The Structure and Host Entry of an Invertebrate Parvovirus

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The 3.5-Å resolution X-ray crystal structure of mature cricket parvovirus (Acheta domestica densovirus [AdDV]) has been determined. Structural comparisons show that vertebrate and invertebrate parvoviruses have evolved independently, although there are common structural features among all parvovirus capsid proteins. It was shown that raising the temperature of the AdDV particles caused a loss of their genomes. The structure of these emptied particles was determined by cryo-electron microscopy to 5.5-Å resolution, and the capsid structure was found to be the same as that for the full, mature virus except for the absence of the three ordered nucleotides observed in the crystal structure. The viral protein 1 (VP1) amino termini could be externalized without significant damage to the capsid. In vitro, this externalization of the VP1 amino termini is accompanied by the release of the viral genome.

Parvoviruses are small (~250- to 300-Å-diameter), single-stranded DNA (ssDNA), icosahedral (T = 1), nonenveloped viruses whose genomes are approximately 5 kb long (1). The Parvoviridae family has been subdivided into viruses that infect vertebrates (Parvovirinae) and those that infect invertebrates (Densovirinae) (2). Parvoviruses replicate in dividing cells such as in tissues from insect larvae and fetuses. Densoviruses are highly pathogenic, and those that use insect hosts usually kill 90% of the larvae within a few days (2). Densoviruses pose a threat to commercial invertebrates such as shrimp (3), silkworms (4), and crickets (5, 6). Some highly pathogenic densoviruses are potential selective pesticides for vectors that transmit mosquito-borne diseases (7). Parvovirinae generally have three types of proteins (VP1, VP2, and VP3) in their capsids (8), whereas Densovirinae generally have four types of proteins (VP1 to VP4) in their capsids (2). In densoviruses there are 200 additional amino acids in VP1 at the N terminus. These different proteins result from different initiation sites for translation of the capsid gene and from posttranslational modification of their N termini (8). Generally, each of the 60 subunits within a capsid has the same amino acid sequence and is structurally the same, except that the different proteins start at different amino acids. The VP2s of some densoviruses are unique among VP2s of paroviruses since they are not completely contained within corresponding VP1s (Fig. 1A).

Parvoviruses enter cells by dynamin-dependent receptor-mediated endocytosis and escape the endosome by the phospholipase (PLA2) activity within the amino-terminal domain of VP1 (9–13). Although there is often less than 5% amino acid identity among the structural proteins of paroviruses, the sequence of the PLA2 N-terminal domain of VP1 has more than 30% amino acid identity (Fig. 1A and B). The PLA2 domain is not exposed in assembled, full paroviruses such as minute virus of mice (MVM) (13) and human parovirus B19 (14), and it therefore has to be exposed during endocytosis (9, 11, 13–15). However, the mechanism by which the VP1 amino-terminal PLA2 domain is exposed has not been elucidated in detail (16).

The structures of six autonomous vertebrate paroviruses (carnine parovirus [CPV] [17], feline parovirus [FPV]) [18], porcine parovirus [PPV] [19], MVM [20], H-1 parovirus [H-1PV] [21], and human parovirus B19 [22]) and three invertebrate paroviruses (Galleria mellonella densovirus [GmDV] [23], Bombyx mori densovirus [BmDV] [24], and Penaeus stylirostris densovirus [PstDV] [25]) have been determined (Table 1). Furthermore, extensive studies have been made of the human adenovirus-associated dependoviruses (26, 27). The structures of these paroviruses consist of 60 structurally equivalent capsid proteins assembled with icosahedral symmetry. Each capsid protein has a “jelly roll” fold, a motif that is common to many viruses, including the nonenveloped RNA picornaviruses (28) and small RNA plant viruses (29) as well as larger double-stranded DNA (dsDNA) adenoviruses (30), the enveloped bacteriophage PRD1 (31), the fungal virus Paramecium bursaria chlorella virus 1 (PBCV-1) (32), vaccinia virus (33), and probably also mimivirus (34). The jelly roll fold is a β-barrel consisting of two opposed antiparallel β-sheets with adjacent-strand BIDG and CHEF, where the strands along the polypeptide chain are named A and B to H. The interior of the barrel is exceedingly hydrophobic.

Paroviruses have a channel along the 5-fold axes formed by five symmetry-related DE loops (the “DE” loop is between the β-strands D and E). Residues lining the channel are mostly hydrophobic and guide the externalization of a conserved glycine-rich sequence near the amino ends of the VP5s (35–37). The loops connecting the β-strands of the jelly roll fold are usually exceptionally large in paroviruses compared with the loops in picornaviruses (28, 38) and form the exterior of the virus and intersubunit contacts (Fig. 1C). These loops are more variable in sequence than the core jelly roll structure.

Here, we describe the crystal structure of mature virions of cricket parovirus (Acheta domestica densovirus [AdDV]) at 3.5-Å resolution and the cryo-electron microscopic (cryoEM) structure of the emptied virus at 5.5-Å resolution. We also report on the externalization of the VP1 N-terminal region and subsequent genome release by an increase in temperature.

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MATERIALS AND METHODS

Virus purification and preparation of the emptied virus particles. The original virus was isolated from infected crickets (5). Further purification was achieved using CsCl equilibrium density gradient centrifugation. Of the two bands with different densities, the lower band contained the full particles and represented 99% of all the particles. The upper band contained empty particles assembled mainly from VP4. The two bands were separately transferred into Tris-buffered saline (TBS) (10 mM Tris-Cl, 100 mM NaCl, 1 mM CaCl, and 1 mM MgCl at pH 7.5) for further usage. Aliquots of the full virus particles were incubated at 26, 37, 45, 55, 65, 75, and 100°C for 1 h. The heat-treated emptied particles were frozen on holey carbon (Quantifoil) EM grids and checked by cryoEM. The numbers of full, empty, and broken particles were counted by eye (Fig. 2) and averaged over three holes on two different EM grids. Each hole had roughly 100 particles.

Determination of the crystal structure of the full virus particle. Crystals of the full particles were obtained by hanging-drop vapor diffusion in the presence of 20% polyethylene glycol (PEG) 400 and 100 mM MgCl at 16°C. Further optimization of the crystallization conditions produced crystals of up to 0.5 mm in length. Crystals were soaked for at least 20 min in the presence of 20% glycerol cryoprotectant prior to freezing.

X-ray diffraction data were collected at 100 K at the Advanced Photon Source (APS) beamline 23ID (Table 2). Diffraction data from about 20 crystals were indexed and merged, using the HKL2000 computer program (39) to generate the final 3.5-Å resolution data set. The space group was P42212 with \( a = 412.67 \) Å and \( c = 278.80 \) Å. The Matthews coefficient was 3.64 Å\(^3\)/Da, assuming half a virus particle per crystallographic asymmetric unit. Thus, the virus was located on a crystallographic 2-fold axis. A self-rotation function, calculated with the GLRF program (40) using 8- to 3.5-Å resolution data, gave the accurate orientation of the particle about the crystallographic 2-fold axis. This showed that one of the icosahedral 2-fold axes of the virus was roughly parallel to the \( 4_2 \) crystallographic axis, with a 1.6° rotation away from being exactly parallel. As a consequence, the position of the particle along the crystallographic 2-fold axis could be
determined from the big Patterson peak generated by the large number of parallel equal-length vectors.

The structure was determined using the molecular replacement method (41) with the structure of GmDNV (Protein Data Bank [PDB] code 1DNV) (23) as the initial phasing model to 15-Å resolution. The phases were then extended to 3.5-Å resolution in steps of one reciprocal lattice interval (1/c) at a time. Three cycles of 30-fold noncrystallographic symmetry (NCS) averaging and solvent flattening were performed for each extension step. The averaging and extension processes were performed using the program AVE in the Uppsala Software Factory (42) and FFT, SFALL in CCP4 programs (43). The final overall correlation coefficient between the observed structure amplitudes and the calculated structure factors corresponding to the final averaged and solvent-flattened map was 0.866. The atomic model was built into the 3.5-Å resolution map using COOT (44). The model coordinates were refined with the CNS program (45) while applying NCS constraints and reasonable model restraints, including group temperature factor refinement. No attempt was made to identify water molecules, as the data extended to only 3.5-Å resolution. The structures of the three ordered nucleotides bound to the inside surface of the capsid (see Results and Discussion) were included in TABLE 1 Structural studies of autonomous parvoviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Description of particle</th>
<th>Structural protein(s) in particles</th>
<th>Resolution (Å)</th>
<th>Icosahedral ordered genome structure (bp)</th>
<th>PDB code (reference)</th>
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<tbody>
<tr>
<td><strong>Vertebrate parvoviruses</strong></td>
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<tr>
<td>Canine parvovirus</td>
<td>Full virus</td>
<td>VP1, VP2, VP3</td>
<td>2.9</td>
<td>11</td>
<td>4DPV (17)</td>
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<tr>
<td>Empty particle</td>
<td></td>
<td>VP1, VP2</td>
<td>3.0</td>
<td>None</td>
<td>2CAS (56)</td>
</tr>
<tr>
<td>Feline parvovirus</td>
<td>Empty particle</td>
<td>VP3</td>
<td>3.5</td>
<td>None</td>
<td>1FPV (18)</td>
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<tr>
<td>Porcine parvovirus</td>
<td>Virus-like particle</td>
<td>VP2</td>
<td>3.5</td>
<td>None</td>
<td>1K3V (19)</td>
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<tr>
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<td>VP2</td>
<td>3.5</td>
<td>None</td>
<td>1SS8 (22)</td>
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<tr>
<td>B19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Minute virus of mice</td>
<td>Full virus</td>
<td>VP1, VP2, VP3</td>
<td>3.5</td>
<td>11</td>
<td>1MVM (20)</td>
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<td>Rat H-1 parvovirus</td>
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<td>2.7</td>
<td>10</td>
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<td>3.2</td>
<td>None</td>
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<td>None</td>
<td>1DNV (23)</td>
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<td>VP3</td>
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<td>3POS (24)</td>
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<tr>
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<td>None</td>
<td>3N7X (25)</td>
</tr>
<tr>
<td>AdDNV</td>
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<td>3.5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Induced emptied particle</td>
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<td>VP1, VP2, VP3, VP4</td>
<td>5.5</td>
<td>None</td>
<td></td>
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</tbody>
</table>

FIG 2 Heat treatment and PLA2 activity of the virus particles. (A) CryoEM micrographs showing the virus after incubation for 1 h at 26°C, 45°C, and 65°C, resulting in only full particles (left), about an equal number of full and emptied particles (middle), and emptied particles (right), respectively. (B) Percentage of emptied particles after incubating full particles at different temperatures. (C) Phospholipase activity as a function of temperature with respect to the PLA2 activity of honey bee PLA2 at 26°C.
the final stages of refinement. After more than 5 cycles of refinement and model rebuilding, the R factor had dropped from 34% to 28.9%. In the presence of the 30-fold NCS redundancy, there will be no significant difference between R<sub>free</sub> and R<sub>work</sub> in the Ramachandran plot.

### Detecting the externalized VP1 N termini and their phospholipase A2 activity

Full particles and the heat-treated emptied particles were digested by trypsin at room temperature. About 30 µl of the particle suspension at a concentration of 5 µg/ml was incubated with trypsin for 1 h at 26°C. The trypsin had a final concentration of 1 µg/ml in the mixture. The samples were then checked for VP1 cleavage using a 15% SDS-polyacrylamide gel (Fig. 3).

### CryoEM and three-dimensional structural reconstruction of emptied particles

The optimal condition for obtaining the largest percentage of emptied, unbroken particles was 55°C for 1 h (Fig. 2). Three microliters of the heat-treated emptied particles at a protein concentration of 5 µg/ml was applied to holey grids (Quantifoil) and blotted for 6 s in an FEI Mark 3 Vitrobot chamber at 90% humidity. The grids were then fast-frozen in liquid ethane. Cryo-electron microscopy (CryoEM) images were acquired on an FEI Titan Krios operated at 300 keV. Images were recorded with a 4k × 4k charge-coupled device (CCD) detector. As a control, grids of untreated particles were prepared and viewed in the same way. The assumed magnification of 59,000 was calibrated with respect to a known specimen and was shown to correspond to a pixel separation of 1.51 Å in the image. The electron dose was ~20 e/Å², and the image was defocussed by between ~1.6 and 2.6 µm. About 150 cryoEM micrographs, each showing roughly 100 particles, of the emptied particles were recorded. The defocus and the astigmatism of each micrograph were estimated with the EMAN1 fitcf program (46) and further confirmed with the program cftf. Image processing and three-dimensional reconstruction were performed using the EMAN suite of programs (47). The final reconstruction was computed using ~15,000 particles out of about 17,000 initial boxed images and was found to have 5.5-Å resolution based on the separate structure determinations of two randomly selected independent sets of images using the Fourier shell correlation threshold of 0.143 (Fig. 4) (48).

### Sequence alignment of the PL2A domain and structural comparisons

The sequence of the AdDNV VP1 N-terminal PL2A domain (GI 326392953) was aligned with the corresponding sequences of adeno-associated virus 2 (AAV2) (GI 110645923), human parvovirus B19 (GI 169212578), CPV (GI 116646110), MVM (GI 332290), rat parvovirus (GI 410443463), mimk parvovirus (GI 425696394), PPV (GI 46404508), GmDNV (GI 23343609), and BmDNV (GI 18025360) using Clustal X (49).

The crystal structure of AdDNV was compared with those of other invertebrate densovirus, i.e., GmDNV (23) and BmDNV (24), as well as...
mammalian autonomous parvoviruses CPV (17), PPV (19), FPV (18), MVM (20), and B19 (22) using the HOMOlogy program (50). These structural comparisons do not include the disordered PLA2 domain, whose positions in the virus are random and therefore cannot be observed in the crystal structure.

Accession numbers. The atomic coordinates of the AdDNV crystal structures have been deposited with the Protein Data Bank (www.pdb.org) (PDB code 4MGU); the cryo-EM maps of the emptied AdDNV particle have been deposited with the Electron Microscopy Data Bank (www.emdatabank.org) (EMDB code EMD-2401).

RESULTS AND DISCUSSION

Crystal structure of the full AdDNV particles. The structure of AdDNV was determined to 3.5-Å resolution. The position of the core jelly roll relative to the icosahedral symmetry axes was essentially the same in AdDNV as in other known parvovirus structures (Table 3 and 4).

The four structural proteins VP1 (88.1 kDa), VP2 (65.3 kDa), VP3 (50.8 kDa), and VP4 (46.9 kDa) are in an approximate 1:1:18:30 proportion in AdDNV full particles based on scanning the gel with Kodak Image Station 2000R and analyzing with software Kodak MI (Fig. 1A). The glycine-rich sequence is present in VP1, VP2, and VP3, but is missing in VP4 (Fig. 1A). It may be significant that, compared with vertebrate parvoviruses, there is therefore only one copy of the PLA2 structure per virion. The polypeptide chain of the capsid protein could be traced from residue 23 of VP4 situated at the base of the 5-fold axis channel to residue 418 at the carboxy terminus (Fig. 1C).

The electron density in this channel (σ = 1.5) of AdDNV is weak and discontinuous (Fig. 5C), which is similar to the density in the GmDNV 5-fold channel. The glycine-rich motif in AdDNV consists of about 17 residues, 8 of which are glycines, whereas in GmDNV the same motif has 7 glycines and about 16 residues (Fig. 6). The difference of the sequence length may be partly related to the structure of the channel in the different parvoviruses.

The low density in the 5-fold channel suggests that only several of the 12 5-fold channels are occupied, resulting in externalization of the VP amino termini. A similar lack of amino-terminal externalization was observed in GmDNV, the only other known structure of a mature DNV. The structures of silkworm and shrimp densoviruses (Table 1) were self-assembled from recombinantly expressed VP3 and VP4 capsid proteins, respectively. Hence, these structures are missing the glycine-rich sequence. As there is only one VP1 per virion, some of the 5-fold channels must be occupied by VP2 or VP3. However, in the vertebrate parvoviruses CPV (17)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence identity (%)</th>
<th>RMSD (Å) between Ca atoms</th>
<th>No. of aligned Ca atoms</th>
<th>Total no. of Ca atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine parvovirus</td>
<td>&lt;5</td>
<td>4.8</td>
<td>261</td>
<td>548</td>
</tr>
<tr>
<td>Feline parvovirus</td>
<td>&lt;5</td>
<td>4.9</td>
<td>263</td>
<td>534</td>
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<td>4.9</td>
<td>258</td>
<td>542</td>
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<tr>
<td>Human B19</td>
<td>&lt;5</td>
<td>5.1</td>
<td>260</td>
<td>523</td>
</tr>
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<td>295</td>
<td>415</td>
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<td>&lt;5</td>
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<td>331</td>
<td>412</td>
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<tr>
<td>PrtDNV</td>
<td>8.30</td>
<td>4.1</td>
<td>224</td>
<td>299</td>
</tr>
</tbody>
</table>

FIG 5 Structure of AdDNV emptied particles. (A) CryoEM reconstruction of emptied particles. Surface features with a triangle showing the limits of one icosahedral asymmetric unit are shown. The scale bars represent 2 nm. (B) Center section of the cryoEM reconstruction. The scale bars represent 2 nm. (C) Enlargement of the 5-fold channel density in the X-ray electron density map. The scale bars represent 1 nm. (D) Enlargement of the 5-fold channel density in the cryoEM density map. The scale bars represent 1 nm. (E, F, and G) Fit of the X-ray structure polypeptide backbone into the cryoEM density for the β-sheets of the jelly roll (scale bars represent 5 Å) (E), the BIDG β-sheet (the scale bars represent 3 Å) (F), and the α-helix located in the EF loop (the scale bars represent 3 Å) (G).

FIG 6 Sequence comparisons of the glycine-rich regions of AdDNV and other autonomous parvovirus, including GmDNV (GI 23334609), BmDNV (GI 18025360), canine parvovirus (CPV) (GI 116646110), mink enteritis virus (MEV) (GI 42569394), feline parvovirus (FPV) (GI 335476), porcine parvovirus (FPV), and human parvovirus (B19) (GI 169212578).

TABLE 3 Sequence and structural comparisons of AdDNV capsid protein with other autonomous parvovirus capsid proteins

<table>
<thead>
<tr>
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<td>PrtDNV</td>
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Most paroviruses, including densoviruses, assemble in vivo both as full infectious particles and as empty particles. However, for AdDNV and presumably also for other densoviruses, the small fraction of particles that are empty in a virus preparation consist of only VP4 (Fig. 3) and are missing the glycine-rich sequence, whereas the dominant infectious virus particles contain all four types of subunits (VP1 to VP4) (Fig. 1A). Therefore, after heat treatment, nearly all the emptied particles that have a full complement of all four VP must have been full of genome, whereas empty particles containing only VP4 must have been assembled as empty particles. It had been shown that heating paroviruses to 70°C generated PLA2 activity, suggesting exposure of the VP1 N termini (12, 13). However, it was not clear whether only the VP1 N termini were exposed from intact particles or whether the particles had disassembled. The loss of the genome associated with a presumably transient change in the capsid has some resemblance to the infectious process in picornaviruses (52).

Here we used cryoEM to show that on heating of AdDNV for a defined length of time, the number of emptied particles increased with temperature (Fig. 2A and B). When the temperature was increased beyond 65°C there was also an increase of broken particles. Concomitant with the increase of emptied particles, there was also an increase of PLA2 activity (Fig. 2C). Above about 65°C, the virions disintegrated and had reduced PLA2 activity. Unlike the case for full, infectious AdDNV particles, the VP1 N termini of the heat-treated emptied particles were sensitive to trypsin digestion, whereas the capsids remained intact as determined with

![Unrooted phylogenetic tree of different paroviruses based on the number of inserted residues between β-strands.](https://jvi.asm.org/content/55/12/12528/F1.large.jpg)

**FIG 7** Unrooted phylogenetic tree of different paroviruses based on the number of inserted residues between β-strands.
The density in the channel along the 5-fold axes in the cryoEM map of the emptied particles was similar to that in the crystallographically determined map of the full infectious particle calculated to 5.5-Å resolution (Fig. 5C and D). Thus, the emptied particles still have the glycine-rich region occupying the channel along the 5-fold axes in at least some of the 12 channels of each particle. The externalization of the VP1 termini does not seem to have caused much damage to the particles. In contrast to the case for AdDNV, there is little density along the 5-fold axes of the shrimp (PstDNV) and silkworm (BmDNV) densoviruses. However, these structures are of recombinant particles that contain only VP3 or VP4, respectively, and are therefore missing the glycine-rich region. These observations pose the intriguing question of whether the PLA2 domain has to be refolded to be threaded through the 5-fold pore or whether the pore opens with restoration of the initial capsid structure after extrusion.

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We declare no conflict of interest.

REFERENCES


