Rice dwarf virus is engulfed into and released via vesicular compartments in cultured insect vector cells

Taiyun Wei, Hiroyuki Hibino and Toshihiro Omura

National Agricultural Research Center, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8666, Japan

Vector insect cells infected with Rice dwarf virus had vesicular compartments containing viral particles located adjacent to the viroplasm when examined by transmission electron and confocal microscopy. Such compartments were often at the periphery of infected cells. Inhibitors of vesicular transport, brefeldin A and monensin, and an inhibitor of myosin motor activity, butanedione monoxime, abolished the formation of such vesicles and prevented the release of viral particles from infected cells without significant effects on virus multiplication. Furthermore, the actin-depolymerizing drug, cytochalasin D, inhibited the formation of actin filaments without significantly interfering with formation of vesicular compartments and the release of viruses from treated cells. These results together revealed intracellular vesicular compartments as a mode for viral transport in and release from insect vector cells infected with a plant-infecting reovirus.

In vector cells grown in monolayers (VCMs), Rice dwarf virus (RDV), a phytoreovirus, multiplies and spreads from primarily infected cells to neighbouring cells (Wei et al., 2006a) in addition to spreading via mature, free viral particles. Infection via free viral particles was protected by the addition of neutralizing antibodies to the cell culture medium. As part of integrated studies on RDV proliferation in vector insects, we have focused our study on the accumulation of the virus in cells of the insect vector and on the subsequent release of the virus.

In electron micrographs of thin sections of insect tissues infected with plant-pathogenic reoviruses, viral particles were sequestered in spherical vesicular compartments (Fukushi et al., 1962; Shikata & Maramorosch, 1965; Shikata, 1969; Vidano, 1970; Omura et al., 1985). However, the biological significance of these inclusions in RDV infection has not been clarified because, for the most part, the tissues examined were in the late stage of infection which made it difficult to gather details on the formation of these compartments. Similar vesicular compartments appear to play a role in the release of viral particles from cultured cells infected with animal viruses, such as severe acute respiratory syndrome coronavirus (Ng et al., 2003) and human immunodeficiency virus (HIV) (Nydegger et al., 2003).

A method for the continuous culture of cells from the leafhopper vector Nephrotettix cincticeps, consisting of VCMs, has allowed the study of infection with RDV because such infection results in asymptomatic but persistent infection (Peterson & Nuss, 1985; Kimura, 1986). VCMs have been used to reveal fundamental aspects of viral activity at the cellular level, which suggests details of the transmission, multiplication and cytopathology of RDV in vector insects (Wei et al., 2006a, b, c, 2007, 2008). In the present study with this system, we investigated the role of vesicular compartments in the transport of RDV and its release from infected VCMs by confocal and electron microscopy, and the use of drugs that inhibit vesicular transport.

We examined the intracellular distribution of RDV during the late stages of viral infection when viral particles would be released from the cells. VCMs grown on a coverslip (15 mm in diameter) were inoculated with RDV at an m.o.i. of 10 and fixed 36 h post-inoculation (p.i.) for transmission electron microscopy as described previously (Omura et al., 1998). In these figures, RDV particles, confirmed by immunoelectron microscopy in our earlier study (Wei et al., 2006a), were easily distinguished by their spherical appearance and diameter (70 nm), and they were not found in uninoculated controls (Fig. 1). Further, the viroplasm, the site of viral replication and assembly, reacted specifically with Pns12-specific antibodies as reported earlier (Wei et al., 2006b, c), was easily recognized by its characteristic appearance (Fig. 1a). As reported in our earlier studies (Wei et al., 2006c), viral particles distributed at the periphery of the viroplasm (Fig. 1a). We sometimes observed viral particles in vesicular compartments other than the viroplasm in the cytoplasm of RDV-infected VCMs (Fig. 1a). These compartments varied in size and often reached a diameter of 2 μm. Occasionally, we observed viral particles in vesicular compartments at the periphery of infected cells (Fig. 1b, c). Furthermore, viral

In the present study, we have focused on the role of vesicular compartments in the transport of RDV and its release from infected VCMs by confocal and electron microscopy, and the use of drugs that inhibit vesicular transport.
structures (Fig. 2, untreated panel), as described previously. The viroplasm and the spherical structure often located beside it corresponded to the appearance in Fig. 1(a). These observations revealed that the viroplasms differed distinctly from the spherical structures. In our thorough examination of immunoelectron micrographs of thin sections of RDV-infected cells, we did not find any inclusions other than the spherical structures we observed with immunofluorescence staining that corresponded to the virus-containing vesicular compartments. The similarity in the sizes of the vesicular compartments observed with electron microscopy and the spherical structures observed after immunofluorescence staining support these results. Thus, all our results together suggest a possible pathway whereby viral particles, assembled at the periphery of the viroplasm, are engulfed by vesicular compartments. The occurrence of such compartments at the periphery of infected cells (Fig. 1b, c) also suggests that the virus may be released from the cells upon fusion of the compartments with the cell membranes, rather than be degraded within these compartments.

To confirm the existence of such a pathway, we studied the effects of inhibitors of vesicular transport applied during the viral propagation period. Brefeldin A (BFA) is a fungal metabolite that disrupts the Golgi complex in many cell types, thus inhibiting normal cellular sorting and transport functions (Klausner et al., 1992; Pelham, 1991). Monensin is a carboxylic ionophore that arrests vesicular transport at a site distal to the proximal portion of the Golgi complex (Tartakoff, 1983). These two inhibitors have been extensively used to inhibit vesicular transport of several viruses (Blank et al., 2000; Boulanger et al., 2000; Suikkainen et al., 2003; Kolessnikova et al., 2004; Bugarcic & Taylor, 2006). Two hours p.i. of VCMs with RDV at an m.o.i. of 10, either 0.1 or 0.5 μg BFA (Sigma) ml⁻¹, or 5 or 20 μM monensin (Sigma) was added and incubation was continued for a further 34 h. In preliminary experiments, we tested a range of concentrations of the drugs to determine effective concentrations and to avoid toxic effects of BFA and monensin (data not shown; Wei et al., 2008). Treatment of VCMs with 0.5 μg BFA ml⁻¹ and 20 μM monensin did not interfere with the formation of the viroplasm, composed mainly of Pns12 (Fig. 2; Wei et al., 2006c), while both chemicals reduced the number and size of virus-positive spherical structures in the cytoplasm (Fig. 2).

Next, we examined whether these two drugs had any effects on the replication of RDV in VCMs. Two hours p.i. of VCMs with RDV at an m.o.i. of 10, BFA or monensin was added at a range of concentrations and incubation was continued for a further 34 h. The extracellular medium and the cells were collected. The medium was centrifuged for 30 min at 15 000 g, and the supernatant was collected and frozen at –70 °C before analysis. The cells were subjected to three cycles of freeze–thaw to release viral particles and stored at –70 °C before analysis. The viral titre of each sample was determined in duplicate using VCMs at a

**Fig. 1.** Transmission electron micrographs of virus-containing vesicular compartments in vector cell monolayer (VCM) infected with RDV at 36 h p.i. (a) Typical appearance of viral particles at the periphery of viroplasm (VP) or within vesicular compartments (arrow) inside cells. (b) Virus-containing vesicular compartment (arrow) at the periphery of an infected cell. (c) Virus-containing vesicular compartments (arrows) inside and at the periphery of a cell. Arrowhead points to a cellular remnant containing viral particles outside of the infected cell. Bars, 400 nm.

particles and cellular remnants were often observed outside of infected cells (Fig. 1c). Similar features were never seen in uninfected cells.

To study the appearance of virus-containing vesicular compartments in relation to the viroplasm at the cellular level, we used confocal fluorescence microscopy to visualize the relative localization of the viral antigen and the viroplasm in RDV-infected cells. VCMs were inoculated at an m.o.i. of 10 with RDV and fixed 36 h p.i. for 30 min in 2 % paraformaldehyde. Cells were immunostained with RDV-specific antibodies conjugated with fluorescein isothiocyanate (FITC) and with antibodies raised against the non-structural protein Pns12, a constituent of the viroplasm, conjugated with rhodamine and then examined by confocal fluorescence microscopy as described previously (Wei et al., 2006c). Viral particles were assembled in the peripheral region of the viroplasm and formed ring-like structures (Fig. 2, untreated panel), as described previously (Wei et al., 2006c). RDV-specific antibodies also reacted with numerous spherical structures in the cytoplasm or outside of infected cells (Fig. 2, untreated panel).
magnification of $\times 10$ using a fluorescent focus assay as described previously (Kimura, 1986). End-point titres were calculated as means with standard deviations. As shown in Fig. 3, BFA (0.1 and 0.5 $\mu$g ml$^{-1}$) and monensin (5 and 20 $\mu$M) caused more than 50-fold decrease in viral titre of the medium. By contrast, at doses that significantly affected
the release of viral particles, the inhibitors did not significantly reduce the titres of cell-associated viruses. These results demonstrated that RDV had proliferated in the infected cells but particle release from the cells had been impeded by the inhibitors. Taken together, the evidence that release of RDV from cells was suppressed to a significant extent (Fig. 3) in the presence of the drugs, whereby the formation of virus-positive spherical structures was significantly inhibited (Fig. 2), supported the hypothesis that RDV particles, generated in the viroplasm, were packaged in the spherical structures that correspond to the vesicular compartments and then released from cells.

The actin cytoskeleton is crucial for the intracellular trafficking of vesicular compartments (Gottlieb et al., 1993; Radtke et al., 2006) and for the transport of RDV between cells of the vector insect (Wei et al., 2006a, 2008). To evaluate the involvement of the actin cytoskeleton in the replication process of RDV, we examined the effects of cytochalasin D (CytD), an actin-depolymerizing drug (Sigma) (Goddette & Frieden, 1986; Sampath & Pollard, 1991), and butanedione monoxime (BDM; Sigma), a myosin motor inhibitor (Cramer & Mitchison, 1995), on the assembly of viral particles and their release from infected cells. Fluorescence analysis of the materials revealed well-organized actin filaments in untreated RDV-infected cells, while the majority of cellular filamentous actin was disrupted in CytD (2 μg ml⁻¹)-treated cells. BDM (40 nM) did not affect formation of actin filaments, as described previously (Wei et al., 2008). Immunostaining of viral antigens and Pns12 in infected cells revealed viroplasms and a number of prominent virus-associated spherical structures in the presence of CytD (Fig. 2). Furthermore, smaller virus-associated spherical structures occurred outside of infected cells in the presence of CytD (Fig. 2). By contrast, both the number and size of virus-specific spherical structures were significantly reduced in the presence of BDM (Fig. 2). Taken together, these results indicated that BDM inhibited the association of viral particles with the spherical structures, whereas CytD seemed only to change the pattern of distribution of virus-associated vesicles although it was previously found to interfere with the intercellular transport of RDV (Wei et al., 2008).

Next, we collected the cells and medium separately at 36 h p.i., and the amount of virus in each fraction was quantified by virus infection assay as mentioned. An analysis of extracellular RDV revealed that CytD (0.5 and 2 μg ml⁻¹) slightly reduced the release of RDV (10% of control yield), whereas BDM (20 and 40 nM) reduced the level of detectable virus in the medium to 98% of that in the control (Fig. 3). Both inhibitors had negligible effects on the production of cell-associated viruses (Fig. 3), suggesting that they had little effect on viral replication. These results suggested that myosin has a major role in the release of RDV, in contrast to a less prominent role for actin filaments. Similarly, myosin also plays more important roles than actin in vesicle trafficking (Durán et al., 2003) and HIV release from infected host cells (Sasaki et al., 1995). All our results clearly showed a relationship between the formation of virus-associated spherical structures and the release of progeny viruses from cells, demonstrating that the spherical structures that correspond to vesicular compartments play an important role in the accumulation of viral particles and their release from cells.

As shown in Fig. 3, none of the inhibitors at the various concentrations substantially reduced the titre of cell-associated viruses. These results suggest that the inhibitors had no
significant effect on the RDV replication. Therefore, the reduction in the release of RDV in the medium in the presence of BFA, monensin and BDM was due specifically to interference with post-replication phenomena. CytD, an inhibitor of actin filament formation, interfered with secondary transport of viral particles to neighbouring cells through Pns10 tubules, which need actin for extension (Wei et al., 2008). This phenomenon was confirmed in this study (data not shown) and this drug did not affect spherical structure formation (Fig. 2), viral replication (Fig. 3) or release of viral particles from infected cells (Fig. 3), suggesting that this chemical also worked in specific manners under our conditions.

The apparent restriction of viral particles in spherical structures in untreated and CytD-treated cells was not observed in BFA-, monensin- and BDM-treated cells (Fig. 2), although the virus in BFA-, monensin- and BDV-treated cells proliferated to a level similar to that in CytD-treated and untreated cells (Fig. 3). These results suggest that viruses are concentrated in the spherical structures in untreated and CytD-treated cells, but such accumulation was prevented in the presence of BFA, monensin and BDM. Electron microscopic observation showed that nascent viruses were engulfed in vesicles and released from the surface of cells (Fig. 1). In confocal microscopy, the spherical structure formation (Fig. 2) was accompanied by viral release (Fig. 3), and inhibition of the spherical structure formation (Fig. 2) resulted in the failure of viral release from infected cells (Fig. 3). All these results suggest that the spherical structure, i.e., the vesicular compartment, plays an important role in the release of viral particles from infected leafhopper cells.

It is unclear whether the mechanism involved in the release of viral particles from infected cells is also involved in sequestering a large fraction of infectious viruses within infected VCMs because a high titre of infectious RDV sequesters a large fraction of infectious viruses within infected VCMs. However, this type of sequestration might be a reason for the longevity of infected VCMs because a high titre of infectious RDV remains associated with infected cells. This hypothesis might also hold for viruses in the genera Phytoreovirus and Oryzavirus, which also multiply in both plants and vector insects.

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