Pns12 protein of *Rice dwarf virus* is essential for formation of viroplasms and nucleation of viral-assembly complexes

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INTRODUCTION

Intracellular replication events have been described in detail for animal reoviruses, including reovirus, rotavirus and *Bluetongue virus* (BTV) (Estes, 2001; Nibert & Schiff, 2001; Roy, 2001), and these events are believed to be common to other members of the family *Reoviridae*. Upon infection, vertebrate reoviruses shed their outer capsid and the virus core initiates expression of the viral genome. Segments of the viral genome are transcribed by core-associated RNA polymerases and mRNAs are produced. Viral mRNAs have two functions in an infected cell: they programme the synthesis of viral proteins and they serve as templates for the generation of double-stranded RNA (dsRNA). Replication of the viral genome begins with the sorting and packaging of viral mRNA, which is followed by synthesis of segments of the dsRNA genome. In a coupled process, inner capsid proteins assemble to form core particles. Then, progeny core particles appear to be coated by outer capsid proteins to generate mature virions. Although the precise timing and molecular mechanisms of these processes are largely unknown, it has been proposed that virus replication and the assembly of animal reoviruses occur in discrete, punctate viral inclusions within the cytoplasm of infected cells. These inclusions are commonly called viral factories or viroplasms in the case of reoviruses (Fields et al., 1971; Parker et al., 2002; Broering et al., 2005) and rotavirus (Fabbretti et al., 1999; Mohan et al., 2003), whereas, in the case of BTV, they are called viral inclusion bodies (Thomas et al., 1990; Brookes et al., 1993). Several structural and non-structural proteins have been implicated in the formation of viral
inclusions in cells that have been infected with animal reoviruses (Thomas et al., 1990; Fabbretti et al., 1999; Broering et al., 2002; Becker et al., 2003; Mohan et al., 2003).

Rice dwarf virus (RDV) is an icosahedral, double-layered particle of 69 Å (69.3 nm) diameter that belongs to the genus Phyto Reovirus in the family Reoviridae (Boccardo & Milne, 1984; Nakagawa et al., 2003). The viral genome consists of 12 segmented dsRNAs that encode seven structural (P1, P2, P3, P5, P7, P8 and P9) and five non-structural (Pns4, Pns6, Pns10, Pns11 and Pns12) proteins (Omura & Yan, 1999; Zhong et al., 2003). The core capsid is composed of: P3, the major protein, which encloses P1, a putative RNA polymerase; P5, a putative guanylyl transferase; and P7, a protein with nucleic acid-binding activity. The outer capsid of the virus is composed of: the P2 protein, which is involved in the ability of the virus to infect insect vector cells; the major outer capsid protein, P8; and the minor outer capsid protein, P9. Among the non-structural proteins: Pns4 contains a putative zinc finger and GTP-binding motif (Uyeda et al., 1999); Pns6 might be required for cell-to-cell movement of RDV (Li et al., 2004); Pns11 is known to bind nucleic acids (Xu et al., 1998); and Pns12 is a phosphorylated protein (Suzuki et al., 1999).

Many cytopathological studies of plant reoviruses in infected plants and vector insects were reported in the 1960s and 1970s. Diseased plants and viruliferous vector insects were characterized by the appearance of at least two distinct cytopathological structures: electron-dense inclusions with virus-like particles interspersed within or distributed at the periphery of the matrix, namely, viroplasms; and tubular structures (Fukushi et al., 1962; Shikata, 1969). It is generally considered that the viroplasms are the sites of virus synthesis. However, the details of replication and assembly of RDV have remained largely unknown. We postulated that combining currently available immunocytochemical techniques and cultures of leafhopper vector cells in monolayers (VCMs) would allow us to extend earlier studies of the replication of RDV and the cytopathology of infected cells. Our hypothesis was based on the fact that 100% infection of VCMs can be achieved with a highly diluted inoculum, allowing the synchronous replication and multiplication of the virus to be monitored in detail (Omura & Kimura, 1994).

In the present analysis, confocal immunofluorescence microscopy and immunoelectron microscopy were used to investigate the constituents of viral inclusions and the time course of their formation in RDV-infected VCMs. Our results suggest that the non-structural proteins Pns6, Pns11 and Pns12 of RDV are the major constituents of the matrix of viral inclusions in which the assembly of progeny virions and the synthesis of viral RNA are thought to occur. Heterologous expression systems were used to demonstrate that Pns12 has the intrinsic ability to form aggregates that resemble the matrix of viral inclusion-like structures in the absence of other viral proteins.

**METHODS**

**Cells and viruses.** NC-24 cells, originally established from embryonic fragments dissected from eggs of Nephrotettix cincticeps, were maintained in monolayer culture at 25°C in growth medium that had been prepared as described by Kimura & Omura (1998). The O strain of RDV was purified from infected rice plants without using CCl₄, as described by Zhong et al. (2003). Sucrose-gradient fractions containing purified RDV particles were pooled and stored at −70°C.

Synchronous infection of VCMs by RDV was initiated as described by Kimura (1986). When each cultured monolayer of leafhopper cells on a coverslip (15 mm diameter) reached 80% confluence, cells were washed twice with a solution of 0.1 M histidine that contained 0.01 M MgCl₂ (pH 6.2; His-Mg) and then inoculated with 50 μl virus preparation. Each inoculum was obtained from a series of tenfold dilutions of a preparation of purified virus and cells were incubated with each inoculum for 2 h at 25°C. Then, after removal of the inoculum, each monolayer was washed twice with His-Mg and each coverslip was covered with 0.12–0.2 ml medium. Inoculated monolayers were incubated at 25°C for various times prior to fixation.

**Antibodies.** Rabbit polyclonal antiserum against the products of genome segments S1–S12 were prepared as described previously (Suzuki et al., 1994, 1999; Zhong et al., 2003). The large ORF (Suzuki et al., 1992) was used for antibody preparation using Pns12 expressed in vitro.

**Baculovirus expression of non-structural proteins of RDV.** A baculovirus system was used for expression of Pns6, Pns11 and Pns12, as described by Miyazaki et al. (2005). The coding region of the cDNA that encoded RDV Pns6, Pns11 and Pns12, cloned in the pGADT7 vector (Clontech), was sequenced completely to verify the absence of any misincorporation of nucleotides during amplification. After digestion with the appropriate restriction enzymes, the cDNA was ligated into the pfastBac donor plasmid (Invitrogen). Recombinant pfastBac was then introduced into Escherichia coli DH10Bac cells (Invitrogen) for transposition into the bacmid. The recombinant bacmid was isolated and used to transfect Spodoptera frugiperda (SF9) cells in the presence of Cellfectin (Invitrogen) according to the manufacturer’s instructions. Then, 72 h after transfection, SF9 cells were collected and expression of proteins was examined by immunoblotting with antibodies specific for RDV Pns6, Pns11 and Pns12.

For cytological observations, SF9 cells were seeded the day before transfection at a density of 1.5 × 10⁶ cm⁻² on glass coverslips (15 mm diameter). The culture medium was removed and cells were infected with the recombinant baculovirus. Cells were then incubated for 12–72 h at 27°C before being processed for immunofluorescence microscopy.

**Expression and purification of recombinant Pns12.** The large coding region of the cDNA that encoded RDV Pns12 was cloned as an EcoRI–XhoI fragment into the vectors pMAL-c2X (New England Biolabs) and pET-30a (Novagen), respectively, and expressed in E. coli BL21(DE3) cells (Novagen). Pns12 was then purified as a fusion of Pns12 and maltose-binding protein (MBP–Pns12) according to the instructions from New England Biolabs.

**Filter-binding assay.** The filter-binding assay was performed by a method similar to that described by Ueda et al. (1997). An extract of E. coli cells that expressed histidine-tagged Pns12 of RDV (His-Pns12) was fractionated by electrophoresis on an SDS-PAGE gel (12% polyacrylamide) and bands of protein were transferred to an Immobilon-P (Millipore) PVDF membrane filter. After the filter had been blocked with 5% skimmed milk in PBS (137 mM NaCl,
RESULTS

Non-structural proteins Pns6, Pns11 and Pns12 are the constituents of viral inclusions

To determine whether the non-structural proteins of RDV play a key role in the formation of inclusions, the subcellular localization of non-structural proteins of RDV in infected VCMs was examined by confocal immunofluorescence microscopy. Infected cells were fixed 18 h p.i. and probed with Pns4-, Pns6-, Pns10-, Pns11- or Pns12-specific antibodies. In RDV-infected cells, Pns11 and Pns12 were detected in discrete, punctate inclusions (Fig. 1). Pns6 was distributed diffusely in the cytoplasm of infected cells and was also concentrated in the punctate inclusions (Fig. 1). When the various images were merged, it was observed that three non-structural proteins co-localized, suggesting that the non-structural proteins Pns6, Pns11 and Pns12 are the constituents of the viral inclusions (Fig. 1). In contrast, Pns4 and Pns10 were detected on fibrillar-like structures and tubular structures, respectively (Fig. 1).

Detection of newly made RNA in vivo by immunofluorescence.

To label RNA in vivo at 10 h post-inoculation (p.i.), VCMs grown on glass coverslips were washed with His-Mg and then treated for 30 min with 10 μg actinomycin D ml⁻¹ (Sigma-Aldrich). VCMs were then further incubated for 30 min in the presence of 10 mM bromouridine 5'-triphosphate (BrUTP; Sigma-Aldrich) and 6% Cellfectin transfection reagent (Invitrogen), as described by Silvestri et al. (2004). Cells were fixed at 11 h p.i. and processed for immunofluorescence analysis with monoclonal anti-bromodeoxyuridine (BrdU) from mouse (Sigma-Aldrich) (1:50), followed by an Alexa Fluor 594 donkey anti-mouse IgG (Invitrogen) or Pns12-specific IgG conjugated to FITC (1:50).

Immunoelectron microscopy.

Cells on coverslips were fixed in 2% paraformaldehyde plus 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. Fixed samples were dehydrated through a graded ethanol series at -20°C and embedded in LR gold resin (Bioscience). Polymerization was allowed to proceed for 72 h at -20°C. Sections were stained for 5 min each with 2% uranyl acetate and for 1 h with alkaline phosphatase-conjugated rabbit antibodies raised in goat against rabbit IgG (H+L) (diluted 1:10 000; Biosource International) for 1 h at room temperature. After washing, the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at room temperature. After washing, the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at room temperature. After washing, the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at room temperature. After washing, the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at room temperature. After washing, the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at room temperature.
To confirm our observations, VCMs were infected with RDV, fixed 18 h p.i. and examined by electron microscopy. Examination of ultrathin sections of RDV-infected VCMs revealed the presence of granular, electron-dense inclusions 600–850 nm in diameter in the cytoplasm of infected cells (Fig. 2). In order to determine the composition of the electron-dense inclusions that had formed in cells infected with RDV, the subcellular localization of Pns6, Pns11 and Pns12 of RDV in infected VCMs was examined by immunoelectron microscopy. It was found that the matrix of the inclusions was densely and evenly immunolabelled with antibodies against Pns6, Pns11 and Pns12 (Fig. 2a, b and c, respectively) and the labelling was consistent with the results of immunofluorescence microscopy.

Core proteins P1, P5 and P7 and core capsid protein P3 are located inside viral inclusions

To determine whether the matrix of viral inclusions is required for the formation of the RDV-assembly complex, infected cells were fixed 18 h p.i., stained with P3-specific IgG conjugated to FITC and Pns12-specific IgG conjugated to rhodamine, and then imaged by confocal fluorescence microscopy. In infected cells, core capsid protein P3 was detectable as discrete, punctate inclusions and was also distributed diffusely throughout the cytoplasm (Fig. 3); Pns12 was localized to discrete, punctate inclusions (Fig. 3). When the images were merged, Pns12 and P3 were co-localized as punctate inclusions in infected cells (Fig. 3). Similar results were obtained for core proteins P1, P5 and P7 (Fig. 3).

In order to confirm our observations, infected cells were fixed 18 h p.i. and examined by immunoelectron microscopy using core capsid protein P3-specific antibodies, as well as core protein P1-, P5- and P7-specific antibodies. As shown in Fig. 4, core capsid protein P3, along with core proteins P1, P5 and P7, was distributed in the matrix of the electron-dense inclusions and core-like particles (arrows). In addition, relatively small amounts of core capsid and core proteins were distributed at the periphery of the electron-dense inclusions. Our results suggested that core proteins and core-like particles of RDV could accumulate in the matrix of electron-dense inclusions.

Outer capsid proteins P2, P8 and P9 are located at the periphery of the viral inclusions

To determine whether the matrix of the viral inclusions is required for accumulation of the outer capsid proteins of RDV during virus replication, infected cells were fixed 18 h p.i., stained with P8-specific IgG conjugated to FITC and Pns12-specific IgG conjugated to rhodamine, and imaged by confocal fluorescence microscopy. In infected cells, Pns12 was distributed in discrete, punctate inclusions; P8 was visualized as ring-like structures and was also scattered diffusely in the cytoplasm (Fig. 3). When the images were merged, P8 was clearly localized at the periphery and Pns12 appeared to occupy the central region of each inclusion. The two proteins were also co-localized at the periphery of the inclusions, as indicated by the yellow colour in Fig. 3. Similar results were obtained for the outer capsid proteins P2 and P9 (Fig. 3).

To confirm our observations, infected cells were fixed 18 h p.i. and examined by immunoelectron microscopy using outer capsid protein P2-, P8- and P9-specific antibodies, respectively. As shown in Fig. 4, the three outer capsid proteins P2, P8 and P9 were distributed at the periphery of the electron-dense inclusions where virus-like particles accumulated. All of the results indicated that outer capsid proteins and virus particles were distributed at the periphery of inclusions, whereas the core proteins and core particles were inside the matrix of the electron-dense inclusions, where the three non-structural proteins Pns6, Pns11 and Pns12 accumulated at high levels.

During the course of infection, the number of mature virus particles in the cytoplasm increased significantly and structural proteins could distribute in these virus particles (data not shown).

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**Fig. 2.** Immunogold labelling of electron-dense inclusions with Pns6 (a), Pns11 (b) and Pns12 (c) of RDV in virus-infected VCMs 18 h p.i. Cells were immunostained for Pns6, Pns11 and Pns12 with Pns6-, Pns11- and Pns12-specific polyclonal antibodies in (a), (b) and (c), respectively, as primary antibodies, followed by treatment with 15 nm gold particle-conjugated goat antibodies against rabbit IgG as secondary antibodies. Bars, 300 nm.
Pns12 can form viral inclusion-like structures in vivo

The three non-structural proteins Pns6, Pns11 and Pns12 of RDV appeared to be the major constituents of the viral inclusions in RDV-infected cells. To identify the protein that is mainly responsible for the formation of the matrix of viral inclusions, Sf9 cells were inoculated with recombinant baculovirus that expressed Pns6, Pns11 or Pns12 and incubated for various periods. When analysed by SDS-PAGE and immunoblotting with respective antibodies, the three proteins were first detected 24 h p.i., with increasing levels that reached a maximum 72 h p.i. (data not shown). Immunofluorescence staining of Pns12 in Sf9 cells that had been grown on coverslips revealed the formation of discrete, punctate inclusions within the cells 48 h p.i. (Fig. 5a). When thin sections of these cells were analysed by electron microscopy, large, granular inclusions similar to the electron-dense inclusions in VCMs infected with RDV were identified in the cytoplasm of Sf9 cells that expressed Pns12 (Fig. 5b). Immunogold electron microscopy revealed the presence of Pns12 specifically in these inclusions (Fig. 5b), whereas infection with the Pns6- or Pns11-encoding baculovirus resulted in the diffuse distribution of each respective protein throughout the cytoplasm and no formation of inclusions (data not shown). Our results indicated clearly that expression of only Pns12, even in the absence of the virus-multiplication process, was sufficient for the formation of viral inclusion-like structures in Sf9 cells.

Self-association of RDV Pns12 in vitro

To determine whether Pns12 was capable of self-association to form viral inclusions, a filter-binding assay was performed. Lysates of E. coli cells expressing His-Pns12 were fractionated by SDS-PAGE, the bands of proteins were transferred to a membrane filter and the filter was incubated with MBP or MBP–Pns12. As shown in Fig. 6, MBP–Pns12 bound specifically to His-Pns12 and not to any of the E. coli-derived proteins. In contrast, MBP did not bind to His-Pns12. Thus, it appeared that molecules of Pns12 had the inherent ability to bind to one another and that Pns12 is capable of aggregation for the formation of viral inclusions.

Pns12 precedes P8 in localization to viral inclusions

To see whether Pns12 is an early protein that localizes to viral inclusions, localization of P8 and Pns12 to these structures was examined in cells infected with RDV over a time course of infection. VCMs were inoculated with RDV and cells were fixed at 2 h intervals. Cells were stained with P8-specific IgG conjugated to FITC and Pns12-specific IgG conjugated to rhodamine, and imaged by confocal fluorescence microscopy. Pns12 was first detectable 6 h p.i., when it was scattered throughout the cytoplasm in discrete, punctate inclusions (Fig. 7). P8 was scattered diffusely in the cytoplasm 6 h p.i. At early times after inoculation, inclusions were numerous and small but, with time, these inclusions decreased in number and increased in size. P8 was observed as discrete, punctate inclusions and was also distributed diffusely throughout the cytoplasm 8 h p.i. Moreover, P8 and Pns12 co-localized in punctate inclusions. Then, by 14 h p.i., Pns12 was observed in bigger inclusions,
Fig. 4. Immunogold labelling of RDV core and core capsid proteins P1, P3, P5 and P7 within or outer capsid proteins P2, P8 and P9 at the periphery of the electron-dense inclusions in virus-infected VCMs 18 h p.i. In P7, the inset shows P7 localization on a single core-like particle. Cells were stained for P1, P2, P3, P5, P7, P8 and P9 with P1-, P2-, P3-, P5-, P7-, P8- and P9-specific polyclonal antibodies, respectively, as primary antibodies, followed by treatment with 15 nm gold particle-conjugated goat antibodies against rabbit IgG as secondary antibodies. Arrows indicate core-like particles. Bars, 300 nm.

Fig. 5. Subcellular localization of Pns12 in recombinant baculovirus-infected Sf9 cells 48 h p.i. (a) Immunofluorescence staining of Pns12 revealed the formation of a punctate inclusion. Cells were stained with Pns12-specific IgG conjugated to FITC. (b) Immunogold labelling of Pns12 association with an electron-dense inclusion. Cells were immunostained with Pns12-specific polyclonal antibodies and 15 nm gold particle-conjugated goat antibodies against rabbit IgG as secondary antibodies. Bar, 300 nm.

Fig. 6. Self-association of Pns12 of RDV, as demonstrated by a filter-binding assay. A lysate of E. coli cells that expressed His-Pns12 was fractioned by SDS-PAGE and bands of proteins were transferred to a PVDF membrane. (a) Proteins on the membrane were visualized by staining with Coomassie brilliant blue (CBB). (b) MBP (control) and MBP-fused Pns12 of RDV (MBP–Pns12) were used as probes to detect binding of Pns12 to itself.
whereas P8 was localized to small, ring-like structures and was also scattered diffusely in the cytoplasm. When these images were merged, P8 was found at the periphery of the inclusions. As infection proceeded, larger inclusions were observed, with P8 concentrating at the periphery and Pns12 concentrating at the central regions of these inclusions. These results indicated that Pns12 preceded P8 in localization to the viral inclusions and support the hypothesis that Pns12 is involved in the nucleation of viral inclusions where progeny virions accumulate.

**Viral inclusions are sites of viral RNA synthesis**

To determine whether the viral inclusions are the sites of viral RNA synthesis, RDV-infected cells were treated with actinomycin D to block host replication and transcription and then treated with BrUTP for 30 min beginning at 10-5 h p.i. After fixing the cells, the location of newly made RNA was determined by using anti-BrdU antibody and the location of viral inclusions was determined by using Pns12-specific IgG. The analysis showed that BrU-labelled RNA was distributed in discrete, punctate inclusions that were co-localized with viral inclusions of Pns12 during the 30 min incubation period with BrUTP (Fig. 8), suggesting that these inclusions represented sites of viral RNA synthesis.

**DISCUSSION**

Immunofluorescence and immunoelectron microscopy of RDV-infected VCMs allowed us to characterize the viral components of viral inclusions, namely, the three non-structural proteins Pns6, Pns11 and Pns12 of RDV that co-localized with the matrix of viral inclusions. Expression of Pns12 in Sf9 cells, non-hosts of RDV, resulted in the formation of viral inclusion-like structures that had an appearance similar to that of inclusions in RDV-infected VCMs, whereas Pns6 and Pns11 were distributed diffusely throughout the cytoplasm of Sf9 cells that expressed the respective recombinant proteins. These results suggest that Pns12 is the minimal viral factor required for viral-inclusion formation during RDV infection. The filter-binding assay proved that Pns12 molecules have the intrinsic ability to bind to one another. Thus, it is reasonable to conclude that Pns12 has the ability to aggregate to form the matrix of viral inclusions in infected cells. Pns12 is a phosphoprotein.
obtained by using a confocal microscope. Images were double-labelled with Alexa Fluor 594 donkey anti-mouse IgG. Images were obtained by using a confocal microscope.

Fig. 8. Intracellular sites of RNA synthesis in RDV-infected cells. VCMs were infected with RDV, treated with actinomycin D and maintained in the presence of BrUPT from 10·5 to 11·0 h p.i. Subsequently, cells were fixed and double-labelled with Pns12-specific IgG conjugated to FITC and monoclonal anti-BrdU from mouse. BrUPT-labelled RDV RNA was stained with Pns12-specific IgG conjugated to FITC and monoclonal anti-BrdU from mouse. BrUPT-labelled RDV RNA was stained with Alexa Fluor 594 donkey anti-mouse IgG. Images were obtained by using a confocal microscope.

(Suzuki et al., 1999), but it remains to be determined whether its capacity to form viral inclusion-like structures is related to its phosphorylation.

Comparison of the viral inclusion matrix protein Pns12 of RDV with its functional counterparts μNS of mammalian reovirus, NSP5 of rotavirus and NS2 of BTV reveals striking similarities among them. Similarities include the ability to form viral inclusion-like structures, co-localization with core proteins and phosphorylation (Huismans et al., 1987; Brookes et al., 1993; Poncet et al., 1997; Suzuki et al., 1999; Estes, 2001; Nibert & Schiff, 2001; Roy, 2001; Broering et al., 2002, 2004; Berois et al., 2003; Eichwald et al., 2004; Taraporewala & Patton, 2004; Modrof et al., 2005). These common attributes might be important in the formation and operation of the ‘viral factories’ that serve as the sites of virus replication and the assembly of core particles. In fact, the phosphorylation of NS2 of BTV was shown recently to be essential for the formation of inclusions (Modrof et al., 2005). It will be of interest to determine whether phosphorylation of matrix proteins is a prerequisite for the formation of inclusions by other reoviruses.

Neither of two viral inclusion constituents, Pns6 and Pns11, appeared to be required for viral inclusion-like structure formation in S9 cells. However, the presence of an RNA-binding motif in Pns6, which is similar to that in NS2 of orbivirus (Theron et al., 1996; N. Suzuki, unpublished results), and the capacity of Pns11 to bind RNA (Xu et al., 1998) suggest that Pns6 and Pns11 might play an important role in viral RNA synthesis and replication in viral inclusions. It is reasonable to postulate that Pns6 and/or Pns11 might be recruited to viral inclusions through their association with Pns12. Similar observations regarding the capacity of mammalian reovirus μNS protein to form viral inclusions and to recruit protein σNS to these structures have been reported (Becker et al., 2003; Miller et al., 2003).

Our observations showed that core particles and viral core proteins, including P1, P3, P5 and P7, always localized to the interior regions of inclusions (Fig. 4), in contrast to intact virions and all of the components of viral outer capsid structural proteins, including P2, P8 and P9, which accumulated at the peripheral regions of the inclusions (Fig. 4). It is possible that a peripheral zone of the viral inclusions, shown by the yellow colour in Fig. 3, which contains both viral core and outer capsid proteins, might represent the location of the assembly of progeny virions where outer capsid proteins become attached to core particles. With time, maturing virus particles appeared to bud from the viral inclusions and were scattered in the cytoplasm.

Our observations also showed that the viral inclusion matrix protein Pns12 co-localized with newly synthesized RDV RNA labelled with BrUPT. These results demonstrate that RDV replication occurs on viral inclusion and that the non-structural proteins Pns6, Pns11 and Pns12, as well as the seven structural proteins of RDV, may participate in the formation and functions of the virus-replication complexes.

Our study revealed the dynamic nature of RDV viral inclusions. Pns12 was detected as punctate structures from the beginning of virus infection and these structures increased in size and decreased in number as infection proceeded (Fig. 7). These results suggest that the fusion of smaller inclusions resulted in the formation of larger ones. The homopolymerization property of matrix protein Pns12, as demonstrated by the filter-binding assay, supports this hypothesis. Pns12 was found in viral inclusions prior to the structural proteins of RDV, an observation that suggests that structural proteins might be recruited to the inclusions after non-structural proteins have formed nascent viral inclusions and that morphogenesis of virions occurs in the inclusions. The dynamic nature of viral inclusions, which warrants further examination, might be related to the infection stages in the cell and might contribute to the coordination of the roles of the many players that are involved in virus replication and assembly.

Our analysis suggests that replication and assembly of RDV are also thought to take place in discrete viral inclusions within the cytoplasm of infected cells, as is the case for other members of the family Reoviridae. In addition, the viral inclusions in RDV-infected VCMs that were observed corresponded to the electron-dense inclusions designated ‘viroplasms’ in viruliferous N. cincticeps and diseased plants (Fukushi et al., 1962; Shikata, 1969).

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