Viroplasm matrix protein Pns9 from rice gall dwarf virus forms an octameric cylindrical structure

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The non-structural Pns9 protein of rice gall dwarf virus (RGDV) accumulates in viroplasm inclusions, which are structures that appear to play an important role in viral morphogenesis and are commonly found in host cells infected by viruses in the family Reoviridae. Immunofluorescence and immunoelectron microscopy of RGDV-infected vector cells in monolayers, using antibodies against Pns9 of RGDV and expression of Pns9 in Spodoptera frugiperda cells, demonstrated that Pns9 is the minimal viral factor necessary for formation of viroplasm inclusion during infection by RGDV. When Pns9 in solution was observed under a conventional electron microscope, it appeared as ring-like aggregates of approximately 100 Å in diameter. Cryo-electron microscopic analysis of these aggregates revealed cylinders of octameric Pns9, whose dimensions were similar to those observed under the conventional electron microscope. Octamerization of Pns9 in solution was confirmed by the results of size-exclusion chromatography. Among proteins of viruses that belong to the family Reoviridae whose three-dimensional structures are available, a matrix protein of the viroplasm of rotavirus, NSP2, forms similar octamers, an observation that suggests similar roles for Pns9 and NSP2 in morphogenesis in animal-infecting and in plant-infecting reoviruses.

INTRODUCTION

Rice gall dwarf virus (RGDV) is a member of the genus Phytoreovirus in the family Reoviridae (Omura et al., 1982), and multiplies in both plants and invertebrate insect vectors. RGDV is transmitted propagatively to rice plants by leafhoppers (Morinaka et al., 1982). RGDV has a double-shelled, icosahedral capsid of approximately 65–70 nm in diameter (Omura et al., 1982) and contains 12 segments of dsRNA (S1–S12 in order of their mobility during SDS-PAGE; Hibit et al., 1984). S1, S2, S3, S5, S6 and S8 encode the structural proteins P1, P2, P3, P5, P6 and P8, respectively (Boccadoro et al., 1985; Hibit et al., 1984). The core capsid is composed of P3, the major protein, which encloses P1, P5 and P6 (Ichimi et al., 2002; Omura et al., 1985). The outer capsid of the virus is composed of the P2 protein (Yan et al., 1996) and the major outer-capsid protein P8 (Noda et al., 1991). The other segments of dsRNA encode putative non-structural (Pns) proteins, namely Pns4, Pns7, Pns9, Pns10, Pns11 and Pns12 (Koganezawa et al., 1990; Moriyasu et al., 2000, 2007; Noda et al., 1991). The functions of Pns4, Pns10 and Pns11 are unknown. Pns7 and Pns12 are components of the viroplasm (Wei et al., 2009). The segment S9 encoding Pns9 is 1202 nt and contains a single ORF encoding a protein of 323 aa with a molecular mass of 35.6 kDa (Koganezawa et al., 1990). The deduced amino acid sequence of this protein is only 16% identical to that of the non-structural protein Pns12 of rice dwarf virus (RDV; Moriyasu et al., 2007), which is a major component of the RDV viroplasm, in which viral particles are assembled (Wei et al., 2006).

In the present study, we have shown that Pns9 of RGDV is one of the component proteins of viroplasm-like inclusions on the basis of subcellular localization of the protein in infected insect vector cells and formation of aggregates that resemble the matrix of viral inclusion-like structures in the absence of other viral proteins. In addition, we present the structure of oligomerized Pns9, as determined by cryo-electron microscopy and three-dimensional (3D) reconstruction, and a comparison of RGDV Pns9 with rotavirus
NSP2. This is the first report, to our knowledge, of the ultrastructure of a viroplasm protein of a plant reovirus.

**RESULTS**

**Expression and purification of Pns9**

We expressed the non-structural protein Pns9 of RGDV in *Escherichia coli* as a carboxyl-terminal His-tagged fusion protein. Most of the Pns9 protein was soluble when expressed in cells at 37°C. We purified His-tagged Pns9, with 329 aa, by Ni-NTA affinity chromatography, as confirmed by the mobility of a single protein band during SDS-PAGE (Fig. 1a) and raised antibodies in rabbits against the purified His-tagged protein.

**Subcellular localization of Pns9 in insect cells**

To determine whether the non-structural protein Pns9 of RGDV plays a key role in the formation of inclusions, we examined the subcellular distribution of Pns9 of RGDV in infected vector cell monolayers (VCMs) of the insect *Nephotettix cincticeps* by confocal immunofluorescence microscopy. We detected Pns9 as discrete, punctate inclusions (Fig. 1b) when we fixed infected VCMs at 48 h post-infection (p.i.) and probed them with Pns9-specific antibodies. To confirm our observations, we examined VCMs that had been infected with RGDV and fixed at 48 h p.i. by electron microscopy. Examination of ultrathin sections of RGDV-infected VCMs revealed the presence of granular, electron-dense inclusions of 600–850 nm in diameter in the cytoplasm of infected cells (Fig. 1c). To determine the composition of these electron-dense inclusions, we examined the subcellular localization of Pns9 in infected VCMs by immunoelectron microscopy. As shown in Fig. 1(c), the matrix of the inclusions was immunolabelled with antibodies against Pns9. It should be noted that Pns9 appeared to accumulate towards the edge of viroplasms (Fig. 1b).

**Non-structural proteins Pns7, Pns9 and Pns12 are constituents of viral inclusions**

In a previous study, the non-structural proteins Pns7 and Pns12 were detected as punctate inclusions and were co-localized in the viroplasm (Wei *et al.*, 2009). To determine whether Pns9 also co-localized with Pns7 and Pns12 in the viroplasm, we examined RGDV-infected VCMs by confocal immunofluorescence microscopy. Infected cells were fixed at 48 h p.i. and probed with Pns7-, Pns9- and Pns12-specific antibodies. As shown in Fig. 2, Pns7, Pns9 and Pns12 were detected in discrete, punctate inclusions and these three non-structural proteins co-localized, demonstrating that the non-structural protein Pns9 is a component of the viroplasm, together with Pns7 and Pns12.

**Pns9 forms viral inclusion-like structures in Sf9 cells**

The non-structural protein Pns9 of RGDV thus appears to be one of the major constituents of the viral inclusions in RGDV-infected cells. To identify the protein that is responsible for the formation of the matrix of viral inclusions, we inoculated *Spodoptera frugiperda* (Sf9) cells with recombinant baculovirus expressing Pns9 and confirmed the expression of Pns9 by Western blotting (Fig. 3a). Immunofluorescence staining of Pns9 in Sf9 cells that

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Fig. 1. SDS-PAGE and subcellular localization of Pns9. (a) Expression and purification of His-tagged Pns9. Purified Pns9 was analysed by SDS-PAGE. Lane 1, Cell lysate before induction of the expression of His-tagged Pns9; lane 2, cell lysate after induction of the expression of His-tagged Pns9; lane 3, purified His-tagged Pns9. (b) Subcellular localization of Pns9 of RGDV in virus-infected VCMs. Cells were immunostained with Pns9-specific IgG that had been conjugated to FITC. Images were obtained by confocal microscopy. Immunostaining of Pns9 is shown in green. Bar, 10 μm. (c) Immunogold labelling of Pns9 in electron-dense inclusions in virus-infected VCMs. Cells were immunostained for Pns9 with specific polyclonal antibodies as primary antibodies and subsequent treatment with 15 nm gold particle-conjugated goat antibodies against rabbit IgG as secondary antibodies. Bar, 0.5 μm.
had been grown on coverslips revealed the formation of discrete, punctate inclusions within the cells at 48 h p.i. (Fig. 3b). Our results demonstrated that expression of the viral protein Pns9, by itself and in the absence of viral multiplication, was sufficient for the formation of viral inclusion-like structures in Sf9 cells.

**Pns9 forms octameric ring-like structures**

To examine the possible oligomerization of Pns9 in solution, we performed gel-filtration chromatography. As shown in Fig. 4(a), Pns9 eluted as a major peak that corresponded to a molecular mass of 295 kDa, a value that is eight times the molecular mass of Pns9. To determine the configuration of Pns9 octamers, we examined a solution of Pns9 by conventional electron microscopy. As shown in Fig. 4(b), multimers of Pns9 adopted a characteristic profile that was easily discerned in electron micrographs of negatively stained particles. The ring-like structures formed by Pns9 had an approximate outer diameter of 100 Å.

**Single-particle cryo-electron microscopic examination of Pns9**

The 3D structure of Pns9 is shown in Fig. 5. The projection images calculated from the 3D reconstruction were consistent with the appearance of the structures in cryo-electron micrographs of Pns9 (Fig. 5a). Ring-like and cylindrical structures, as seen in conventional electron micrographs (Fig. 4b), were also observed. We generated class-averaged images from the boxed particles shown in Fig. 5(b). The resultant structure appeared to be a cylinder, with the dimensions $100 \times 100 \times 130$ Å (Fig. 5c). When

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**Fig. 2.** Co-localization of Pns7 and Pns12 with Pns9. VCMs were inoculated with RGDV, fixed at 48 h p.i. and stained with Pns7- or Pns12-specific IgG conjugated to FITC and with Pns9-specific IgG conjugated to rhodamine, and then examined by confocal fluorescence microscopy. Immunostaining of Pns7 and Pns12 is shown in green and that of Pns9 is shown in red. Merged views show the co-localization of Pns7 and Pns12 with Pns9, as shown in yellow. Bars, 10 μm.

**Fig. 3.** Expression of Pns9 in Sf9 cells. (a) Western blot analysis of Pns9 with Pns9-specific IgG. Pns9 was expressed in Sf9 cells using a baculovirus system. Lane 1, Pns9 expressed in Sf9 cells; lane 2, mock-infected Sf9 cells. (b) Distribution of Pns9 in Sf9 cells. Cells were fixed and immunostained with FITC-conjugated Pns9-specific IgG and visualized by confocal microscopy. Bar, 10 μm.
this structure was observed from 'above', it resembled a ring with an inner diameter of 35 Å. When the structure was observed from the 'side', it appeared to have a constriction with an upper tetrameric ring connected to a lower one.

**DISCUSSION**

The co-localization in viral inclusions of Pns7, Pns9 and Pns12 (Fig. 2) of RGDV, which correspond to viroplasm components Pns6, Pns12 and Pns11 of RDV, respectively (Wei et al., 2006), demonstrates that Pns7, Pns9 and Pns12 are constituents of the viroplasm of RGDV. Expression of Pns9 in SF9 cells (Fig. 3), non-hosts of RGDV, in the absence of other viral proteins resulted in the formation of viral inclusion-like structures that resembled the inclusions in RGDV-infected VCMs (host cells). These results indicated that Pns9 has the intrinsic ability to aggregate to form the matrix of viral inclusions in infected cells, and also that Pns9 is the minimal viral factor required for the formation of viral inclusions during infection of host cells by RGDV. Analysis of the role of each of Pns7, Pns9 and Pns12 for viral morphogenesis remains to be clarified in future studies.
When a solution of Pns9 (Fig. 1a) was observed by conventional electron microscopy, ring-shaped aggregates of approximately 100 Å were clearly apparent (Fig. 4b). Cryo-electron microscopic analysis of these aggregates revealed cylinders with an octameric structure (Fig. 5), with dimensions similar to that of structures visualized by conventional electron microscopy. Octamerization of Pns9 was confirmed by the results of size-exclusion chromatography (Fig. 4a).

Aggregates of Pns9 of RGDV resembled those of NSP2 of rotavirus. NSP2 is the only viroplasm protein whose 3D structure is available among those of viruses in the family Reoviridae (Jayaram et al., 2002). By comparison of Pns9 and NSP2 at 20 Å resolution, we found that these proteins both have cylindrical and octameric structures, although we could not find substantial similarity in their primary structures. Similarity in the 3D structures of the proteins of viruses that infect plants and animals implies that an octameric structure with a pore inside may be a basic structure for functioning of the protein as one of the matrix proteins of the viroplasm.

When we compared the dimensions of the octameric and cylindrical aggregates of the two proteins, we found that aggregates of Pns9 were larger than those of NSP2, namely 100 × 100 × 130 Å for Pns9 compared with 85 × 85 × 100 Å for NSP2 (Fig. 6). In addition, the pores of Pns9 aggregates were 35 Å in diameter, whereas those of NSP2 were 20 Å in diameter. The octamer of Pns9 is cylindrical, whilst that of NSP2 is flatter and resembles a doughnut. In each Pns9 octamer, an upper tetramer interacts vertically with a lower one, whilst the orientations of the upper and lower tetramers are twisted relative to one another in the NSP2 octamer. Higher-resolution studies such as X-ray crystallographic analysis of the Pns9 would enable us to make more detailed comparisons such as surface charge distribution (Taraporewala et al., 2006) between viroplasm matrix proteins from plant and animal reoviruses. In the analysis of the structure–function relationship of NSP2, two conserved histidines (H221 and H225) and surrounding basic residues (K188, K223 and R227) were found to contribute the nucleoside triphosphatase (NTPase) activity (Vasquez-Del Carpio et al., 2006), 5′-RNA triphosphatase (RTPase) activity (Vasquez-Del Carpio et al., 2006) and nucleoside diphosphate kinase-like activity (Kumar et al., 2007). These activities were performed inside a cleft between the two domains of NSP2 that exhibits structural similarity to ubiquitous cellular histidine triad (HIT) proteins (Lima et al., 1997). However, the region that corresponds to the HIT-like motif was not found in the primary structure of Pns9. Furthermore, NSP2 is a basic protein (Jayaram et al., 2002), whereas Pns9 is an acidic protein.

NSP2 of rotaviruses forms viroplasms in the presence of NSP5 (Eichwald et al., 2004; Fabbretti et al., 1999), and NSP2 contributes to the binding of the innermost capsid protein to the RNA-dependent RNA polymerase (Kattoura et al., 1994; Prasad et al., 1996), to the binding of ssRNA and to helix-destabilizing (Taraporewala & Patton, 2001; Taraporewala et al., 1999), NTPase and RTPase (Vasquez-Del Carpio et al., 2006) activities. In addition, structural information at the atomic level has suggested a hydrolytic mechanism for nucleotide binding by NSP2 (Kumar et al., 2007). Moreover, regulation of the binding of NSP2 to RNA by NSP5 has been clarified by single-particle analysis of octamers of NSP2 by electron microscopy (jiang et al., 2006). The various properties of NSP2 suggest that this viral protein contributes to packaging and replication of the viral genome by relaxing the secondary structures in viral template dsRNAs that impede polymerase function and by facilitating the translocation of viral RNAs into pre-virion cores. In plant reoviruses, the P9-1 protein of Mal de Rio Cuarto virus (MRCV) belonging to the genus Fijivirus has cytopathological properties similar to RGDV Pns9 and has some biochemical properties similar to rotavirus NSP2 (Maroniche et al., 2010). Similar cytopathological properties and structural features of RGDV Pns9 and rotavirus NSP2, and similar cytopathological properties of RGDV Pns9 and MRCV P9-1, suggest that RGDV Pns9 might also exhibit the various biochemical and molecular biological properties attributed to rotavirus NSP2 and MRCV P9-1, and might contribute to virus replication. Confirmation of this hypothesis will be the focus of future studies.

Fig. 6. Comparison of the structures of octamers of Pns9 and of rotavirus NSP2. (a) Structure of octamers of Pns9. The image on the right was generated by rotation of the image on the left through 90°. (b) Structure of octamers of rotavirus NSP2, calculated from atomic coordinates at 20 Å resolution from Protein Data Bank entry number 1L9V (Jayaram et al., 2002). The image on the right was generated by rotation of the image on the left through 90°. Bar, 50 Å.
Viruses in the family *Reoviridae* are grouped into 12 genera and have a wide range of hosts, such as mammals, fishes, insects, plants, fungi and bacteria. Among viruses in the family *Reoviridae*, those reported to form viroplasms are as follows: rotaviruses (McNulty et al., 1976), bluetongue virus (Eaton et al., 1987), animal reoviruses (Sharpe et al., 1982), avian reoviruses (Touris-Otero et al., 2004), fijiviruses (Maronica et al., 2010; Zhang et al., 2008), RDV (Wei et al., 2006) and RGDV. The basically similar, but in many ways different, structures of the viroplasms of viruses that infect plants and animals suggest that the structures of reoviral viroplasms have evolved to adapt to the environments of the respective hosts.

**METHODS**

**Cells and viruses.** NC-24 cells derived from *N. cincticeps* were maintained in monolayer cultures as described elsewhere (Kimura, 1986; Omura et al., 1988). Inocula of RGDV were prepared as described previously (Omura et al., 1988) and stored at −80 °C. Synchronous infection of VCMs by RGDV was initiated as described previously (Omura et al., 1988). In brief, cells grown on a coverslip (15 mm diameter) were inoculated with 50 µl of a preparation of RGDV (m.o.i. of 1) for 2 h at 25 °C. After removal of the inoculum, inoculated monolayers were incubated at 25 °C for 2 days prior to fixation.

**Construction of the Pn9 expression plasmid.** For expression of Pn9 in *E. coli*, the cDNA encoding Pn9 was amplified with KOD-plus DNA polymerase (Toyobo) from a cDNA clone (Koganezawa et al., 1990) using forward primer 5′-TGGACATATGTTTACATCTT-CTGCAGGCAA-3′ and reverse primer 5′-TGCTCGAGAGGGTT-CTTCCAGGTCTGTAATA-3′, and inserted at the Ndel/Xhol sites of pET21a (Novagen) to generate pET21a-Pn9, which encoded Pn9 with a carboxyl-terminal 6×His tag. The integrity of all constructs was assessed by DNA sequence analysis using a 3130 Genetic Analyzer (Applied Biosystems). For expression of Pn9 in insect cells, the cDNA encoding Pn9 was amplified as described above using forward primer 5′-GGGAGCAGGTTGTACAAAAAGCGGGTTAATGTACATCTTCTCGAGGCAA-3′ and reverse primer 5′-GGGACCTC-ACITTGTCACAAAAAGCGGGTGTTCAAGGTATCCTCCAGGTCTGTAATA-3′. It was subcloned into the pDONR-221 vector (Invitrogen) to generate pENTR-Pn9 as an entry vector. Subsequently, the cDNA encoding Pn9 in this vector was cloned into pDEST-Pn9 (Invitrogen) using Gateway technology (Invitrogen), according to the manufacturer’s instructions, to generate pDEST-Pn9. The integrity of all constructs was assessed by DNA sequencing, as described above.

**Expression and purification of Pn9.** *E. coli* Rosetta-gami BBL21(DE3) cells (Novagen) were transformed with pET21a-Pn9 and cultured in 1 litre of Luria–Bertani medium containing 50 µg ampicillin ml⁻¹ and 15 µg tetracycline ml⁻¹ at 37 °C to an optical density at 600 nm of 0.6–0.9. Expression of the His-tagged protein was induced by addition of IPTG to a final concentration of 0.1 mM. After cultivation for 4 h at 37 °C, cells were harvested by centrifugation. The His-tagged Pn9 protein was purified by affinity chromatography on Ni-NTA resin (Qiagen), as recommended by the manufacturer. Pn9 that had bound to the resin was eluted with buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 100 mM imidazole. Fractions containing Pn9 were identified by SDS-PAGE, pooled and dialysed against buffer containing 10 mM Tris/HCl (pH 7.4) and 150 mM NaCl for 16 h at 4 °C. Approximately 5 mg Pn9 was obtained from a 1 litre culture.

**Expression of Pn9 in S9 cells.** The pDEST-Pn9 plasmid was introduced into *E. coli* DH10Bac (Invitrogen) for transposition into the bacmid. The recombinant bacmid was isolated and used to transfect S9 cells in the presence of Cellfectin (Invitrogen), according to the manufacturer’s instructions. After 72 h transfection, S9 cells were collected and expression of proteins was examined by immunoblotting with antibodies specific for Pn9. For cytological observations, S9 cells that were infected with the recombinant baculovirus and the expression of proteins was monitored as described previously (Wei et al., 2006).

**Immunofluorescence staining.** The rabbit polyclonal antiserum against Pn9 and Pns12 that we have used were described previously (Moriyasu et al., 2007). IgG, isolated from specific polyclonal antisera on a protein A–Sepharose affinity column, was conjugated directly to FITC or rhodamine according to the manufacturer’s instructions (Invitrogen). At various times after inoculation, VCMs and S9 cells that had been cultured on glass coverslips were stained with these conjugates and visualized under a confocal laser-scanning microscope (LSM 510; Carl Zeiss MicroImaging), as described elsewhere (Wei et al., 2006).

**Immunoelectron microscopy.** Cells on coverslips were fixed, dehydrated and embedded in LR gold resin (Oken Shoji Co.), as described previously (Wei et al., 2006). Polymerization of the resin was allowed to proceed for 72 h at −20 °C. Samples were sectioned on an ultramicrotome (LKB) with a diamond knife and then incubated with the appropriate rabbit antisera and immunogold-labelled goat antibodies against rabbit IgG that had been conjugated with 15 nm gold particles (GAR 15; British Bifocals International), as described previously (Wei et al., 2006). Sections were stained for 2 h with 2% uranyl acetate and 5 min with lead citrate and observed under an electron microscope (H-7000; Hitachi High-Technologies).

**Size-exclusion chromatography.** Size-exclusion chromatography was performed using a Superdex 200 pg column (GE Healthcare). The column was equilibrated with storage buffer. Size-exclusion chromatography was performed at a flow rate of 0.5 ml min⁻¹ with the same buffer. Elution was monitored in terms of absorbance at 280 nm. The following standards were used to calibrate the column: blue dextran (to define the excluded volume), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa) and ovalbumin (43 kDa) (all from GE Healthcare). Distribution coefficients (Kd) were calculated from the equation 

\[
K_d = \frac{V_e}{V_i} - 1
\]

where \( V_e \) is the elution volume for the protein of interest, \( V_i \) is the void volume and \( V_c \) is the included volume.

**Cryo-electron microscopy and imaging procedures.** Samples of Pn9 at 0.2 mg ml⁻¹ were embedded in vitreous ice and examined at 40–70 K with a cryo-electron microscope (IEM-3200FSC/BU; JEOL), which incorporated a field emission gun, a liquid helium stage and an omega-type energy filter operated at 200 keV and a nominal magnification of ×80 000. A slit width of 20 eV was used to obtain a zero energy loss electron beam. Images were recorded on a 8K×8K pixel complementary metal-oxide semiconductor camera (twofold binning; TVIPS) with a dose of ~20 e⁻/Å² applied at underfocus values of approximately 3.0 μm. Single-particle cryo-electron microscopy analysis, including particle selection and 3D reconstruction, was performed using the IMAGIC suite (Ludkte et al., 1999) and IMAGIC (van Heel et al., 1996). Particles were selected from individual charged-coupled device frames (with an effective pixel size of 2.1 Å) using the program Boxer in the IMAGIC suite. The particle images were rotationally and translationally aligned by a multireference alignment procedure and subjected to multivariate statistical analysis specifying 20 classes using the IMAGIC program. The 20 class-averaged images were used to generate an initial model with C4 symmetry, as described by Ludkte et al. (1999). Reference-based iterative refine-
ment using D4 symmetry was then performed to obtain the final reconstruction. The final reconstruction of Ps9 octamers was computed from 6821 particles, and the resolution was assessed to 20 Å with a 0.5 threshold in the Fourier shell correlation between two reconstructions, which was calculated from two halves of each dataset. Thus, the map was filtered to 20 Å resolution.

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