera (63).

Fluorescent labeling of VLPs. Volumes of 300 to 500 μ l of purified HBoV1, HBoV2, AAV2, and AAV5 VLPs (0.5 to 0.8 mg/ml) were labeled using the DyLight 488 antibody labeling kit (Thermo Fisher) as described previously (71). Unbound dye was removed by repeated dialysis. The success of capsid labeling was confirmed by SDS-PAGE and analysis of the gel on a UV transilluminator. Labeled VLPs were aliquoted and stored until usage at -80°C .

Cell lines and cell binding assay. The CHO cell lines Pro5, Lec2, and Lec8 were cultured as monolayers in minimum essential medium (MEM- α) (Gibco) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Gibco) in a 5% CO $_2$, 37°C incubator. For cell binding assays, the cells were diluted to 5×10^5 /ml, prechilled for 30 min at 4°C, and aliquoted in 500- μ l fractions. Each tube of cells was then incubated with the fluorescently labeled VLPs at an MOI of 10^6 under constant rotation for 3 to ~ 4 h at 4°C. Following the incubation, the cells were pelleted at 2,000 rpm for 10 min and the supernatant was discarded. Unbound VLPs were removed by washing the cells with 300 μ l of ice-cold 1 $\times$ PBS, followed by another centrifugation. Pellets were resuspended in 300 μ l of 1 $\times$ PBS and analyzed by fluorescence-activated cell sorting (FACS) utilizing a FACSCalibur (Becton & Dickinson).

Heparin binding assay. Microspin columns (Bio-Rad) were washed with 200 μ l of TNTM buffer followed by 1 ml of 1 $\times$ TD buffer (1 $\times$ PBS with 1 mM MgCl $_2$ and 2.5 mM KCl). Then 50 μ l of heparin-conjugated agarose type I resin (Sigma) was loaded into the columns. The affinity columns were equilibrated with 1 ml of 1 $\times$ TD buffer and charged by washing with 500 μ l of 1 $\times$ TD buffer/1 M NaCl buffer followed by three sequential washes with 1 ml of 1 $\times$ TD buffer. Subsequently, 10 μ g of HBoV1, HBoV2, AAV5, and AAV2 VLPs was diluted in 60 μ l of 1 $\times$ TD buffer, and 30 μ l of the samples was loaded onto the columns, followed by sequential collection of flowthrough, five column washes with 1 $\times$ TD buffer (30 μ l each), and five elution fractions with 1 $\times$ TD buffer/1 M NaCl buffer (30 μ l each). All fractions were analyzed by SDS-PAGE.

DSF stability assay. Purified HBoV and AAV5 VLPs (72) were diluted to ~ 0.2 mg/ml in 1 $\times$ PBS buffer and then dialyzed in universal buffer to a pH of 4.0, 5.5, 6.0, or 7.4 or in glycine HCl at pH 2.6, respectively. For the differential scanning fluorescent (DSF) assay, 2.5 μ l of 1% SYPRO orange dye (Molecular Probes, Invitrogen) was added to 22.5 μ l of dialyzed sample. The assay was conducted in a thermocycler (Bio-Rad CFX Connect) with the temperature ramped from 30 to 99°C, increasing by 0.1°C every 6 s. The melting temperature (T_m) for each sample was defined as the vertex of the first derivative (dF/dT) of relative fluorescence unit (RFU) values.

Accession number(s). The HBoV1 and HBoV2 cryo-EM reconstructed density maps and models built for their capsids were deposited in the Electron Microscopy Data Bank (EMDB) with accession numbers [EMD-23106/PDB ID 7LOW](#) (HBoV1 pH 5.5), [EMD-23108/PDB ID 7LOY](#) (HBoV1 pH 2.6), [EMD-23105/PDB ID 7LOV](#) (HBoV2 pH 7.4), [EMD-23104/PDB ID 7LOU](#) (HBoV2 pH 5.5), and [EMD-23107/PDB ID 7LOX](#) (HBoV2 pH 2.6), respectively.

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M.L. was responsible for VLP production, purification, and dialysis to the desired pH condition, fluorescent labeling of VLPs, the execution of the cell and heparin binding assays, the DSF assay, initial structure determinations using reconstruction by cryo-EM, and writing of the first draft of the manuscript. M.M. was responsible for cell binding assay analysis, cryo-reconstruction, structure refinement and analysis, model building and refinement, and manuscript preparation. P.C. vitrified sample, frozen, and screened cryo-EM grids. C.X., J.S., and D.S. collected cryo-EM data. R.M. and M.S.-V. contributed to interpretation of the results and manuscript preparation. M.A.-M. conceived and designed the project, analyzed all results, and contributed to manuscript preparation. All authors have read and agreed to the published version of the manuscript.

We declare no conflict of interest.

REFERENCES

- Cotmore SF, Agbandje-McKenna M, Canuti M, Chiorini JA, Eis-Hubinger AM, Hughes J, Mietzsch M, Modha S, Ogliastro M, Penzes JJ, Pintel DJ, Qiu J, Soderlund-Venermo M, Tattersall P, Tijssen P, ICTV Report Consortium. 2019. ICTV virus taxonomy profile: Parvoviridae. *J Gen Virol* 100:367–368. <https://doi.org/10.1099/jgv.0.001212>.
- Abinanti FR, Warfield MS. 1961. Recovery of a hemadsorbing virus (HADEN) from the gastrointestinal tract of calves. *Virology* 14:288–289. [https://doi.org/10.1016/0042-6822\(61\)90206-9](https://doi.org/10.1016/0042-6822(61)90206-9).
- Storz J, Leary JJ, Carlson JH, Bates RC. 1978. Parvoviruses associated with diarrhea in calves. *J Am Vet Med Assoc* 173:624–627.
- Binn LN, Lazar EC, Eddy GA, Kajima M. 1970. Recovery and characterization of a minute virus of canines. *Infect Immun* 1:503–508. <https://doi.org/10.1128/IAI.1.5.503-508.1970>.
- Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc Natl Acad Sci U S A* 102:12891–12896. <https://doi.org/10.1073/pnas.0504666102>.
- Arnott A, Vong S, Rith S, Naughtin M, Ly S, Guillard B, Deubel V, Buchy P. 2013. Human bocavirus amongst an all-ages population hospitalised with acute lower respiratory infections in Cambodia. *Influenza Other Respir Viruses* 7:201–210. <https://doi.org/10.1111/j.1750-2659.2012.00369.x>.
- Arthur JL, Higgins GD, Davidson GP, Givney RC, Ratcliff RM. 2009. A novel bocavirus associated with acute gastroenteritis in Australian children. *PLoS Pathog* 5:e1000391. <https://doi.org/10.1371/journal.ppat.1000391>.
- Schildgen O, Muller A, Allander T, Mackay IM, Volz S, Kupfer B, Simon A. 2008. Human bocavirus: passenger or pathogen in acute respiratory tract infections? *Clin Microbiol Rev* 21:291–304. <https://doi.org/10.1128/CMR.00030-07>.
- Fabbiani M, Terrosi C, Martorelli B, Valentini M, Bernini L, Cellesi C, Cusi MG. 2009. Epidemiological and clinical study of viral respiratory tract infections in children from Italy. *J Med Virol* 81:750–756. <https://doi.org/10.1002/jmv.21457>.
- Albuquerque MC, Pena GP, Varella RB, Gallucci G, Erdman D, Santos N. 2009. Novel respiratory virus infections in children, Brazil. *Emerg Infect Dis* 15:806–808. <https://doi.org/10.3201/eid1505.081603>.
- Khamrin P, Malasao R, Chaimongkol N, Ukarapol N, Kongsricharoern T, Okitsu S, Hayakawa S, Ushijima H, Maneekarn N. 2012. Circulating of human bocavirus 1, 2, 3, and 4 in pediatric patients with acute gastroenteritis in Thailand. *Infect Genet Evol* 12:565–569. <https://doi.org/10.1016/j.meegid.2012.01.025>.
- Smuts H, Workman L, Zar HJ. 2008. Role of human metapneumovirus, human coronavirus NL63 and human bocavirus in infants and young children with acute wheezing. *J Med Virol* 80:906–912. <https://doi.org/10.1002/jmv.21135>.
- Christensen A, Kesti O, Elenius V, Eskola AL, Døllner H, Altunbulakli C, Akdis CA, Söderlund-Venermo M, Jartti T. 2019. Human bocaviruses and paediatric infections. *Lancet Child Adolesc Health* 3:418–426. [https://doi.org/10.1016/S2352-4642\(19\)30057-4](https://doi.org/10.1016/S2352-4642(19)30057-4).
- Guo L, Wang Y, Zhou H, Wu C, Song J, Li J, Paranhos-Baccala G, Vernet G, Wang J, Hung T. 2012. Differential seroprevalence of human bocavirus species 1–4 in Beijing, China. *PLoS One* 7:e39644. <https://doi.org/10.1371/journal.pone.0039644>.
- Kantola K, Hedman L, Tanner L, Simell V, Mäkinen M, Partanen J, Sadeghi M, Veijola R, Knip M, Ilonen J, Hyöty H, Toppa J, Simell O, Hedman K, Söderlund-Venermo M. 2015. B-cell responses to human bocaviruses 1–4: new insights from a childhood follow-up study. *PLoS One* 10:e0139096. <https://doi.org/10.1371/journal.pone.0139096>.
- Chen AY, Cheng F, Lou S, Luo Y, Liu Z, Delwart E, Pintel D, Qiu J. 2010. Characterization of the gene expression profile of human bocavirus. *Virology* 403:145–154. <https://doi.org/10.1016/j.virol.2010.04.014>.
- Qiu J, Soderlund-Venermo M, Young NS. 2017. Human parvoviruses. *Clin Microbiol Rev* 30:43–113. <https://doi.org/10.1128/CMR.00040-16>.
- Nuesch JP, Rommelaere J. 2006. NS1 interaction with CKII alpha: novel protein complex mediating parvovirus-induced cytotoxicity. *J Virol* 80:4729–4739. <https://doi.org/10.1128/JVI.80.10.4729-4739.2006>.
- Li Q, Zhang Z, Zheng Z, Ke X, Luo H, Hu Q, Wang H. 2013. Identification and characterization of complex dual nuclear localization signals in human bocavirus NP1: identification and characterization of complex dual nuclear localization signals in human bocavirus NP1. *J Gen Virol* 94:1335–1342. <https://doi.org/10.1099/vir.0.047530-0>.
- Sukhu L, Fasina O, Burger L, Rai A, Qiu J, Pintel DJ. 2013. Characterization of the nonstructural proteins of the bocavirus minute virus of canines. *J Virol* 87:1098–1104. <https://doi.org/10.1128/JVI.02627-12>.
- Sun Y, Chen AY, Cheng F, Guan W, Johnson FB, Qiu J. 2009. Molecular characterization of infectious clones of the minute virus of canines reveals unique features of bocaviruses. *J Virol* 83:3956–3967. <https://doi.org/10.1128/JVI.02569-08>.
- Mietzsch M, Penzes JJ, Agbandje-McKenna M. 2019. Twenty-five years of structural parvovirology. *Viruses* 11:362. <https://doi.org/10.3390/v11040362>.
- Chapman MS, Agbandje-McKenna M. 2006. Atomic structure of viral particles, p 107–123. In Kerr JR, Cotmore SF, Bloom ME, Linden RM, Parrish CR (ed), *Parvoviruses*. Hodder Arnold, London, United Kingdom.
- Chen KC, Shull BC, Moses EA, Lederman M, Stout ER, Bates RC. 1986. Complete nucleotide sequence and genome organization of bovine parvovirus. *J Virol* 60:1085–1097. <https://doi.org/10.1128/JVI.60.3.1085-1097.1986>.
- Lederman M, Bates RC, Stout ER. 1983. In vitro and in vivo studies of bovine parvovirus proteins. *J Virol* 48:10–17. <https://doi.org/10.1128/JVI.48.1.10-17.1983>.
- Gurda BL, Parent KN, Bladik H, Sinkovits RS, DiMattia MA, Rence C, Castro A, McKenna R, Olson N, Brown K, Baker TS, Agbandje-McKenna M. 2010. Human bocavirus capsid structure: insights into the structural repertoire of the parvoviridae. *J Virol* 84:5880–5889. <https://doi.org/10.1128/JVI.02719-09>.
- Zádori Z, Szelei J, Lacoste M-C, Li Y, Gariépy S, Raymond P, Allaire M, Nabi IR, Tijssen P. 2001. A viral phospholipase A2 is required for parvovirus infectivity. *Dev Cell* 1:291–302. [https://doi.org/10.1016/S1534-5807\(01\)00031-4](https://doi.org/10.1016/S1534-5807(01)00031-4).
- Mietzsch M, Kailasan S, Garrison J, Ilyas M, Chipman P, Kantola K, Janssen ME, Spear J, Sousa D, McKenna R, Brown K, Söderlund-Venermo M, Baker T, Agbandje-McKenna M. 2017. Structural insights into human bocaviruses. *J Virol* 91:e00261–17. <https://doi.org/10.1128/JVI.00261-17>.

29. Kailasan S, Halder S, Gurda B, Bladec H, Chipman PR, McKenna R, Brown K, Agbandje-McKenna M. 2015. Structure of an enteric pathogen, bovine parvovirus. *J Virol* 89:2603–2614. <https://doi.org/10.1128/JVI.03157-14>.
30. Tu M, Liu F, Chen S, Wang M, Cheng A. 2015. Role of capsid proteins in parvovirus infection. *Virol J* 12:114. <https://doi.org/10.1186/s12985-015-0344-y>.
31. Halder S, Ng R, Agbandje-McKenna M. 2012. Parvoviruses: structure and infection. *Future Virol* 7:253–278. <https://doi.org/10.2217/fvl.12.12>.
32. Dudleeanmijil E, Lin CY, Dredge D, Murray BK, Robison RA, Johnson FB. 2010. Bovine parvovirus uses clathrin-mediated endocytosis for cell entry. *J Gen Virol* 91:3032–3041. <https://doi.org/10.1099/vir.0.024133-0>.
33. Blackburn SD, Cline SE, Hemming JP, Johnson FB. 2005. Attachment of bovine parvovirus to O-linked alpha 2,3 neuraminic acid on glycoprotein A. *Arch Virol* 150:1477–1484. <https://doi.org/10.1007/s00705-005-0496-y>.
34. Lins-Austin B, Patel S, Mietzsch M, Brooke D, Bennett A, Venkatakrishnan B, Van Vliet K, Smith AN, Long JR, McKenna R, Potter M, Byrne B, Boye SL, Bothner B, Heilbronn R, Agbandje-McKenna M. 2020. Adeno-associated virus (AAV) capsid stability and liposome remodeling during endo/lysosomal pH trafficking. *Viruses* 12:668. <https://doi.org/10.3390/v12060668>.
35. Nam HJ, Gurda BL, McKenna R, Potter M, Byrne B, Salganik M, Muzyczka N, Agbandje-McKenna M. 2011. Structural studies of adeno-associated virus serotype 8 capsid transitions associated with endosomal trafficking. *J Virol* 85:11791–11799. <https://doi.org/10.1128/JVI.05305-11>.
36. Dorsch S, Liebis G, Kaufmann B, von Landenberg P, Hoffmann JH, Drobnik W, Modrow S. 2002. The VP1 unique region of parvovirus B19 and its constituent phospholipase A2-like activity. *J Virol* 76:2014–2018. <https://doi.org/10.1128/jvi.76.4.2014-2018.2002>.
37. Farr GA, Zhang LG, Tattersall P. 2005. Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. *Proc Natl Acad Sci U S A* 102:17148–17153. <https://doi.org/10.1073/pnas.0508477102>.
38. Hattne J, Shi D, Glynn C, Zee CT, Gallagher-Jones M, Martynowicz MW, Rodriguez JA, Gonen T. 2018. Analysis of global and site-specific radiation damage in cryo-EM. *Structure* 26:759–766.e754. <https://doi.org/10.1016/j.str.2018.03.021>.
39. Huang LY, Patel A, Ng R, Miller EB, Halder S, McKenna R, Asokan A, Agbandje-McKenna M. 2016. Characterization of the adeno-associated virus 1 and 6 sialic acid binding site. *J Virol* 90:5219–5230. <https://doi.org/10.1128/JVI.00161-16>.
40. Bell CL, Gurda BL, Van Vliet K, Agbandje-McKenna M, Wilson JM. 2012. Identification of the galactose binding domain of the adeno-associated virus serotype 9 capsid. *J Virol* 86:7326–7333. <https://doi.org/10.1128/JVI.00448-12>.
41. Emmanuel SN, Mietzsch M, Tseng YS, Smith JK, Agbandje-McKenna M. 21 April 2020. Parvovirus capsid-antibody complex structures reveal conservation of antigenic epitopes across the family. *Viral Immunol* <https://doi.org/10.1089/vim.2020.0022>.
42. Fakhiri J, Schneider MA, Puschhof J, Stanifer M, Schildgen V, Holderbach S, Voss Y, El Andari J, Schildgen O, Boulant S, Meister M, Clevers H, Yan Z, Qiu J, Grimm D. 2019. Novel chimeric gene therapy vectors based on adeno-associated virus and four different mammalian bocaviruses. *Mol Ther Methods Clin Dev* 12:202–222. <https://doi.org/10.1016/j.omtm.2019.01.003>.
43. Fakhiri J, Linse KP, Mietzsch M, Xu M, Schneider MA, Meister M, Schildgen O, Schnitzler P, Soderlund-Venermo M, Agbandje-McKenna M, Grimm D. 2020. Impact of natural or synthetic singletons in the capsid of human bocavirus 1 on particle infectivity and immunoreactivity. *J Virol* 94:e00170–20. <https://doi.org/10.1128/JVI.00170-20>.
44. Summerford C, Samulski RJ. 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 72:1438–1445. <https://doi.org/10.1128/JVI.72.2.1438-1445.1998>.
45. Kaludov N, Brown KE, Walters RW, Zabner J, Chiorini JA. 2001. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J Virol* 75:6884–6893. <https://doi.org/10.1128/JVI.75.15.6884-6893.2001>.
46. North SJ, Huang HH, Sundaram S, Jang-Lee J, Etienne AT, Trollope A, Chalabi S, Dell A, Stanley P, Haslam SM. 2010. Glycomics profiling of Chinese hamster ovary cell glycosylation mutants reveals N-glycans of a novel size and complexity. *J Biol Chem* 285:5759–5775. <https://doi.org/10.1074/jbc.M109.068353>.
47. Graziano VR, Wei J, Wilen CB. 2019. Norovirus attachment and entry. *Viruses* 11:495. <https://doi.org/10.3390/v11060495>.
48. Afione S, DiMattia MA, Halder S, Di Pasquale G, Agbandje-McKenna M, Chiorini JA. 2015. Identification and mutagenesis of the adeno-associated virus 5 sialic acid binding region. *J Virol* 89:1660–1672. <https://doi.org/10.1128/JVI.02503-14>.
49. Kern A, Schmidt K, Leder C, Muller OJ, Wobus CE, Bettinger K, Von der Lieth CW, King JA, Kleinschmidt JA. 2003. Identification of a heparin-binding motif on adeno-associated virus type 2 capsids. *J Virol* 77:11072–11081. <https://doi.org/10.1128/jvi.77.20.11072-11081.2003>.
50. Leisi R, Di Tommaso C, Kempf C, Ros C. 2016. The receptor-binding domain in the VP1u region of parvovirus B19. *Viruses* 8:61. <https://doi.org/10.3390/v8030061>.
51. Fischer H, Widdicombe JH. 2006. Mechanisms of acid and base secretion by the airway epithelium. *J Membr Biol* 211:139–150. <https://doi.org/10.1007/s00232-006-0861-0>.
52. Manson JM, Rauch M, Gilmore MS. 2008. The commensal microbiology of the gastrointestinal tract. *Adv Exp Med Biol* 635:15–28. https://doi.org/10.1007/978-0-387-09550-9_2.
53. Bennett A, Patel S, Mietzsch M, Jose A, Lins-Austin B, Yu JC, Bothner B, McKenna R, Agbandje-McKenna M. 2017. Thermal stability as a determinant of AAV serotype identity. *Mol Ther Methods Clin Dev* 6:171–182. <https://doi.org/10.1016/j.omtm.2017.07.003>.
54. Venkatakrishnan B, Yarbrough J, Domsic J, Bennett A, Bothner B, Kozyreva OG, Samulski RJ, Muzyczka N, McKenna R, Agbandje-McKenna M. 2013. Structure and dynamics of adeno-associated virus serotype 1 VP1-unique N-terminal domain and its role in capsid trafficking. *J Virol* 87:4974–4984. <https://doi.org/10.1128/JVI.02524-12>.
55. Zhang R, Cao L, Cui M, Sun Z, Hu M, Zhang R, Stuart W, Zhao X, Yang Z, Li X, Sun Y, Li S, Ding W, Lou Z, Rao Z. 2019. Adeno-associated virus 2 bound to its cellular receptor AAVR. *Nat Microbiol* 4:675–682. <https://doi.org/10.1038/s41564-018-0356-7>.
56. Hafenstein S, Palermo LM, Kostyuchenko VA, Xiao C, Morais MC, Nelson CD, Bowman VD, Battisti AJ, Chipman PR, Parrish CR, Rossmann MG. 2007. Asymmetric binding of transferrin receptor to parvovirus capsids. *Proc Natl Acad Sci U S A* 104:6585–6589. <https://doi.org/10.1073/pnas.0701574104>.
57. Jose A, Mietzsch M, Smith JK, Kurian J, Chipman P, McKenna R, Chiorini J, Agbandje-McKenna M. 2019. High-resolution structural characterization of a new adeno-associated virus serotype 5 antibody epitope toward engineering antibody-resistant recombinant gene delivery vectors. *J Virol* 93:e01394–18. <https://doi.org/10.1128/JVI.01394-18>.
58. Péñzes JJ, Pham HT, Chipman P, Bhattacharya N, McKenna R, Agbandje-McKenna M, Tijssen P. 2020. Molecular biology and structure of a novel penaeid shrimp densovirus elucidate convergent parvoviral host capsid evolution. *Proc Natl Acad Sci U S A* 117:20211–20222. <https://doi.org/10.1073/pnas.2008191117>.
59. Spear JM, Noble AJ, Xie Q, Sousa DR, Chapman MS, Stagg SM. 2015. The influence of frame alignment with dose compensation on the quality of single particle reconstructions. *J Struct Biol* 192:196–203. <https://doi.org/10.1016/j.jsb.2015.09.006>.
60. Zheng SQ, Palovcak E, Armache JP, Verba KA, Cheng Y, Agard DA. 2017. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods* 14:331–332. <https://doi.org/10.1038/nmeth.4193>.
61. Grant T, Rohou A, Grigorieff N. 2018. cisTEM, user-friendly software for single-particle image processing. *Elife* 7:e35383. <https://doi.org/10.7554/eLife.35383>.
62. Emsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60:2126–2132. <https://doi.org/10.1107/S0907444904019158>.
63. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. 2004. UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* 25:1605–1612. <https://doi.org/10.1002/jcc.20084>.
64. Schwede T, Kopp J, Guex N, Peitsch MC. 2003. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res* 31:3381–3385. <https://doi.org/10.1093/nar/gkg520>.
65. Carrillo-Tripp M, Shepherd CM, Borelli IA, Venkataraman S, Lander G, Natarajan P, Johnson JE, Brooks IIC, Reddy VS. 2009. VIPERdb2: an enhanced and web API enabled relational database for structural virology. *Nucleic Acids Res* 37:D436–D442. <https://doi.org/10.1093/nar/gkn840>.
66. Emsley P, Lohkamp B, Scott WG, Cowtan K. 2010. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66:486–501. <https://doi.org/10.1107/S0907444910007493>.

67. Tang G, Peng L, Baldwin PR, Mann DS, Jiang W, Rees I, Ludtke SJ. 2007. EMAN2: an extensible image processing suite for electron microscopy. *J Struct Biol* 157:38–46. <https://doi.org/10.1016/j.jsb.2006.05.009>.
68. Kleywegt GJ, Jones TA. 1996. xDIPMAN and xDIDATAMAN—programs for reformatting, analysis and manipulation of biomacromolecular electron-density maps and reflection data sets. *Acta Crystallogr D Biol Crystallogr* 52:826–828. <https://doi.org/10.1107/S0907444995014983>.
69. Afonine P, Headd J, Terwilliger T, Adams P. 2013. New tool: phenix.real_space_refine. *Computat Crystallogr Newsl* 4:43–44.
70. Krissinel E, Henrick K. 2004. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. *Acta Crystallogr D Biol Crystallogr* 60:2256–2268. <https://doi.org/10.1107/S0907444904026460>.
71. Mietzsch M, McKenna R, Väisänen E, Yu JC, Ilyas M, Hull JA, Kurian J, Smith JK, Chipman P, Lasanajak Y, Smith D, Söderlund-Venermo M, Agbandje-McKenna M. 2020. Structural characterization of cuta- and tusavirus: insight into protoparvoviruses capsid morphology. *Viruses* 12:653. <https://doi.org/10.3390/v12060653>.
72. Rayaprolu V, Kruse S, Kant R, Venkatakrishnan B, Movahed N, Brooke D, Lins B, Bennett A, Potter T, McKenna R, Agbandje-McKenna M, Bothner B. 2013. Comparative analysis of adeno-associated virus capsid stability and dynamics. *J Virol* 87:13150–13160. <https://doi.org/10.1128/JVI.01415-13>.
73. DeLano WL. 2002. The PyMOL molecular graphics system. DeLano Scientific, San Carlos, CA.
74. Kalynych S, Füzik T, Pridal A, de Miranda J, Plevka P. 2017. Cryo-EM study of slow bee paralysis virus at low pH reveals iflavirus genome release mechanism. *Proc Natl Acad Sci U S A* 114:598–603. <https://doi.org/10.1073/pnas.1616562114>.