NOTES

Association of *Rice Gall Dwarf Virus* with Microtubules Is Necessary for Viral Release from Cultured Insect Vector Cells[▽]

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Vector insect cells infected with *Rice gall dwarf virus*, a member of the family *Reoviridae*, contained the virus-associated microtubules adjacent to the viroplasms, as revealed by transmission electron, electron tomographic, and confocal microscopy. The viroplasms, putative sites of viral replication, contained the nonstructural viral proteins Pns7 and Pns12, as well as core protein P5, of the virus. Microtubule-depolymerizing drugs suppressed the association of viral particles with microtubules and prevented the release of viruses from cells without significantly affecting viral multiplication. Thus, microtubules appear to mediate viral transport within and release of viruses from infected vector cells.

Rice gall dwarf virus (RGDV), *Rice dwarf virus* (RDV), and *Wound tumor virus*, members of the genus *Phytoreovirus* in the family *Reoviridae*, multiply both in plants and in invertebrate insect vectors. Each virus exists as icosahedral particles of approximately 65 to 70 nm in diameter, with two concentric layers (shells) of proteins that enclose a core (1, 13). The viral genome of RGDV consists of 12 segmented double-stranded RNAs that encode six structural (P1, P2, P3, P5, P6, and P8) and six nonstructural (Pns4, Pns7, Pns9, Pns10, Pns11, and Pns12) proteins (reference 21 and references therein). The core capsid is composed of P3, the major protein, which encloses P1, P5, and P6 (12). The outer layer consists of two proteins, namely, P2 and P8 (10, 12).

Cytoplasmic inclusion bodies, known as viroplasms or viral factories, are assumed to be the sites of replication of viruses in the family *Reoviridae*. After infecting insect vector cell monolayers (VCMs) in culture with RDV, Wei et al. (19) examined the generation of RDV particles in and at the periphery of such viroplasms. VCMs are also useful for studies of RGDV, allowing detailed analysis of the synchronous replication and multiplication of this virus (14). In order to identify the viroplasms in RGDV-infected VCMs, we examined the subcellular localization of Pns7, Pns12, P5, and RGDV particles by confocal immunofluorescence microscopy. Pns7 and Pns12 of RGDV correspond to Pns6 and Pns11, respectively, which are components of the viroplasm of RDV (12, 19). RGDV P5 is a counterpart of RDV P5, a core protein that locates inside the viroplasm in RDV-infected cells. We inoculated VCMs with RGDV, purified by the method reported in reference 15, at a

multiplicity of infection (MOI) of 1; fixed them 48 h postinfection (p.i.); probed the cells with Pns7-, Pns12-, P5-, and viral-antigen-specific antibodies (11, 12) that had been conjugated to fluorescein isothiocyanate (FITC) (Sigma, St. Louis, MO) or rhodamine (Sigma); and examined them by confocal microscopy, as described previously (19). In RGDV-infected cells, Pns7, Pns12, and P5 were detected as punctate inclusions (Fig. 1). Immunostained viral antigens formed ringlike structures around the punctate inclusions. When the images were merged, Pns7, Pns12, and P5 were colocalized in the punctate inclusions, indicating that these proteins were constituents of the viral inclusions (Fig. 1). Our observations revealed the similar respective localizations of the corresponding nonstructural proteins, core proteins, and viral particles of two phytoreoviruses, RGDV and RDV, in infected cells. Thus, Pns7 and Pns12 of RGDV had attributes common to their functional counterparts—Pns6 and Pns11, respectively—of RDV (19). The core protein P5 was located inside the viroplasms, and the viral antigens were distributed at the periphery of the viroplasms. The results, together, suggest that RGDV and RDV exploit similar replication strategies. Specific fluorescence was not detected in noninfected cells after incubation with Pns7-, Pns12-, P5-, and viral-antigen-specific antibodies (data not shown).

In addition to the viral location at the periphery of the viral inclusions visualized as immunostained Pns12 (Fig. 1), the antigens were distributed as bundles of fibrillar structures, a form not observed in RDV-infected cells. To analyze the entity of the bundles of fibrillar structures, VCMs on coverslips were inoculated with RGDV at an MOI of 1, fixed at 48 h p.i., and examined by electron microscopy (EM), as described previously (19). We observed viral particles of approximately 70 nm in diameter in close association with the free ends, as well as along the edges, of tubules of approximately 25 nm in diameter (Fig. 2A to D). The abundant bundles of tubules with closely

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FIG. 1. Subcellular localization of Pns7, Pns12, and P5 of RGDV and viral antigens in RGDV-infected VCMs 48 h p.i. Arrowheads show ringlike profiles of viral antigens that surround viral inclusions, which have been immunostained with the Pns12-specific antibodies. Arrows show the fibrillar profiles of immunostained viral antigens. Bars, 5 μ m.

associated viral particles were clearly in contact with the periphery of granular, electron-dense inclusions of 800 to 1,200 nm in diameter (Fig. 2B), namely, viroplasms. The dimensions and appearance of the tubular structures resembled those of microtubules (Fig. 2C) (17). Transverse sections of tubules revealed arrays of closed circles of approximately 25 nm in diameter, with viral particles attached directly or via a filament to the circumference (Fig. 2D).

Our observations suggested that RGDV particles might attach to microtubules in infected cells. To examine this possibility, we inoculated VCMs with RGDV at an MOI of 1, fixed the cells 48 h p.i., immunostained them with α -tubulin-specific antibodies conjugated to FITC and with viral-antigen-specific antibodies conjugated to rhodamine, and analyzed them by confocal microscopy, as described previously (19). Viral antigens were visualized as ringlike and fibrillar structures (Fig. 2E). Double immunostaining of the infected cells revealed that a network of microtubule-based filaments colocalized with most of the fibrillar structures that represented viral antigens, confirming the association of viral particles with the microtubule-like inclusions visualized by EM (Fig. 2A). Nonspecific reactions were not detected with either of the stainings (data not shown). Our results suggested that RGDV particles, which assembled at the periphery of viroplasms, might be transported along microtubules. Due to the lack of RGDV infectious clones fused with green fluorescent protein and the effective gene transfection system for VCMs, we could not observe the trafficking of RGDV particles along microtubules in living cells.

We then used three-dimensional (3-D) electron tomographic microscopy (ET) to reveal a new level of morphological detail about the association of RGDV with microtubules.

To produce 3-D reconstructions of RGDV-infected cells, we fixed, embedded, and sectioned infected leafhopper cells as described previously (5). We chose a representative region that showed numerous RGDV particles close to bundles of microtubules for this novel tomographic analysis. A single-axis tilt series was collected manually from -60° to 60° with 2° increments using an H9500SD EM (Hitachi, Tokyo) operated at 200 kV. These tomographic data were recorded at a defocus of 3.6 μ m on the TVIPS 2k $\times$ 2k charge-coupled-device camera (TVIPS, Gauting, Germany). Microscopic magnification of $\times 15,000$, providing 1.28 nm/pixel, was enough to view the microtubules and virus particles following tomographic reconstruction of the tilt series using IMOD (7). As shown in the 3-D tomogram in Fig. 3, most of the RGDV particles were bound to the edges of bundles of microtubules. The RGDV particles along the edges of microtubules were arrayed in an orderly but uncrowded manner (Fig. 3). Our ET analysis also revealed that some viral particles were linked to filaments of approximately 10 nm in diameter (Fig. 3B). Morphologically, these filaments resembled vimentin intermediate filaments (4). In many lines of cultured cells, vimentin intermediate filaments partially overlap the microtubules, and there is evidence that the two filament systems interact (3, 9, 20). Unfortunately, vimentin-specific monoclonal antibodies from mouse and rabbit did not react specifically with our leafhopper cells (data not shown), but the nature of the intermediate filaments was apparent from their dimensions, intracellular location, and organization. Thus, our ET analysis indicated that RGDV particles were able to associate directly and/or via intermediate filaments with microtubules.

To examine the role of the microtubules for RGDV activity, we added a microtubule-disrupting agent, either nocodazole

FIG. 2. Association of RGDV particles with microtubules. (A) Electron micrograph showing RGDV particles associated with microtubules in virus-infected VCMs 48 h p.i. Bar, 300 nm. VP, electron-dense inclusion. (B) Virus-associated microtubules in contact with the periphery of the electron-dense inclusion indicated by a white rectangle in panel A. Bar, 300 nm. (C) Viral particles along the edges of tubules of approximately 25 nm in diameter. Bar, 150 nm. (D) Transverse sections of arrays of closed circles of approximately 25 nm in diameter with viral particles attached to their circumference directly (arrow) or via a filament (arrowhead). Bar, 150 nm. (E) Confocal micrograph showing the association of viral

FIG. 3. ET analysis showing the association of RGDV particles with microtubules either directly or via intermediate filaments. (A) Translucent representation of the reconstructed viruses lining up with microtubules. (B) Slice of the reconstructed volumes from the inset of A to show the association of RGDV particles with intermediate filaments (arrows). Bars, 150 nm.

(Sigma) or colchicine (Sigma), 2 hours after inoculation of VCMs with RGDV at an MOI of 1 and then continued the incubation for a further 46 h. Cells were fixed 48 h p.i. and stained with α -tubulin-specific antibodies conjugated to FITC (Sigma) and viral-particle-specific antibodies conjugated to rhodamine, with subsequent confocal fluorescence microscopy, as described previously (19). We tested a range of drug concentrations in preliminary experiments (data not shown) and determined optimal concentrations. Treatment of infected cells with 10 μ M nocodazole or 5 μ g/ml colchicine resulted in the complete disassembly of microtubules, with the accumulation of ringlike structures exclusively and no fibrillar structures representative of viral antigens in the cytoplasm (Fig. 4A). These ringlike aggregates of viral antigens were confirmed to surround viroplasms when the latter were immunostained for Pns12, as described above and shown in Fig. 1. Nonspecific reactions were not detected with either staining (data not shown). These results suggest that RGDV particles multiply around the viroplasm but are unable to distribute along the microtubules in the presence of the chemicals.

During the process of infection, microtubules play important roles in viral entry, intracellular trafficking, and extracellular release (2, 8, 16). We next investigated the effects of the microtubule-disrupting agents on the production in and release of viruses from virus-infected cells by the method described previously (18). Nocodazole or colchicine was added 2 h after inoculation of VCMs with RGDV at an MOI of 1, and incubation was continued for a further 46 h. The extracellular medium and the cells were collected separately. The medium was centrifuged for 30 min at 15,000 $\times g$, and the supernatant was collected. The cells were subjected to three cycles of freezing and thawing to release viral particles. The viral titer of each

sample was determined, in duplicate, by the fluorescent focus assay as described previously (6), with VCMs and a magnification of $\times 10$. As shown in Fig. 4B, nocodazole (20 μ M) and colchicine (10 μ g/ml) caused a fivefold reduction in the number of released viruses, compared to that from untreated control infected cells. In contrast, each inhibitor at the selected dose failed to significantly reduce the titer of cell-associated viruses (less than 5% compared to that from untreated control). These results suggest that the inhibitors impeded the release of viruses into the medium without affecting viral production in infected cells. We do not yet understand why the viral titer was not elevated in drug-treated cells from which viral release was inhibited. However, our data show clearly that disruption of microtubules directly inhibited the release of mature viral particles from infected cells.

In conclusion, EM, ET, immunofluorescence staining, and experiments with two inhibitors support the hypothesis that the transport of RGDV from viroplasms to the plasma membrane and into the medium is dependent on microtubules. In the case of RDV, vesicular compartment-containing viral particles that locate adjacent to the viroplasms were considered to play an important role in the transport and release of the virus from the viroplasm to the culture medium in infected VCMs (18). On the other hand, a fibrillar structure (Fig. 1 and 2), not observed in RDV-infected cells, was considered to function in the trafficking of RGDV from viroplasm into the culture medium (Fig. 4) in the present study. RGDV and RDV, both members of the *Phytoreovirus* genus, have some common biological and biochemical properties but are distinct from each other (13). For example, viruses are restricted to phloem-related cells in RGDV-infected plants but distributed in many types of cells in RDV-infected plants, and a P2 protein with a

particles with microtubules in virus-infected VCMs 48 h p.i. Microtubules were stained with α -tubulin-specific antibodies conjugated to FITC; viral particles were stained with viral-antigen-specific antibodies conjugated to rhodamine. Arrowheads indicate the ringlike organization of viral antigens. Arrows show the colocalization of fibrillar profiles of viral antigens with microtubules. The insets show ringlike and fibrillar profiles of immunostained viral antigens. The circular areas inside the ringlike structures are viroplasms. Bar, 5 μ m.

FIG. 4. (A) Effects of microtubule-disrupting agents on the formation of microtubules and fibrillar profiles of immunostained viral antigens. Bars, 5 μm . The insets show the ringlike profiles of immunostained viral antigens after treatment with inhibitors, suggesting that viral replication occurs in the presence of each inhibitor. (B) Effects of drugs on the production of cell-associated (gray bars) and extracellular (black bars) viruses in VCMs infected with RGDV. The error bars indicate standard deviations.

function to adsorb to and/or penetrate into insect vector cells is present in RGDV and absent in RDV in particles purified using carbon tetrachloride. The present molecular cytopathological study revealed one more difference between the viruses: they have different means for transporting and releasing infectious particles to the cell exterior. The presence of such a molecular mechanism may accelerate the secondary infections by the viruses in infected vector insects, and the high propagation speed would allow the viruses to complete infection cycles through insects and plants.

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