Electron microscopic imaging revealed the flexible filamentous structure of the cell attachment protein P2 of *Rice dwarf virus* located around the icosahedral 5-fold axes

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Naoyuki Miyazaki^{1,2,*}, Akifumi Higashiura¹,
Tomoko Higashiura¹, Fusamichi Akita^{3,4},
Hiroyuki Hibino³, Toshihiro Omura³,
Atsushi Nakagawa¹ and Kenji Iwasaki^{1,†}

¹Institute for Protein Research, Osaka University, Suita, Osaka, Japan;

²Supportive Center for Brain Research, National Institute for Physiological Sciences, Okazaki, Aichi, Japan; ³Laboratory of Virology, National Agricultural Research Center, Tsukuba, Ibaraki, Japan; and ⁴Photosynthesis Research Center, Graduate School of Natural Science and Technology, Okayama University, Okayama, Okayama, Japan

*Naoyuki Miyazaki, National Institute for Physiological Sciences, 38 Nishigonaka, Myodaiji, Okazaki, Aichi 444-8585, Japan. Tel: +81-56-455-7872, Fax: +81-56-452-7913, email: naomiya@nips.ac.jp

†Kenji Iwasaki, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel: +81-66-879-8608, Fax: +81-66-879-8609, email: ikenji@protein.osaka-u.ac.jp

The minor outer capsid protein P2 of *Rice dwarf virus* (RDV), a member of the genus *Phytoreovirus* in the family *Reoviridae*, is essential for viral cell entry. Here, we clarified the structure of P2 and the interactions to host insect cells. Negative stain electron microscopy (EM) showed that P2 proteins are monomeric and flexible L-shaped filamentous structures of ~20 nm in length. Cryo-EM structure revealed the spatial arrangement of P2 in the capsid, which was prescribed by the characteristic virion structure. The P2 proteins were visualized as partial rod-shaped structures of ~10 nm in length in the cryo-EM map and accommodated in crevasses on the viral surface around icosahedral 5-fold axes with hydrophobic interactions. The remaining disordered region of P2 assumed to be extended to the radial direction towards exterior. Electron tomography clearly showed that RDV particles were away from the cellular membrane at a uniform distance and several spike-like densities, probably corresponding to P2, connecting a viral particle to the host cellular membrane during cell entry. By combining the *in vitro* and *in vivo* structural information, we could gain new insights into the detailed mechanism of the cell entry of RDV.

Keywords: cryo-electron microscopy/electron tomography/*Phytoreovirus*/*Rice dwarf virus*/viral cell entry.

Abbreviations: CCD, charge-coupled device; CPV, *Cytoplasmic polyhedrosis virus*; cryo-EM, cryo-electron microscopy; dsRNA, double-stranded RNA; RDV, *Rice dwarf virus*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 3D, three-dimensional.

Reoviridae is the largest and most diverse family of non-enveloped double-stranded RNA (dsRNA) viruses, infecting plants, vertebrates, insects and fungi (1). Except for the single-layered *Cytoplasmic polyhedrosis virus* (CPV), all known reoviruses have two or three multi-layered capsids of 60–80 nm in diameter. The capsid shells enclose segmented dsRNA as a viral genome and their own transcriptional enzymes. They have the conserved innermost capsid shells in spite of the absence of sequence homology, while the outer capsid shells including the cell attachment proteins exhibit diverse morphologies among viruses of different genera. This is probably related to the wide range of host species associated with the different host-cell recognition mechanisms among the reoviruses (2, 3). Viral cell attachment to the host cell surface, mediated mostly through specific molecules, is required for the successful adsorption and subsequent entry of the virus into the host cell for viral replication. To understand the molecular mechanisms of how viruses infect and replicate in cells, structural information on the viruses and the molecular interactions between the viral and cellular factors are essential. Several structures of viral cell attachment proteins (4, 5) and the formation of membrane pores by viral cell-penetration proteins (6, 7) are known for animal reoviruses. However, little or no knowledge is available so far regarding the cell attachment and entry mechanisms of plant and insect reoviruses.

Rice dwarf virus (RDV), a member of the genus *Phytoreovirus* in the family *Reoviridae* (1), multiplies in both plants and invertebrate insect vectors. RDV has an icosahedral capsid composed of seven proteins organized in two concentric protein layers that surround a genome of 12 segmented dsRNAs (8). The inner layer consists of 120 copies of P3 protein, while the outer layer consists of three proteins; namely, P2, P8 and P9 (8, 9). The major outer capsid protein P8 forms trimers and constructs the hexagonal network in a $T = 13\ell$ icosahedral lattice on the viral surface to protect viral genome, while the minor outer capsid protein P2 of RDV is essential for the viral infection to insect vectors (10–12). Therefore, P2 is a putative cell attachment protein to interact with undefined receptors on insect vector cells, and this interaction is required for the host recognition of RDV (12). Moreover, the entry of RDV involves clathrin-mediated endocytosis (13), and P2 induces membrane fusion in insect vector cells (14). Atomic structures of the major capsid components P3 and P8 have been determined by X-ray crystallography (15), and a hierarchical capsid assembly mechanism has been

proposed based on the structures, which was confirmed by several biochemical experiments (16, 17). In addition, structures inside the capsid shell were also analysed by cryo-electron microscopy (cryo-EM) (18). However, no structural information about the cell attachment P2 protein, including that of the location in the capsid shell, is available, because the samples used in the previous structural analyses lacked the P2 protein, which has restricted our understanding of the cell entry process of RDV.

In this study, we determined the overall structure of P2 proteins isolated from the purified intact RDV particles, using negative stain EM and single-particle image processing, which exhibited the flexible structure of P2. Furthermore, we determined the intact RDV virion structure containing the P2 protein by cryo-EM, which visualized the partial structure of P2 and its location in the capsid shell. Furthermore, in addition to these *in vitro* studies, we examined the three-dimensional (3D) structure of RDV at various stages of the viral entry process in the cellular context, by electron tomography. These results obtained by combining the *in vitro* and *in vivo* studies could provide new insights into the cell attachment and entry of RDV.

Materials and Methods

Purification of intact RDV particles

All RDV particles (O strain) used in this study were purified without using CCl_4 , as described by Omura *et al.* (19). Infected rice leaves were macerated with a meat chopper in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.01 M MgCl_2 . After differential centrifugations of the slurry of chopped leaves, the viral particles were purified by sucrose density gradient centrifugation on 10–40% sucrose and then on 40–70% sucrose. The banded material containing the viral particles was pelleted by high-speed centrifugation and resuspended in 0.1 M histidine containing 0.01 M MgCl_2 , pH 6.2 (His-Mg buffer). The presence of P2 protein in the viral particles was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis with RDV P2-specific antibodies.

Isolation of P2 protein from intact RDV particles and imaging of P2 protein by negative stain EM

P2 protein was isolated from intact RDV particles purified without CCl_4 treatment as described earlier. The intact RDV particles containing P2 protein were first dialyzed against His-Mg buffer containing 0.8 M MgCl_2 for 12 h at 4°C to disassemble the outer capsid proteins (P2, P8 and P9 proteins) from the virus particles (17, 20, 21). When the partially disassembled particles were dialyzed against His-Mg buffer for 12 h at 4°C, P8 and P9 proteins would reassemble on the outer surface of the disassembled particles, while P2 proteins remained in the solution. The reassembled particles were removed as pellets by three successive centrifugations at 230,000 $\times g$ for 10 min. Then, the P2 protein in the supernatant was further purified by anion-exchange chromatography (HiTrapQ HP), followed by gel filtration on a Superdex 200 pg column (1.6 $\times$ 60 cm; GE Healthcare, USA) equilibrated with His-Mg buffer. The purity of the P2 protein was analysed by SDS-PAGE and identified by western blotting with RDV P2-specific antibodies. The sample was negatively stained with 2% (w/v) uranyl acetate and examined under an electron microscope (H7650; Hitachi, Japan) operated at 80 kV and a nominal magnification of $\times 50,000$. Images were recorded on a 1,024 $\times$ 1,024 pixels charge-coupled device (CCD) camera (TVIPS, Germany). Single-particle electron microscopic analysis, including particle selection and 2D classification and averaging, was performed using the EMAN EM analysis suite (22) and the IMAGIC software (23). Particles were selected from individual frames (with an effective pixel size of 0.59 nm) using the program *Boxer* in the EMAN suite. The particle images were rotationally and

translationally aligned by a multireference alignment procedure and subjected to multivariate statistical analysis (specifying 50 classes) using IMAGIC.

Cryo-EM of intact RDV particles

Samples of intact RDV particles were embedded in vitreous ice and examined at ~ 100 K with a cryo-electron microscope (Titan-Krios; FEI, Netherlands) operated at 200 kV and at a nominal magnification of $\times 29,000$ (0.293 nm/pixel). Images were recorded with a 4,096 $\times$ 4,096 pixels CCD at underfocus values ranging from 0.5 to 3.0 μm . Viral particles were boxed out, and the individual images were corrected for the contrast transfer function. Three-dimensional reconstruction was performed using the EMAN2 suite (24). The final reconstruction of intact RDV was computed from $\sim 1,034$ particles, and the resolution was assessed at 13 Å with a 0.143 threshold and at 16 Å with a 0.5 threshold in the Fourier shell correlation between two reconstructions, which was calculated from two halves of each data set (25). Atomic structures of RDV (Protein Data Bank (PDB) ID: 1UF2) were fitted into the cryo-EM map to compare the structures.

Electron tomography

Thin sections for electron tomography were prepared as described previously (13, 26, 27). Briefly, NC24 cells, established originally from embryonic fragments dissected from the eggs of the leafhopper *Nephotettix cincticeps* (the insect host of RDV), were maintained in monolayer culture at 25°C in growth medium that had been prepared as described by Kimura (28). The NC24 cells on coverslips were inoculated with several dilutions of RDV concentrations to reach 100% infection (13, 28). Post inoculation, the cells were fixed at different times and were then cut into ultrathin sections for electron tomography (12, 13). The thin sections were examined with an electron microscope (H7650; Hitachi, Japan) equipped with a 1,024 $\times$ 1,024 CCD camera for identification of regions that contained virus particles entering the host cell. Once a region of interest had been identified, colloidal gold of 15 nm in diameter was placed on the sections to facilitate the alignment required for tomographic analysis. Single-axis tilt series of thin sections were collected manually, at 2° intervals between -60° and $+60^\circ$, using an electron microscope (H9500SD; Hitachi, Japan) that was operated at an acceleration voltage of 200 kV and a nominal underfocus of 4.3 μm . Data were acquired at a microscopic magnification of $\times 20,000$ (at 0.88 nm/pixel) with a 2,048 $\times$ 2,048 pixels CCD camera (TVIPS). Image processing was performed with the IMOD software package (29), and the Amira software platform (FEI Visualization Science Group, USA) was used for the segmentation.

Results

Purification of intact RDV particles

Analyses by SDS-PAGE and western blotting with RDV P2-specific antibodies indicated that the RDV particles purified without CCl_4 treatment definitely possessed the P2 protein as shown previously (Fig. 1a Lane 2 and 1b Lane 2) (10). The purity of intact RDV virions containing P2 was almost the same as that of virions without P2 that were used in the previous X-ray crystallographic study (15), and no other protein band except for the viral components was visible on the SDS-PAGE gel stained with Coomassie Brilliant Blue (Fig. 1a). Based on the density of the band, the number of copies of P2 in one viral particle was roughly estimated to be 10–30 derived from multiple experiments.

Purification of P2 protein

P2 protein was isolated from the intact RDV virions purified without CCl_4 treatment, as described earlier, by disassembling outer capsid proteins with 0.8 M MgCl_2 . At the final purification step, the P2 protein was applied to size-exclusion chromatography. The P2

Fig. 1 Overall structure of the isolated P2 protein. (a) SDS-PAGE of the structural proteins of RDV with and without CCl_4 treatment. Intact RDV has seven kinds of proteins (Lane 2; P1–P3, P5, P7–P9 proteins). Lane 1, RDV particles lacking the P2 protein by CCl_4 treatment used in the previous study (15); Lane 2, intact RDV particles containing the P2 protein (without CCl_4 treatment) used in this study. P9 protein is appeared as several bands in SDS-PAGE (30). P9* is processed or degraded from a large polypeptide of P9 protein (P9). (b) SDS-PAGE and western blot analysis of the isolated P2 protein from intact RDV particles. The isolated P2 protein was separated by SDS-PAGE and then stained with Coomassie Brilliant Blue (left) or analysed by western blotting with RDV P2-specific antibodies (right). Lane 1, molecular weight markers; Lane 2, intact RDV particle; Lane 3, isolated P2 protein. (c) Electron micrograph of negatively stained P2 proteins isolated from intact RDV particles, which was recorded at a magnification of $\times 50,000$ with an H7650 electron microscope (Hitachi, Japan). Small aggregated P2 proteins are also observed in the micrographs, which are indicated by arrowheads. The bar represents 50 nm. (d) Selected class-averaged images. The width of each image is 40.1 nm. A histogram of the lengths of the P2 proteins is presented later. The lengths of the P2 proteins were measured by tracing the centre of the filament in each class-averaged image. All 50 class-averaged images are shown in the Supplementary Data (Supplementary Fig. S2). (e) Schematic representation of the P2 structure.

protein eluted slightly faster as a single peak that corresponded to a molecular mass of 154 kDa and thus behaved like a somewhat larger molecule than the molecular mass of the P2 monomer (127 kDa; Supplementary Fig. S1). SDS-PAGE and western blotting of the peak fraction using RDV P2-specific antibodies showed that it contained only the P2 protein (Fig. 1b).

Overall structure of P2

The isolated P2 protein was examined by negative stain EM. A total of 2,219 particles were boxed out and averaged after being classified into 50 classes. All class-averaged images clearly showed monomeric extended structures with the length of 20.7 ± 1.9 nm (standard deviation; Fig. 1c and d; Supplementary Fig. S2), which agreed with the result from the size-exclusion chromatography. P2 was bent and basically L-shaped. Moreover, the class-averaged images exhibited the flexible nature of the P2 structure, where the angles between two ends were considerably different among class-averaged images (Fig. 1e).

Position and structure of P2 in the outer capsid shell

To examine the position and structure of P2 in the capsid shell, we determined the 3D structure of the intact RDV particle by cryo-EM (Supplementary Fig. S3) and atomic structures of the outer capsid protein P8 and the inner capsid protein P3 (15) were fitted into the cryo-EM map. The atomic structures were accommodated well into the cryo-EM map without any positional adjustments (Supplementary Fig. S4). The cryo-EM structure of the intact RDV clearly showed that unique densities that the crystal structure lacks (Fig. 2a and b), which form starfish-like structure sticking to the vertices of the viral particle. Their relatively low densities are located above the ditch between adjacent P8 trimers. Compared with the samples used in the previous X-ray crystallographic study, the samples in this cryo-EM study have an additional component that is the outer capsid P2 protein (Fig. 1a). Other outer capsid P8 and P9 proteins exist in both samples, although P9 structures are invisible in the X-ray density map. Therefore, those additional densities in the cryo-EM map were certainly attributed to the P2 proteins. However, even when contoured at a lower density level, the size of the rod-shaped structures was 2–3 nm in diameter and 10 nm in length, which was much shorter than that of the isolated P2 protein (20 nm in length). Moreover, the volume was ~ 71 nm³ (diameter: 3 nm; length: 10 nm), which was also much smaller than the estimated volume of 156 nm³ from an average volume of $1.23 \text{ \AA}^3/\text{Da}$ for proteins (molecular mass of the P2 protein: 127 kDa) (33), which corresponded to 46% of the total volume of the P2 protein. These rod-shaped structures on the viral surface were docked in crevasses formed by two P8 trimers located around 5-fold axes, designated as P trimers (Fig. 2a–c) (15, 33). Contouring at a lower density level showed the rod-shaped structures to be connected to the P8 structures, which indicated that the hydrophobic amino acid residues of P8 (Pro²⁰³, Ala²⁰⁴, Gly²⁰⁵ and Ala²⁰⁶) were located at the contact

site and might be involved in the interaction between the P2 and P8 proteins (Fig. 2b).

Interactions between RDV and the host cell as visualized by electron tomography

To investigate the interactions between RDV and the host cell, electron tomography was performed at various stages of the viral entry into the host cell (Fig. 3). We observed that the RDV particles and the cellular membrane were separated by a uniform distance of ~ 15 nm and the RDV particles had several spike-like densities around themselves during cell entry, some of which attached to the host cellular membrane continuously (Fig. 3b, c, e and f). The size of the spike-like structures was similar to that of the disordered region of P2 protein in the cryo-EM structure as described earlier. Moreover, the spike-like structures were observed even in the direction facing away from the cellular membrane (Supplementary Fig. S5).

Discussion

Flexible structure of the P2 protein

The viral host-cell recognition system involves viral cell attachment and entry, which are the first essential processes in the viral life cycle. In the case of RDV, the P2 protein in the outer capsid has an important role in viral cell attachment and entry (11, 12), and cell entry involves clathrin-mediated endocytosis (13). However, the detailed cell attachment and entry mechanism and the structure of P2 have been unknown. In this study, we revealed the structure of P2 and its position in the outer capsid shell. The negative stain electron microscopic observations revealed that the isolated P2 protein has monomeric and a flexible L-shaped filamentous structure of ~ 20 nm in length, which agrees well with the result in the size-exclusion chromatography. The P2 proteins were located around the icosahedral 5-fold axis in the capsid. However, their density levels were lower than those of P3 and P8 proteins, and only partial rod-shaped structures of 10 nm in length were visible in the cryo-EM map of the intact RDV virion. These results may be caused by the enforcement of the icosahedral symmetry for the heterologous structures of P2 due to their flexibility during the imaging process and/or the low occupancy of P2. Where does the remaining portion of P2 disappeared in the 3D reconstruction exist? One end of the observed density corresponding to the partial structure of P2 faced the exterior at the Hollow I, and the other end faced the interior at the Hollow II (Fig. 2a). Together with the overall L-shaped structure of P2, there are two possibilities to extend the partial structure in the cryo-EM map: going inside at the Hollow II, and going outside at the Hollow I. As there is no space to accommodate the remaining portion of the P2 protein inside the Hollow II, we assume that the remaining portion of P2 is extended to the direction towards the exterior at the Hollow I (Fig. 2e).

The monomeric P2 protein is quite different from the trimeric cell-attachment proteins of animal reoviruses, such as VP4 of rotavirus (5, 34, 35) and VP2 of bluetongue virus (4, 36). *Reoviridae* is the largest

Fig. 2 Location and structure of the P2 protein in the capsid shell. (a) Surface representation of the overall structure of intact RDV. The P2 proteins are coloured in orange. The P2 proteins were located in the large crevasses between P8 trimers, designated as P trimer around the icosahedral 5-fold axes. The outer capsid shell of RDV is mainly composed of 260 trimers of the P8 protein, and the P8 trimers are designated P, Q, R, S and T trimers according to their positions in the $T = 13\ell$ icosahedral lattice (15, 31). (b) The P2 protein makes contact with two P8 trimers, indicated by the black arrowhead in the upper panel. Hydrophobic residues (Pro²⁰³, Ala²⁰⁴, Gly²⁰⁵ and Ala²⁰⁶) of P8 are located at the contact site and might be involved in the interactions. (c) Interactions between P8 trimers. There are seven kinds of interactions between P8 trimers in the outer capsid shell of RDV (32). Side views of contacts between adjacent P8 trimers (P-P and S-S contacts among them) are shown. The P-P contacts deviate markedly from other contacts since they have a large convex tilt angle. The upper domains of adjacent P trimers are more distantly separated from each other, with a tilt angle of $\sim 41^\circ$ as described previously (32). (d) Schematic representation of the P2 binding mechanism around icosahedral 5-fold axes. The P2 protein is docked in the crevasse formed by the P-P contact. (e) Proposed overall P2 structural models in the intact virion. The rod-shaped structure of 10 nm in length in the cryo-EM map is considered to be a partial structure of P2, because the size is much shorter than total length of P2 visualized by negative stain EM. The remaining structure of P2 is assumed to be disordered in the cryo-EM map due to the flexibility and to be extended outward at icosahedral axes based on the overall L-shaped structure of P2. The proposed structural models of P2 are shown in five different colours (magenta, green, yellow, sky blue and yellow green).

Fig. 3 Electron tomographic images showing the entry of RDVs into insect vector cells. (a–c) The virus attached to the plasma membrane by interaction of the P2 protein with cellular receptors. (d–f) The virus enclosed in a clathrin-coated pit. (a, d) Non-tilted images of the region used for tomography. (b, e) Slices of tomographic reconstructions, showing the dense forms connecting the viruses to the cellular membrane (indicated by arrows). (c, f) Schematic drawings corresponding to (b) and (e), respectively. The bars represent 200 nm.

and most diverse family of dsRNA viruses, whose hosts include plants, vertebrates, insects and fungi (1). All known reoviruses have a multi-layered capsid, with the exception of the single-layered CPV. The morphologies of the innermost capsid shells are quite similar in spite of the absence of significant sequence homology, while the morphologies of the outer capsid shells vary among genera (2, 3). Animal reoviruses like rotavirus and bluetongue virus are known to strip their outermost capsid shell from the virion

(the triple-layered particle) during infectious entry, and deliver into the cytosol an inner capsid particle (the double-layered particle), but the uncoating mechanism remains unclear (37). Unlike animal reoviruses, the RDV virion has a double-layered capsid that is similar to the core particles of animal reoviruses. RDV does not shed its outermost capsid shell upon host cell entry and retains the double-layered particle in the cytoplasm (13). These differences may reflect the diversity of the viral host-cell recognition mechanism,

Fig. 4 Proposed model of cell entry by *Rice dwarf virus* (RDV) P2 proteins with an L-shaped structure are docked in the large crevasses around icosahedral 5-fold axes. RDV particles attach to the host cellular membrane via interactions between the P2 proteins and as-yet unidentified receptor molecules. Then, the RDV particles enter the host cell via the clathrin-mediated endocytosis pathway (13).

in that the reoviruses probably acquired additional host-cell attachment proteins in the outer capsid shell during their evolution (2, 3).

Regulation of the spatial arrangement of P2 proteins

The spatial arrangement of P2 in the capsid is prescribed by the characteristic overall structure of RDV, in which P2 proteins anchor only at the circumference of the 5-fold axis of the viral surface. As described in previous studies (16, 32), the overall structure of RDV is more like a pronounced polyhedron, unlike other reoviruses such as rotavirus (38) and blue-tongue virus (31) that have spherical core particles. The overall polyhedral shape of RDV is caused by the shape of the outer surface of the inner capsid layer of the P3 protein, which forms the flat surface around the icosahedral 3-fold axis and protrudes

around the icosahedral 5-fold axis. Five icosahedrally unique P8 trimers (P, Q, R, S and T trimers) sit on the outer surface of the inner capsid layer at distinct positions and form the hexagonal network on the surface (Fig. 2a). Thus, the hexagonal pattern is considerably distorted at the edges and vertexes of the icosahedron (20), in that the interactions deviate from the quasi-equivalent theory (39). Indeed, the P-P contacts around the 5-fold axis deviate markedly from other contacts, because they have a large convex tilt angle, and the upper domains of adjacent P trimers are extremely separated from one another and hydrophobic residues are exposed, which result in the formation of large crevasses on the outer surface of RDV (Fig. 2c and d) (20). Thus, the rod-shaped structures of P2 are accommodated within the crevasses around the icosahedral 5-fold axes probably through the hydrophobic

interactions (Fig. 2a and b). The hydrophobic nature of the crevasses coincides well with the previous result calculating an electrostatic potential surface of the P8 trimer (15). Furthermore, the expected hydrophobic interactions between P2 and P8 also agree well with evidence that RDV loses P2 proteins after treatment with organic solvent, CCl₄.

Interactions between the P2 proteins of RDV and the host cell

Electron tomography clearly showed that RDV particles attached to host cells were away from the cellular membrane at a uniform distance (~15 nm; Fig. 3). Furthermore, several spike-like densities connecting the RDV particles and the host cellular membrane were observed, which were projected radially from the viral particles. The size of the structures was consistent with the size of the disordered region of P2 protein in the cryo-EM structure, which was assumed to be protruded outward from the RDV particle as described earlier (Figs 2e and 4). Thus, we attributed these spike-like structures to the P2 proteins on the viral surface, rather than receptor molecules on the host cell surface. The number of P2 proteins in one viral particle was estimated 10–30, based on SDS-PAGE analysis of purified RDV particles, which agree with previous studies that the P2 band intensity appears to be much less than one-half the intensity of the P3 band (120 copies in one particle) and slightly more than the intensity of the P1 band (12 copies in one particle) (10–11). Assuming that each particle has the P2 protein uniformly, then only a few P2 molecules bind around one icosahedral 5-fold axis of the viral particle. However, the estimation of the molecular number based on the SDS-PAGE of purified viruses is not always coincident with the actual value, owing to the removal of some proteins during virus purification, heterogeneity of viral particles or other unknown reasons. Therefore, we cannot exclude the possibility that five fragments of P2 distribute at each icosahedral 5-fold axis and that the number of P2 proteins in one RDV particle is 60. Thus, we left the 60 averaged structures of P2 protein in the RDV virion as they are. Further experiments are necessary to determine the correct copy number of P2 protein in one RDV particle in future.

Our results demonstrate that the combined approach of *in vitro* and *in vivo* structural studies has great utility in revealing the structural basis of the viral host-cell entry mechanism. When the virus or the macromolecular complex is highly purified and the structure is considerably rigid, we can determine the structure at an atomic or near-atomic resolution by X-ray crystallography or cryo-EM single-particle analysis (38, 40). However, flexible molecules like the P2 protein of RDV pose special difficulties for structural determination by these methods. By contrast, negative staining and single-particle image processing can explore dynamic properties of flexible and smaller macromolecules owing to their higher contrast (41, 42). In this study, we analysed the flexible structure of the P2 protein by negative stain EM. Furthermore, by combining the results with the partial

structure of the P2 protein in the capsid shell determined by cryo-EM, we were able to consider the whole structure of the flexible P2 protein on the viral surface. In addition to these *in vitro* structural analyses, we investigated the interactions between RDV and the host cell by electron tomography. Although the resolution of electron tomography is lower than that achieved by the *in vitro* structural analyses, electron tomography allows us to visualize and analyse the virus structures that actually carry out the interactions with the cellular components (43, 44). By combining *in vivo* structural information about the interactions between the viruses and the host cells and *in vitro* structural information at higher resolution, we can gain new insights into the detailed mechanism of viral attachment and invasion into the host cell (Fig. 4). Furthermore, if the dynamic events are followed or correlated by integrating high-resolution snapshots captured by electron tomography with other complementary methodologies, such as confocal light microscopy, then a far more accurate picture of the cellular events and a greater insight into the molecular architecture and cellular mechanisms would be obtained than by using any single method alone. We believe that these combined approaches will give us new routes for investigating the interactions between viruses and their host cells in the complex cellular context.

Accession numbers

The reconstruction has been deposited in the Electron Microscopy Database at the European Bioinformatics Institute with the accession number EMD-2782.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest

None declared.

References

1. Attoui, H., Mertens, P.P.C., Becnel, J., Belaganahalli, S., Bergoin, M., Brussaard, C.P., Chappell, J.D., Ciarlet, M., del Vas, M., Dermody, T.S., Dormitzer, P.R., Duncan, R., Fang, Q., Graham, R., Guglielmi, K.M., Harding, R.M., Hillman, B., Makkay, A., Marzachi, C., Matthijnssens, J., Milne, R.G., Mohd Jaafar, F.,

- Mori, H., Noordeloos, A.A., Omura, T., Patton, J.T., Rao, S., Maan, M., Stoltz, D., Suzuki, N., Upadhyaya, N.M., Wei, C. and Zhou, H. (2012) Family Reoviridae in *Virus Taxonomy: Ninth Report of the International Committee for the Taxonomy of Viruses*. (King A. M. Q., Adams M. J., Carstens E. B., and Lefkowitz E. J., eds.) pp. 541–637, Elsevier, Academic Press, New York
2. Hill, C.L., Booth, T.F., Prasad, B.V., Grimes, J.M., Mertens, P.P., Sutton, G.C., and Stuart, D.I. (1999) The structure of a cypovirus and the functional organization of dsRNA viruses. *Nat. Struct. Biol.* **6**, 565–568
 3. Miyazaki, N., Ichiki-Uehara, T., Xing, L., Bergman, L., Higashiura, A., Nakagawa, A., Omura, T., and Cheng, R.H. (2008) Structural evolution of *Reoviridae* revealed by *Oryzavirus* in acquiring the second capsid shell. *J. Virol.* **82**, 11344–11353
 4. Zhang, X., Boyce, M., Bhattacharya, B., Zhang, X., Schein, S., Roy, P., and Zhou, Z.H. (2010) Bluetongue virus coat protein VP2 contains sialic acid-binding domains, and VP5 resembles enveloped virus fusion proteins. *Proc. Natl. Acad. Sci. USA* **107**, 6292–6297
 5. Settembre, E., Chen, J.Z., Dormitzer, P.R., Grigorieff, N., and Harrison, S.C. (2011) Atomic model of an infectious rotavirus particle. *EMBO J.* **30**, 408–416
 6. Agosto, M.A., Ivanovic, T., and Nibert, M.L. (2006) Mammalian reovirus, a nonfusogenic, nonenveloped virus, forms size-selective pores in a model membrane. *Proc. Natl. Acad. Sci. USA* **103**, 16496–16501
 7. Zhang, L., Agosto, M.A., Ivanovic, T., King, D.S., Nibert, M.L., and Harrison, S.C. (2009) Requirements for the formation of membrane pores by the reovirus myristoylated μ 1N peptide. *J. Virol.* **83**, 7004–7014
 8. Omura, T. and Yan, J. (1999) Role of outer capsid proteins in transmission of *Phytoreovirus* by insect vectors. *Adv. Virus Res.* **54**, 15–43
 9. Zhong, B., Kikuchi, A., Moriyasu, Y., Higashi, T., and Hagiwara, K. (2003) A minor outer capsid protein, P9, of *Rice dwarf virus*. *Arch. Virol.* **148**, 2275–2280
 10. Yan, J., Tomaru, M., Takahashi, A., Kimura, I., Hibino, H., and Omura, T. (1996) P2 protein encoded by genome segment S2 of rice dwarf phytoreovirus is essential for virus infection. *Virology* **224**, 539–541
 11. Tomaru, M., Maruyama, W., Kikuchi, A., Yan, J., Zhu, Y., Suzuki, N., Isogai, M., Oguma, Y., Kimura, I., and Omura, T. (1997) The loss of outer capsid protein P2 results in nontransmissibility by the insect vector of rice dwarf phytoreovirus. *J. Virol.* **71**, 8019–8023
 12. Omura, T., Yan, J., Zhong, B., Wada, M., Zhu, Y., Tomaru, M., Maruyama, W., Kikuchi, A., Watanabe, Y., Kimura, I., and Hibino, H. (1998) The P2 protein of rice dwarf phytoreovirus is required for adsorption of the virus to cells of the insect vector. *J. Virol.* **72**, 9370–9373
 13. Wei, T., Chen, H., Ichiki-Uehara, T., Hibino, H., and Omura, T. (2007) Entry of *Rice dwarf virus* into cultured cells of its insect vector involves clathrin-mediated endocytosis. *J. Virol.* **81**, 7811–7815
 14. Zhou, F., Pu, Y., Wei, T., Liu, H., Deng, W., Wei, C., Ding, B., Omura, T., and Li, Y. (2007) The P2 capsid protein of the nonenveloped *rice dwarf phytoreovirus* induces membrane fusion in insect host cells. *Proc. Natl. Acad. Sci. USA* **104**, 19547–52
 15. Nakagawa, A., Miyazaki, N., Taka, J., Naitow, H., Ogawa, A., Fujimoto, Z., Mizuno, H., Higashi, T., Watanabe, Y., Omura, T., Cheng R.H., and Tsukihara, T. (2003) The atomic structure of *Rice dwarf virus* reveals the self-assembly mechanism of component proteins. *Structure* **11**, 1227–1238
 16. Hagiwara, K., Higashi, T., Miyazaki, N., Naitow, H., Cheng, R.H., Nakagawa, A., Mizuno, H., Tsukihara, T., and Omura, T. (2004) The amino-terminal region of major capsid protein P3 is essential for self-assembly of single-shelled core-like particles of *Rice dwarf virus*. *J. Virol.* **78**, 3145–3148
 17. Miyazaki, N., Hagiwara, K., Naitow, H., Higashi, T., Cheng, R.H., Tsukihara, T., Nakagawa, A., and Omura, T. (2005) Transcapsidation and the conserved interactions of two major structural proteins of a pair of phytoreoviruses confirm the mechanism of assembly of the outer capsid layer. *J. Mol. Biol.* **345**, 229–237
 18. Miyazaki, N., Wu, B., Hagiwara, K., Wang, C.Y., Xing, L., Hammar, L., Higashiura, A., Tsukihara, T., Nakagawa, A., Omura, T., and Cheng, R.H. (2010) The functional organization of the internal components of *Rice dwarf virus*. *J. Biochem.* **147**, 843–850
 19. Omura, T., Morinaka, T., Inoue, H., and Saito, Y. (1982) Purification and some properties of *Rice gall dwarf virus*, a new *Phytoreovirus*. *Phytopathology* **72**, 1246–1249
 20. Takahashi, Y., Tomiyama, M., Hibino, H., and Omura, T. (1994) Conserved primary structures in core capsid proteins and reassembly of core particles and outer capsids between *Rice gall dwarf* and *Rice dwarf phytoreoviruses*. *J. Gen. Virol.* **75**, 269–275
 21. Hagiwara, K., Higashi, T., Namba, K., Uehara-Ichiki, T., and Omura, T. (2003) Assembly of single-shelled cores and double-shelled virus-like particles after baculovirus expression of major structural proteins P3, P7 and P8 of *Rice dwarf virus*. *J. Gen. Virol.* **84**, 981–984
 22. Ludtke, S.J., Baldwin, P.R., and Chiu, W. (1999) EMAN: semiautomated software for high-resolution single-particle reconstructions. *J. Struct. Biol.* **128**, 82–97
 23. van Heel, M., Harauz, G., Orlova, E. V., Schmidt, R., and Schatz, M. (1996) A new generation of the IMAGIC image processing system. *J. Struct. Biol.* **116**, 17–24
 24. Tang, G., Peng, L., Baldwin, P.R., Mann, D.S., Jiang, W., Rees, I., and Ludtke, S.J. (2007) EMAN2: An extensive image processing suite for electron microscopy. *J. Struct. Biol.* **157**, 38–46
 25. Grigorieff, N. and Harrison, S.C. (2011) Near-atomic resolution reconstructions of icosahedral viruses from electron cryo-microscopy. *Curr. Opin. Struct. Biol.* **21**, 265–273
 26. Wei, T., Ichiki-Uehara, T., Miyazaki, N., Hibino, H., Iwasaki, K., and Omura, T. (2009) Association of *Rice gall dwarf virus* with microtubules is necessary for viral release from cultured insect vector cells. *J. Virol.* **83**, 10830–10835
 27. Wei, T., Miyazaki, N., Ichiki-Uehara, T., Hibino, H., Shimizu, T., Netsu, O., Kikuchi, A., Sasaya, T., Iwasaki, K., and Omura, T. (2011) Three-dimensional analysis of the association of viral particles with mitochondria during the replication of *Rice gall dwarf virus*. *J. Mol. Biol.* **410**, 436–446
 28. Kimura, I. (1986) A study of *Rice dwarf virus* in vector cell monolayers by fluorescent antibody focus counting. *J. Gen. Virol.* **67**, 2119–2124
 29. Kremer, J.R., Mastronarde, D.N., and McIntosh, J.R. (1996) Computer visualization of three-dimensional image data using IMOD. *J. Struct. Biol.* **116**, 71–76
 30. Zhong, B., Kikuchi, A., Moriyasu, Y., Higashi, T., Hagiwara, K., and Omura, T. (2003) A minor outer capsid protein P9, of *Rice dwarf virus*. *Arch. Virol.* **148**, 2275–2280

31. Grimes, J.M., Burroughs, J.N., Gouet, P., Diprose, M., Malby, R., Zientara, S., Mertens, P.P., and Stuart, D.I. (1998) The atomic structure of the bluetongue virus core. *Nature* **395**, 470–478
32. Iwasaki, K., Miyazaki, N., Hammar, L., Zhu, Y., Omura, T., Wu, B., Sjöborg, F., Yonekura, K., Murata, K., Namba, K., Caspar, D.L., Fujiyoshi, Y., and Cheng, R.H. (2008) Pleomorphic configuration of the trimeric capsid proteins of *Rice dwarf virus* that allows formation of both outer capsid and tubular crystals. *J. Mol. Biol.* **383**, 252–265
33. Kantardjiev, K.A. and Rupp, B. (2003) Matthews coefficient probabilities: improved estimates for unit cell content of proteins, DNA, and protein-nucleic acid complex crystals. *Protein Sci.* **12**, 1865–1871
34. Kraschnefski, M.J., Bugarcic, A., Fleming, F.E., Yu, X., von Itzstein, M., Coulson, B.S., and Blanchard, H. (2009) Effects on sialic acid recognition of amino acid mutations in the carbohydrate-binding cleft of the rotavirus spike protein. *Glycobiology* **19**, 194–200
35. Li, Z., Baker, M.L., Jiang, W., Estes, M.K., and Prasad, B.V. (2009) Rotavirus architecture at subnanometer resolution. *J. Virol.* **83**, 1754–1766
36. Nason, E.L., Rothagel, R., Mukherjee, S.K., Kar, A.K., Forzan, M., Prasad, B.V., and Roy, P. (2004) Interactions between the inner and outer capsids of bluetongue virus. *J. Virol.* **78**, 8059–8067
37. Baker, M. and Prasad, B.V. (2010) Rotavirus cell entry. *Curr. Top. Microbiol. Immunol.* **343**, 121–148
38. Zhang, X., Settembre, E., Xu, C., Dormitzer, P.R., Bellamy, R., Harrison, S.C., and Grigorieff, N. (2008) Near-atomic resolution using electron cryomicroscopy and single-particle reconstruction. *Proc. Natl. Acad. Sci. USA* **105**, 1867–1872
39. Caspar, D.L. and Klug, A. (1962) Physical principles in the construction of regular viruses. *Cold Spring Harbor Symp. Quant. Biol.* **27**, 1–24
40. Yu, X., Jin, L., and Zhou, Z.H. (2008) 3.88 Å structure of *cytoplasmic polyhedrosis virus* by cryo-electron microscopy. *Nature* **453**, 415–419
41. Burgess, S.A., Walker, M.L., Thirumurugan, K., Trinick, J., and Knight, P.J. (2004) Use of negative stain and single-particle image processing to explore dynamic properties of flexible macromolecules. *J. Struct. Biol.* **147**, 247–258
42. Ohi, M., Li, Y., Cheng, Y., and Walz, T. (2004) Negative staining and image classification – powerful tools in modern electron microscopy. *Biol. Proced. Online* **6**, 23–34
43. Iwasaki, K. and Omura, T. (2010) Electron tomography of the supramolecular structure of virus-infected cells. *Curr. Opin. Struct. Biol.* **20**, 632–639
44. Miyazaki, N., Nakagawa, A., and Iwasaki, K. (2013) Life cycle of phytoreoviruses visualized by electron microscopy and tomography. *Front. Microbiol.* **4**, 306