Hierarchical structure assembly model of rice dwarf virus particle formation

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Abstract

Rice dwarf virus (RDV) of the family *Reoviridae* and genus *Phytoreovirus*, is the cause of rice dwarf disease, a major negative effector of rice production throughout East Asia, including Japan. RDV has an icosahedral double-layered shell of approximately 70 nm diameter. The structural proteins constituting the capsid can self-assemble into a correct particle without requiring the help of any external factors in vitro. A total of more than 900 components assemble to make the full particle. A series of structural and functional studies of RDV, including X-ray crystallography and cryo-electron microscopy, suggest a hierarchical self-assembly mechanism involving both homologous and heterologous interactions. As such, systems for the recognition of each component should be essential for particle formation.

Keywords Virus assembly · Multi-layered virus · *Reoviridae* · Rice dwarf virus · Capsid structure

Introduction

Rice dwarf virus (RDV), the causal agent of rice dwarf disease, infects rice plants and negatively affects rice production in East Asia, including Japan. RDV is transmitted to rice plants by vector insects known as leafhoppers (*Nephotettix* spp). RDV is a member of the genus *Phytoreovirus* of the family *Reoviridae*. The *Reoviridae* has a wide host range, including humans, animals, insects and plants. RDV has an icosahedral double-layered capsid shell with the diameter of approximately 70 nm and contains 12-segmented double-stranded RNAs (S1 to S12) as the genome. The genome encodes 7 structural (P1, P2, P3, P5, P7, P8, P9) and 5 non-structural (Pns4, Pns6, Pns10, Pns11, Pns12) proteins (Omura and Yan 1999; Suzuki et al. 1992, 1996). The inner capsid shell formed by P3 proteins contains the genome and

transcription complexes (TC), which are composed of an RNA polymerase (P1), a capping enzyme which shows guanylyltransferase activity (P5) and nucleic acid binding proteins (P7). The outer capsid shell is mainly composed of P8 proteins as well as minor components of P2 and P9 proteins which are attached on the outer shell. The P2 protein is essential for the infection of insect cells (Omura and Yan 1999), but the function of P9 is still unknown. The capsid consists of a double-shelled structure; the inner core, which contains 120 copies of the P3 protein, and the outer shell, which consists of 780 copies of the P8 protein which form a tight trimer structure. Therefore, a total of 900 copies of various capsid proteins, along with other protein types and double-stranded RNAs, form the full virus particle. In vitro experiments show that a mixture of the protein inner core and outer shell proteins form a double-shell structure (Hagiwara et al. 2003; Miyazaki et al. 2005). Therefore, the capsid components have intrinsic abilities to assemble into a correct double-layered particle without any help (e.g., scaffold proteins) but with only subunit–subunit interactions. The precise atomic structure of an RDV particle determined by X-ray crystallography showed the details of the atomic interactions in the virus particle (Nakagawa et al. 2003). The interactions between subunits based on the high-resolution atomic structure of an RDV particle, some other biochemical studies and electron micrograph observations suggested hierarchical structural organization of the virus particles.

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Atomic structure of the RDV particle

The atomic structure of a double-shelled capsid of RDV was determined by X-ray crystallography. The electron density map was obtained by non-crystallographic symmetry averaging phase improvement and phase extension using a 26-Å resolution cryoEM map as a starting model. The phases were extended to 3.5 Å resolution and the resulting map showed clear electron density features of the molecules (Nakagawa et al. 2003). The crystal structure of RDV particle showed an inner core structure which consists of P3 proteins and P7 proteins, and an outer shell structure which consists of P8 proteins. As the 222 symmetry axis of the icosahedral symmetric virus particle shares the 222 symmetry axis of the crystal lattice, a quarter of a particle's structure was determined and then expanded to create the full particle structure (by 222 symmetry). The electron density map showed a fragment of peptides, which formed a beta-sheet structure with the inner core protein P3, around 5-fold axis of icosahedral symmetry. This region could be assigned as a part of P7, an RNA binding protein, from the structural feature of the electron density. In the crystal structure, 1986 out of 2028 residues of P3, 5436 out of 5473 residues of P8 and 12 out of 506 residues of P7 were identified and modeled. The final *R*-factor and free-*R*-factor at 230–3.5 Å resolution were 0.301 and 0.305, respectively.

The inner core is composed of 120 copies of the P3 protein with a icosahedral triangulation number, $T = 1$; this means that two independent P3 subunits form the inner core particle (Fig. 1a). The outer shell is composed of 780 copies of P8 subunits, which are arranged into a trimer structure in the capsid. The trimer structure of P8 shows tight interaction among the subunits by domain swapping (Fig. 1b). The P8 proteins show $T = 13I$ symmetry as shown in the low-resolution structure of RDV (Naitow et al. 1999). This means that five different trimers of P8 were found on the outer shell of a RDV particle (Fig. 1b). This symmetry is also found in

other viruses of the *Reoviridae* family (Grimes et al. 1998; Reinisch et al. 2000). However, the overall shape of RDV particles seems to be an icosahedron with sharp outer edges, whereas the structures of other *Reoviridae* family viruses, such as rotavirus (Prasad et al. 1996), reovirus (Reinisch et al. 2000) and bluetongue virus (Grimes et al. 1998), show more of a spherical shape.

The double-shelled structure of RDV consists of 120 copies of P3 and 780 copies of P8. The RDV particle can be assembled into a double-shell structure in vitro using only expressed P3 and P8 proteins (Hagiwara et al. 2003). It is mysterious how so many components can be assembled into a correct structure without the help of other factors. However, the structure of the full particle provides valuable information with regard to the self-assembly mechanism of RDV.

Structure organization of the double shell capsid

Expression of the P3 in a baculovirus system resulted in the formation of a single-shelled core-like particle. Furthermore, co-expression of P3 and P8 formed double-shell structures as observed by negative stain electron microscopy (Hagiwara et al. 2003). These results showed that P3 and P8 could form double-shelled particles without the help of any other components. These results also suggested that the inner core is self-assembled first, and then the outer capsid proteins attach after the completion of a virus particle formation. To propose a self-assembly mechanism, we assume that the tighter interactions found in the full particle cause earlier formation of the oligomeric structures.

Interactions of the inner core subunits

The potential energy of interaction between the subunits in the inner core can be estimated by summation of the potential energy contributions of the individual interactions (such as

Fig. 1 Structure of Rice dwarf virus. **a** Inner core structure formed by P3 proteins shown in Co-trace model. Icosahedrally-independent two structures of P3 are shown in different colors. **b** P8 trimer structure shown in the outer shell represented by cartoon model. Three different subunits are

shown in different colors. **c** Outer shell structure formed by P8 proteins shown in Co-trace model. Icosahedrally-independent five P8 trimers are shown in different colors

hydrogen bonds, salt bridges, van der Waals interactions) found in the RDV atomic structures. Among six different interactions between P3 subunits found in an inner core, the tightest interaction was found between the two adjacent icosahedrally independent subunits around icosahedral 5-fold axis (Fig. 2a). These two subunits have identical primary structures but show slightly different tertiary structures. Comparison of these structures showed that the N-terminal 50 residues of one of the subunits (subunit B) interacts with subunit A and changes its conformation (Fig. 2b). N-terminal 49 residues of subunit A are disordered and could not be fit into the electron density map, whereas the corresponding region of subunit B was interacted with subunit A and was well ordered. Comparison of the structures of subunit A and B suggested that the N-terminal 10 residues of subunit B blocked the extension of the N-terminal arm of subunit A (Fig. 3). Reconstruction experiments of the deletion mutants of N-terminal region showed that a 10-residue deletion decreased capsid particle formation and a 52-residue deletion could not form a particle at all (Hagiwara et al. 2004). Therefore, only 50 residues at the N-terminal out of the 1019-residue P3 subunit are essential for the formation of the particle structure, and the formation of the dimer structure seemed to be a starting point of the particle assembly. The formation of the inner core particle may be caused by the organization of the icosahedral symmetry. Assembly of P3 dimers may take place around either a 5-fold or a 3-fold axis. A low-resolution X-ray crystallography map (Naitow et al. 1999) and a CryoEM map (Miyazaki et al. 2010) showed that the transcriptional complex is attached around the 5-fold axis of icosahedral symmetry of the inner core. Even though the inner core protein P3 can be self-assembled into a particle, the attachment of transcriptional complex may facilitate the formation of the intermediate structure around the 5-fold axis during the formation of the inner core particle in vitro.

Interaction of the outer shell subunits

As described above, P8 forms a tight trimeric structure via domain swapping interactions and this trimer structure can

be considered as a unit of the assembly of the outer shell. This P8 trimer showed unique electrostatic features on the trimer–trimer interaction surface (Fig. 4); the right-hand half on the interaction surface showed positively charged and the left-hand half on the interaction surface showed negatively charged. This electrostatic feature, together with good shape complementarities (Lawrence and Colman 1993), may cause electrostatic attraction of side-by-side interaction of P8 trimers. P8 trimer itself forms a 2-dimensional monolayer (Zhu et al. 1997) or tubular structure (Iwasaki et al. 2008) using side-by-side interactions.

Interaction of inner core and outer shell subunits

Expression of the P3 in baculovirus system resulted in the formation of single-shelled core-like particles, and co-expression of P3 and P8 formed a double-shell structure (Hagiwara et al. 2003). These results suggested that the P8 trimer might be attached on the outer surface of the inner core after formation of the inner core structure. Comparison of the interactions between the inner core surface and the five different P8 trimers showed that the P8 trimer on the 3-fold axis of icosahedral symmetry showed the tightest interaction. In addition, electrostatic complementarities between the P8 trimer and the 3-fold axis area of the inner core showed good agreement (Fig. 5). Indeed, the P8-trimers on the icosahedral 3-fold axes are resistant even at the high salt conditions (Wu et al. 2000). This interaction results in a unique mode of attachment of P8 trimer on the 3-fold axis of the inner core and may trigger the formation of the outer shell structure.

Structural analysis of RDV with the minor outer capsid protein P2

The RDV X-ray crystal structure just described (Nakagawa et al. 2003) lacked the minor outer capsid protein P2, which is required for infection of insect cells, by the chemical treatment in the purification from infected plants. The structure of RDV with P2 proteins was revealed by single-particle analysis of cryoEM images (Miyazaki et al. 2016). In the cryo-EM map, the rod-

Fig. 2 Interactions of most tightly interacted dimer in a inner core. **a** Dimer structure of P3 proteins which show the tightest interaction in an inner core. **b** N-terminal tail of P3 is tightly interacted with the adjacent molecule

Fig. 3 N-terminal 10 residues of one of the subunit in the tightest interaction dimer blocked the extension of the N-terminal arm of the other subunit. **a** Close-up view of the N-terminal region of P3B (magenta). 1–50 residues of P3A (green) are completely disordered and could not build the model. **b** Superposition of a P3B (pink) onto P3A (green). If these two

subunit have similar folding, N-terminal tail of P3A must be more extended than the actual conformation found in a particle. **c** However, N-terminal 10 residues of P3B (magenta) blocks the extension of the P3A (pink) and change the conformation of P3A (green)

shaped densities attached to the surface of the outer capsid were clearly visualized at the lower density level. The rod-shaped structures were localized in crevasses formed by two P8 trimers located around 5-fold axes, designated as P trimers. The crystal

structure of the outer capsid protein P8 was fitted into the cryoEM map, and the fitted model 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. 4 Interaction between P8 trimers in an outer shell. **a** Electrostatic potential on the P8 trimer calculated by APBS (Baker et al. 2001) showing P8-P8 interface. **b** schematic representation of side-by-side interactions among P8 trimers view from a normal direction of a surface. Yellow triangles represent the P8 trimers. Positively charged patches (blue) and negatively charged patches (red) on the interfaces of the adjacent P8 trimers make electrostatic attractive interactions

Proposed model of structure assembly of Rice dwarf virus particle

From the observations described above, we propose a hierarchical structure assembly model of RDV particles. First, formation of a dimeric structure of P3 initiates the structure assembly of the inner core particle. During the process of dimer formation, the N-terminal 50 residues of a P3 subunit interact with the other P3 molecule to form a tight interaction between two subunits. The N-terminal 10 residues on the N-terminal interacting tail block the extension of the N-terminal tail of the other subunit and causes structural changes for dimerization. This dimer structure of P3 subunits acts as a piece of a jigsaw puzzle of an inner core structure. Then, this dimer of P3 subunits may assemble to form a decameric structure having 5-fold symmetry of icosahedral symmetry. In this process, a transcriptional complex may attach around a 5-fold axis in the formation of an inner core of an intact virus particle. After the formation of an inner core particle, the first P8 trimer will attach on the icosahedral 3-fold axis using electrostatic interactions between the inner core and a P8 trimer, then a total of 12 P8 trimers will be attached on all the 3-fold axes of the inner core particle. Using the side-by-side interactions of the P8 trimers, they may attach sequentially on the surface of an inner core. Finally, P8 trimers around icosahedral 5-fold axes will attach to form a full particle of RDV (Fig. 6). The interaction of P8 trimers around 5-fold is the weakest among the interactions between P8 trimers and an inner core. X-ray structure analysis also revealed that P8 trimers around the 5-fold axis show higher B-factors (Nakagawa et al. 2003). After the formation of the outer capsid, the P2

Fig. 5 Tightest interaction sites between a P8 trimer and an inner core is found at the icosahedral 3-fold axis with good electrostatic complementarity

Fig. 6 Proposed model of hierarchical structure assembly of RDV particle. **a** Dimer formation of P3 proteins initiate the structural assembly. **b** Dimer of P3 assemble around 5-fold axis to form decamer structure. Attachment of a TC and genome may help the assembly in vivo. **c** Icosahedral symmetry may support inner core particle formation to form an inner core structure. **d** P8 trimers attach at the 3-fold axis position on the surface of an inner core. **e** Side-by-side interaction between P8 trimers may support attachment of P8 trimers on the surface of an inner core. **f** Attachment of P8 trimers on the surface of an inner core by side-by-side interactions between P8 trimers and electrostatic interactions between P8 trimers and the inner core surface form full RDV particle

proteins are inserted into the crevasses formed by P8 trimers around 5-fold symmetry via hydrophobic interactions (Miyazaki et al. 2016).

Discussion

To form the correct double-shelled structure of RDV from many numbers of two different subunits (P3: 120; P8: 780), a systematic assembly pathway is required. Hierarchical structure assembly models based on the atomic structure of a full particle suggested ordered subunit interactions at the atomic level. The attachment of the first P8 trimers at a 3-fold axis of an inner core symmetry can be confirmed by the attempted formation of the particle from an inner core and P8 trimers in which the side-by-side interaction is interfered with via mutation. The electron microscopy showed layered structures of RNA in an RDV full particle (Miyazaki et al. 2010); however, it is still a large problem whether each particle contains 12 different segmented RNAs or the RNAs are encapsidated randomly in a particle. High-resolution cryoEM observations of the intermediate structure of virus particle assembly may be needed to solve this question. The actual process of the structure assembly should be more complex than the basic picture presented here. Attachment of other structural proteins, especially TC, encapsulation of genomes and attachment of other outer shell protein subunits, may support the assembly of the particle. In addition, virus assembly occurs inside and outside the viroplasma. Interaction between virus proteins and viroplasma proteins may support or enhance the formation of the inner core particle.

Conclusion

A virus particle is formed by many numbers, but limited kinds, of protein components. As these components self-assemble into the correct virus particle structure, a systematic recognition and assembly pathway should be essential. The rice dwarf virus particle is composed of 7 different proteins and 12 segments of dsRNA, and a total of more than 900 components are assembled into a full particle. In vitro expression of two protein components form a double-shell structure. Therefore, formation of a particle requires at least these two components. Hierarchical structure assembly models were proposed based on the atomic interactions of the subunits found in a particle. This suggested that systematic and hierarchical structure assembly might be essential to form the correct particle structure from many numbers of two different subunits. The packaging mechanism of other protein components, which form TC, and genomic RNA and attachments of outer capsid

minor protein components are still unknown. A combination of in vitro structural analysis of X-ray crystallography and in vivo structural analysis of cryoEM technique, combined with CEMOVIS (cryo-electron microscopy of vitreous sections) and/or FIB (focused ion beam) dissection, may be necessary to reveal the mechanism of full virus particle formation.

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Compliance with ethical standards

Conflict of interest Atsushi Nakagawa declares that he has no conflicts of interest. Naoyuki Miyazaki declares that he has no conflicts of interest. Akifumi Higashiura declares that he has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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