The P2 capsid protein of the nonenveloped rice dwarf phytoreovirus induces membrane fusion in insect host cells

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Insect transmission is an essential process of infection for numerous plant and animal viruses. How an insect-transmissible plant virus enters an insect cell to initiate the infection cycle is poorly understood, especially for nonenveloped plant and animal viruses. The capsid protein P2 of rice dwarf virus (RDV), which is nonenveloped, is necessary for insect transmission. Here, we present evidence that P2 shares structural features with membrane-fusogenic proteins encoded by enveloped animal viruses. When RDV P2 was ectopically expressed and displayed on the surface of insect *Spodoptera frugiperda* cells, it induced membrane fusion characterized by syncytium formation at low pH. Mutational analyses identified the N-terminal and a heptad repeat as being critical for the membrane fusion-inducing activity. These results are corroborated with results from RDV-infected cells of the insect vector leafhopper. We propose that the RDV P2-induced membrane fusion plays a critical role in viral entry into insect cells. Our report that a plant viral protein can induce membrane fusion has broad significance in studying the mechanisms of virus entry into insect cells and insect transmission of nonenveloped plant and animal viruses.

heptad repeat | transmembrane | entry

Virus entry into a host cell is a critical step to initiate infection. Elucidating the mechanisms involved in this process has broad implications in basic biology, agriculture, and medicine. Host cell entry has been well studied for enveloped viruses. For these viruses, the viral-encoded fusion proteins mediate fusion between the viral and cellular membranes, allowing the viral genomes to enter the host cells (1). Among these proteins, the type I fusion proteins from a group of widely disparate viruses have been well characterized. These include the paramyxovirus F protein, the HIV gp160 protein, the retroviral Env protein, and the influenza virus HA (2, 3). Although they differ in their overall structures, many of these proteins share a number of common features. For example, they all contain multiple glycosylation sites, must be trimeric, and undergo proteolytic cleavage to be fusogenically active. Proteolytic cleavage releases the subunit that contains the transmembrane domain, whose N terminus is extremely hydrophobic and is called the fusion peptide. Furthermore, all of these fusion proteins contain ≈3–4 heptad repeat (HR) sequences near both the fusion peptide and the transmembrane domain. The two discontinuous HR motifs always form a central core trimer consisted of three antiparallel coiled-coil heterodimers (4). Presumably, entry of a nonenveloped virus into a host cell is accompanied by conformational changes in the virus particle, which presumably confer hydrophobic, membrane interaction properties (5–10). However, the mechanisms remain poorly understood.

Some nonenveloped reoviruses that are fusogenic encode a distinct class of membrane fusion proteins, called fusion-associated small transmembrane (FAST) proteins, which are always N-terminal-myristoylated and function in the process of viral cell-to-cell movement but not in the process of viral entry into host cells (11–13). The VP5 of bluetongue virus acts not only as a membrane penetration protein but also as a fusion protein that induces syncytium formation when it is fused to a transmembrane anchor and expressed on the cell surface. By short exposure to a low pH, the VP5 undergoes conformational changes that enable it to interact with cellular membranes (14).

We used rice dwarf virus (RDV) as a model system to address the mechanisms of how nonenveloped viruses enter insect cells. RDV is a member of the genus *Phytoreovirus* of the family *Reoviridae*. Its icosahedral double-shelled particle is ≈700 Å in diameter (15, 16). The core particle contains the full genome of 12 segments of dsRNAs, each as a single copy, and four structural proteins, P1, P3, P5, and P7. This core is enclosed within an outer capsid comprising another three structural proteins, P2, P8, and P9 (17, 18). RDV-infected rice plants exhibit growth stunting and severe disease symptoms in China, Korea, and Japan (19). RDV is transmitted in nature by insect vectors such as leafhoppers (*Nephotettix cincticeps or Recilia dorsalis*). It multiplies in insect cells and plant cells.

The minor outer capsid protein P2 encoded by segment 2 (S2) of RDV is a multifunctional protein. It is essential for RDV infection of insect vectors, influences transmission of RDV by the insect vectors (20), and contributes to the development of dwarf phenotype in infected rice plants by interfering with gibberellic acid synthesis (21). It was postulated that P2 interacts with a receptor encoded by the insect vector cells, leading to the recognition of viral particles by the insect cells (22). The evidence in support of this hypothesis is still outstanding, and whether P2 can alter the biology of cell membranes is unknown.

To establish a foundation for mechanistic studies on the mechanisms of phytoreovirus entry into insect cells for replication, we investigated the role of RDV P2 in membrane biology. We report here that the RDV P2 contains an N-terminal hydrophobic peptide, two HRs, and a transmembrane region, features of type I fusion proteins of enveloped viruses. When ectopically expressed and displayed on the surface of insect cells, the P2 caused syncytium formation, indicative of membrane fusion as has been established from studies on several enveloped viruses (23). The N-terminal and

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Abbreviations: RDV, rice dwarf virus; HR, heptad repeat; VCM, vector cells in monolayers; MOI, multiplicity of infection; Sf9, *Spodoptera frugiperda*; VSV, vesicular stomatitis virus.

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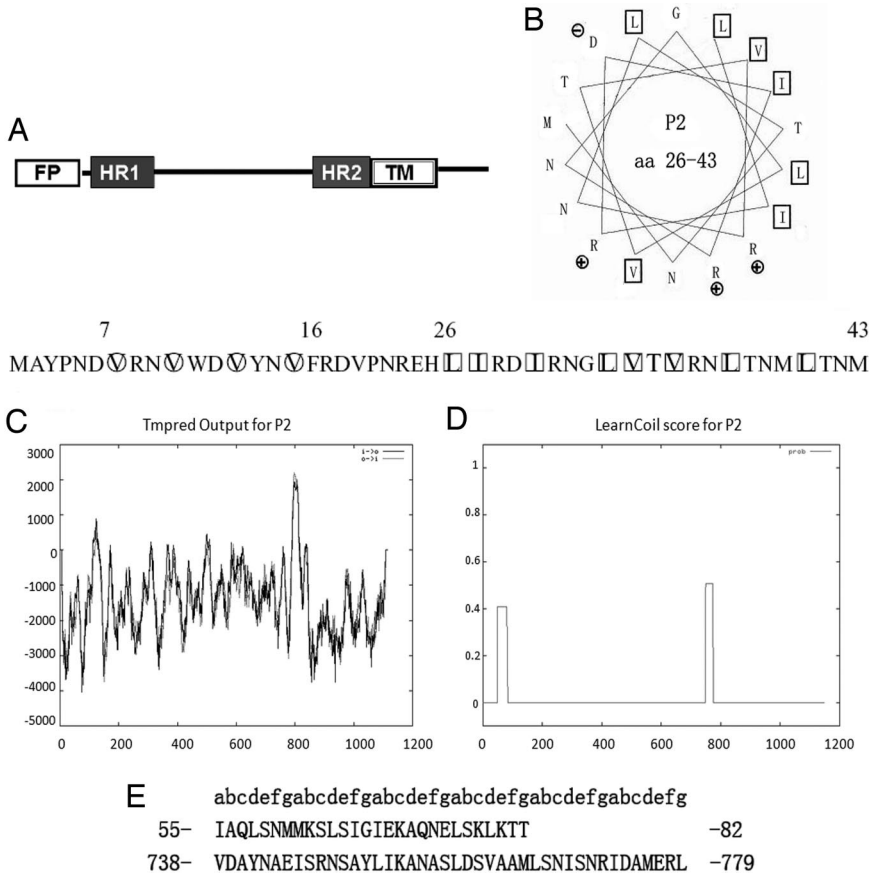

Fig. 1. Diagram of P2 configuration predicted from primary amino acid analysis. (*A*) Structural features and domains of P2 identified by using computer-assisted programs. (*B*) Helical wheel representation of amino acids 26–43 of P2. Each panel represents an α-helix viewed along the helix axis with the indicated amino acid residues. The P2 sequence was searched for the presence of amphipathic structures initially by using the program Moment of the GCG software package. The program Helical Wheel was then used to plot a helical wheel representation of the N-terminal amino acids of P2. (*C*) Transmembrane domain prediction of P2 using PSORT version 6.4 (WWW). Amino acids 802–818 surrounded with solid line have a high possibility to be a transmembrane domain. (*D* and *E*) LearnCoil-VMF detects two HR regions in P2 (*D*), and sequence analysis shows these two regions contain additional leucine zipper motifs (*E*).

HR regions were found to be critical for inducing membrane fusion. Finally, RDV also induces cell–cell fusion, resulting in syncytium formation in infected leafhopper vector cells in monolayers (VCM) under conditions of a low multiplicity of infection (MOI). These findings establish RDV P2 as a plant viral protein that has membrane fusion-inducing activities. They have broad significance in studying the mechanisms of cell entry by nonenveloped viruses and further in comparative studies on the common and distinct mechanisms that the enveloped and nonenveloped viruses have evolved to enter host cells for multiplication.

Results

RDV P2 Shares Structural Features with the Fusion Proteins of Enveloped Viruses. Almost all of the fusion proteins studied to date contain an N-terminal fusion peptide that can insert into the lipid bilayer. The fusion peptide segment is often hydrophobic, rich in glycine (G), and located at the N-terminal end of the fusion protein. By protein sequence domain/motif analysis, we found that the RDV P2 contains the following features (Fig. 1*A*). First, the N-terminal residues 7–16 of P2 are rich in regularly distributed hydrophobic amino acid valine. Second, residues 26–43 form an amphipathic helix (Fig. 1*B*). These two features indicate that the P2 segment encompassing amino acids 1–43 are structurally similar to the fusion peptides of many enveloped viruses (4). Third, sequence analysis with PSORT version 6.4 revealed a putative transmembrane domain (amino acids 802–818) near the carboxyl-terminal domain of P2 (Fig. 1*C*). Fourth, analysis with the LearnCoil-VMF program (24) identified two HR regions (Fig. 1*D*). HR1 is located at the C terminus of the fusion peptide, and HR2 is located adjacent to the N terminus of the transmembrane domain. Finally, the RDV P2 contains putative leucine zipper motifs located in the N- and C HRs (Fig. 1*E*). The positions "a" and "d" on both HR1 and HR2, which are important for coiled-coil structure formation, often consist of typical hydrophobic amino acids, e.g., leucine (L), isoleucine (I), or valine (V).

These structural features showed that P2 is similar to the fusion peptides of many enveloped viruses such as Moloney murine leukemia virus, HIV, and influenza virus. The following experiments were performed to test directly whether P2 plays a role in influencing the membrane biology of insect cells.

P2 Induced Membrane Fusion in Insect Cells. Based on the observation that P2-free RDV particles lose the ability to infect insect vector cells (20) and on the above structural information, we postulate that RDV P2 functions in membrane fusion to facilitate viral particle entry into insect cells. To test this hypothesis, we investigated P2–membrane interactions by expressing P2 in insect cells and displaying it on the cell surface, by using a vector that was used to study the bluetongue virus VP5 fusion activities (14). By using pAcVSVG, we prepared a recombinant baculovirus that expressed RDV P2 or P8 after a signal peptide derived from the baculovirus gp64 signal peptide and fused at the C terminus to the transmembrane domain of the vesicular stomatitis virus (VSV) G protein (Fig. 2*A*). Expression of P2 or P8 protein in infected

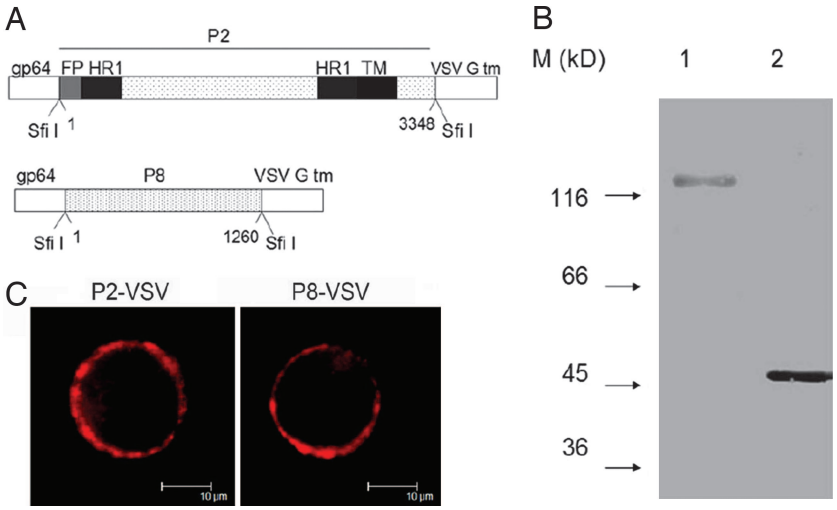

Fig. 2. Construction and expression of membrane-anchored P2 and P8. (*A*) Baculovirus transfer vectors were constructed in which the coding sequences of P2 and P8 of RDV were fused in-frame to the signal peptide of the baculovirus gp64 and VSV G at the N terminus and the C terminus, respectively. (*B*) Western blotting of insect cells infected with AcP2-VSV (lane 1) and AcP8-VSV (lane 2). (*C*) Immunofluorescence assay. Sf9 cells were infected with baculovirus encoding P2 (*Left*) or P8 (*Right*) at an MOI of 2.5 for 42 h. The cells were then labeled under nonpermeabilizing conditions with polyclonal antisera against P2 or P8, followed by TRITC-conjugated secondary antibodies. Pictures were taken on a Zeiss LSM510 microscope.

Spodoptera frugiperda (Sf9) cells with recombinant baculoviruses was confirmed by Western blotting using anti-P2 and anti-P8 sera, respectively (Fig. 2*B*). To verify that the recombinant proteins were displayed on the cell surface, we stained the cells that express P2-VSV or P8-VSV with P2- or P8-specific IgG conjugated to TRITC. Confocal microscopy showed distinct fluorescence rings around the cells, confirming display of both P2 and P8 on the cell surface (Fig. 2*C*).

To test whether the RDV P2 has a membrane fusion activity at low pH, the cells infected with P2-VSV virus were exposed to pH 5.0 for 2 min followed by incubation at the normal pH 6.2. A high number of syncytia were observed within 4 h. Both the number and size of the syncytia increased over a period of 7–8 h after pH shift (Fig. 3*A*). As a control, no membrane fusion was observed in cells infected with P8-VSV at this pH (Fig. 3*B*). As further controls, when the cells infected with either P2-VSV or P8-VSV were incubated at pH 8.0 for 2 min and then returned to normal pH condition, there was no syncytium formation even after a long period of incubation of 60 h (data not shown). In addition, when insect cells coinfected with both P2-VSV and P8-VSV were treated with low pH shift, the syncytium formation was observed (Fig. 3*C*). These results demonstrated that the RDV P2 has a specific role in inducing membrane fusion at low pH, and RDV outer capsid protein P8 has no such an activity.

The N-Terminal Fusion Peptide-Like Region of P2 Was Critical for Fusogenic Activity. Based on studies from enveloped viral fusion proteins, we postulated that the N-terminal hydrophobic sequence and the putative amphipathic helix of P2 likely function as the fusion peptide to mediate the P2 fusogenic activity. To test this, we constructed a series of truncated derivative of P2, the stable expression of these truncated mutants was examined by Western blotting assay (Fig. 4 *A* and *B*). In the mutant, in which aminos acid 1–49 covering the potential fusion peptide was deleted (Fig. 4*A*, line 2), the fusogenic activity of the truncated mutant of P2(50-1110)-VSV protein was examined in Sf9 cells infected with the recombinant baculovirus virus. As shown in Fig. 4 *C2* and *D*, bar 2, the deletion of amino acids 1–49 greatly reduced the number of syncytia. Thus, the N-terminal fusion peptide-like region of P2 contributed to the fusogenic activity of P2.

The HR Region of P2 Was Essential for Its Fusogenic Activity. The envelope of enveloped virus contains two important glycoproteins, attachment glycoprotein and fusion glycoprotein. The fusion glycoprotein contains two highly conserved HRs, HR1 and HR2 (3). When membrane fusion occurs, HR1 and HR2 form a six-helix bundle structure. Deletion of HR1 or HR2 results in different abilities to mediate membrane fusion. To test whether the HR regions of RDV P2 play a role in P2 fusogenic activity, we constructed a series of mutants in which the two HR regions of P2 were deleted individually or together (Fig. 4 *A* and *B*). As shown in

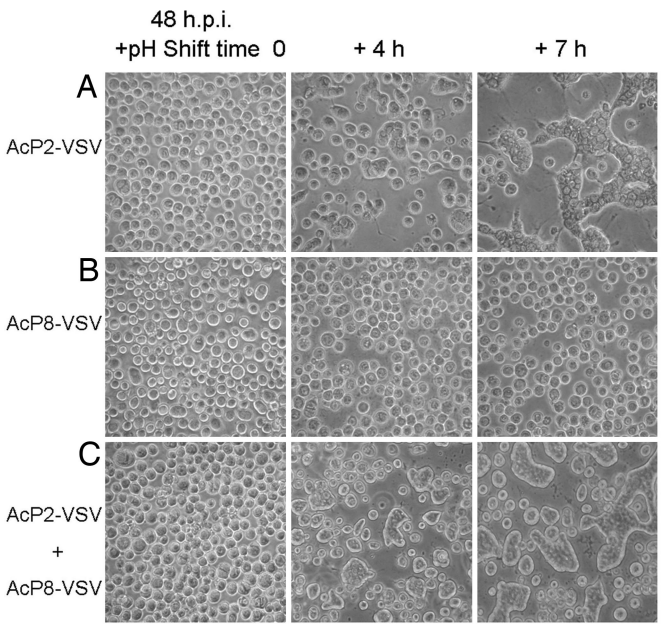

Fig. 3. Fusogenic activity of P2-VSV. Sf9 cells were infected with AcP2-VSV (*A*) or AcP8-VSV (*B*) or coinfected with AcP2-VSV and AcP8-VSV (C) for 48 h at an MOI of 2.5 and then exposed to pH 5.0 for 2 min, after which the low-pH buffer was replaced by normal growth medium. Pictures were taken on an inverted light microscope at different time points after the pH shift. Infected cells after 0 h (*Left*), 4 h (*Center*), and 7 h (*Right*) pH shift are shown.

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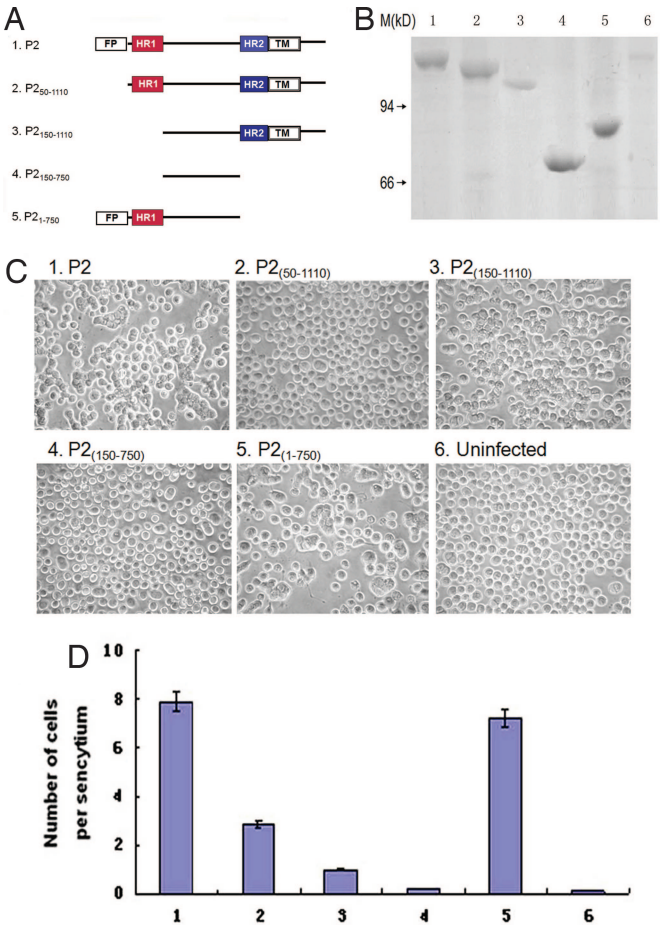

Fig. 4. Expression and fusogenic activity of P2-truncated mutants by recombinant baculoviruses in insect cells. (*A*) Schematic representation of P2 and various deletion mutants. The different structural features and domains of P2 are indicated by different shadings. (*B*) Stable expression of P2 and its mutants in Sf9 cells verified by Western blotting analysis with an anti-VSVG polyclonal antiserum. Labels on the top indicate the number of recombinant baculoviruses used for infection. Lane 6 contained an uninfected cell lysate as a control. Insect cells were infected with the recombinant baculovirus expressing P2 mutants for 48 h. (*C*) Syncytium formation pictures were taken 4 h after exposure to pH 5, and the numbers of cells per syncytium were counted. (*D*) These mutations exhibited different fusion activities, in comparison with full-length AcP2-VSV.

Fig. 4 *C* and *D*, deletion of HR1 in mutant P2 (150-1110) and deletion of HR1 and HR2 in mutant P2 (150-750) abolished the fusogenic activity of P2. Deletion of HR2 in mutant P2 (1-750) did not affect fusogenic activity. These results indicated that the N-terminal HR1 played an important role in the fusogenic activity of P2.

The Transmembrane Domain of P2 Could Anchor P2 in the Cell Membrane. To test whether the putative transmembrane domain of P2 has the function of displaying P2 on the cell membrane, we prepared a recombinant baculovirus that expresses RDV P2 without the transmembrane domain of the VSV G protein at the C terminus by adding stop codon TGA at the 3′ end of P2 ORF. Expression of P2(TGA) protein in infected Sf9 cells was confirmed by Western blotting using P2 antibody. To examine that the recombinant proteins were displayed on the cell surface, we stained the cells that express P2(TGA) with P2-specific IgG conjugated to FITC. Confocal microscopy showed distinct fluorescence rings around the cells, confirming display of P2 on the cell surface (Fig. 5*A*). Cell-to-cell fusion was observed after low-pH shift (Fig. 5*B*). These results indicate that the transmembrane domain of P2 is functional in anchoring P2 in the cell membrane.

RDV Induced Membrane Fusion in Its Insect Host Vector Cells VCMs. Based on the observation that RDV P2 could induce membrane fusion in insect cells Sf9, we postulated that RDV could also induce membrane fusion in its insect host vector cells (VCMs). To test this hypothesis, we inoculated VCMs, i.e., NC-24 cells (25), with RDV. VCMs were fixed 5 days after inoculation, stained with viral particle-specific IgG conjugated to FITC, and visualized by fluorescence microscopy. When the cells were inoculated with RDV and cultured for 5 days, a high level of cell fusion was observed, which resulted in a remarkable increase in both the number and size of syncytia, including large number of nuclei within these foci by fluorescence microscopy (Fig. 6). On the other hand, no cell fusion was found in mock-inoculated healthy cells. These results indicated that RDV infection induced cell–cell fusions and syncytium formation in VCMs.

Discussion

A number of enveloped and nonenveloped viruses enter cells by receptor-mediated endocytosis. In some cases the membrane of an enveloped virus undergoes fusion with the membrane of an intracellular vesicle, resulting in delivery of the viral nucleocapsid to the cytoplasm (26, 27). Many fusion proteins (or its homologues) are responsible for the fusion between viral and cellular membranes. The fusion proteins usually undergo a conformational change to become fusogenically active, triggered by the acidic endosome or an elevated temperature (28). The formation of coiled-coil bundles, via HR1–HR2 interaction, is often a key conformational change in the transition of the fusion protein from the fusion-inactive to the fusion-active state (29).

Nonenveloped viral particles must breach the membrane of a target host cell to gain access to its cytoplasm (30). How a nonenveloped virus enters a cell is poorly understood. It is postulated that nonenveloped viral entry into a cell is also accompanied by conformational changes in the viral particles triggered by certain conditions, such as a low pH environment for mammalian reoviruses or neutral to alkaline pH for avian reovirus (25). The protein factors and mechanisms involved remain to be fully understood.

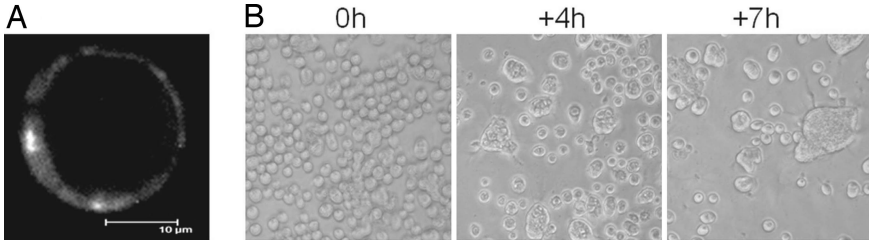

Fig. 5. Fusogenic activity of P2 (TGA)-VSV. Sf9 cells were infected with recombinant baculovirus expressing P2 (TGA) at an MOI of 2.5 for 42 h. (*A*) The cells were then labeled under nonpermeabilizing conditions with a polyclonal antiserum against P2, followed by FITC-conjugated secondary antibodies. (*B*) The fusogenic activity was checked at 48 h after infection as described above, and pictures were taken 0 (*Left*), 4 (*Center*), and 7 (*Right*) h after the pH shift.

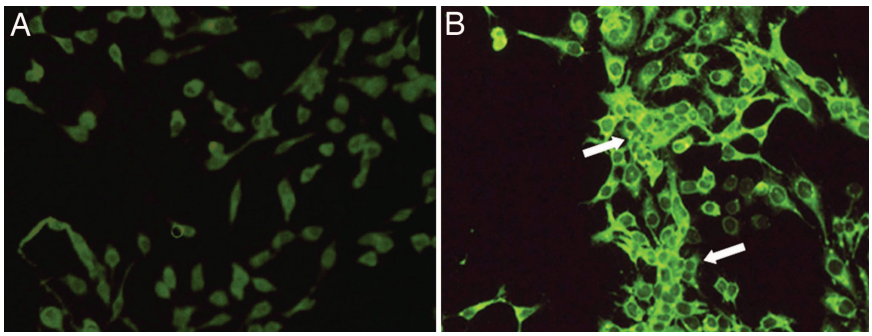

Fig. 6. Syncytia formation in RDV-infected VCMs of insect host cells. (*A*) Mock-inoculated healthy cells 5 days after seeding. (*B*) RDV-infected VCM cells 5 days after inoculation. The cells were stained with viral particle-specific IgG conjugated to FITC. Green fluorescence shows infected cells. Arrows show fused cells. (Magnification: ×500.)

We showed in this study that the RDV P2 contains structural features similar to those of type I fusion proteins of enveloped viruses, P2 has a direct role in triggering membrane fusion when expressed and displayed on the surface of insect cells, and the N-terminal region and HR1 are necessary for fusogenic activity. The specific role of P2 in membrane fusion is also supported by the absence of such an activity in P8. Thus, P2 and P8 appear to play different roles in viral entry into host cells. P8 was found to interact with rice glycolate oxidase, a typical enzyme of peroxisomes, and this interaction may target RDV into peroxisomes (31). Membrane fusion was also found in the RDV-infected insect host cells, i.e., the leafhopper VCM cells (Fig. 6). This result is consistent with P2-mediated cell fusion. It is important to note that Omura *et al.* (20) exposed monolayer cells to intact RDV virions and subsequently observed RDV double-layer particles on the surface of all cell membranes and in the vesicles of these monolayer cells under an electron microscope. A recent study (32) showed that RDV enters insect vector cells through receptor-mediated, clathrin-dependent endocytosis and is sequestered in a low-pH-dependent endosomal compartment. These microscopic observations are fully consistent with a role of P2 in membrane fusion. Intriguingly, unlike the fusion proteins of other nonenveloped viruses, the RDV P2 contains additional transmembrane domains similar to the fusion proteins of enveloped viruses. The entry of enveloped viruses into host cells requires the viral membrane to fuse with the target cell membrane. Experimental data suggest that the transmembrane domain of viral fusion glycoproteins, which is inserted into the viral envelope, is required for later steps of membrane fusion, the formation and enlargement of the aqueous fusion pore. By contrast, nonenveloped viruses have no viral membranes, and it is unlikely the transmembrane domain of the outer capsid protein has a role in the viral capsid structure. Instead, this domain might have evolved for insertion into the host cell membrane to trigger changes in the membrane dynamics to form endocytotic vesicle that enclose viral particles.

RDV P2 does not show significant amino acid sequence similarities to the fusion peptides of many enveloped viruses such as Moloney murine leukemia virus, HIV, and influenza, even the VP5 of bluetongue virus. P2 does show significant amino acid sequence similarities to P2 of rice gall dwarf *Phytoreovirus* (RGDV) (33). The function of RGDV P2 in inducing membrane fusion is not clear.

Based on results from this and previous studies, we advance the following hypothesis. RDV enters an insect vector cell through receptor-mediated, clathrin-dependent endocytosis (32). RDV P2 may be involved in the recognition of viral particles by host cell receptors and the formation of virus-containing endocytotic vesicle. Within the cell a low-pH endosomal entry pathway exists and P2 plays key roles in the release of viral particles into the cytoplasm from the endocytotic vesicles and the fusion of host cell membrane with the membrane of endocytotic vesicles. Because enveloped viruses use the same mechanism to mediate the membrane interactions involved in both virus entry and syncytium formation (33), the syncytium-inducing ability of RDV in VCMs suggests that RDV P2 is required to promote the membrane interactions necessary for both virus entry and syncytium formation. P2 may also play an important role in RDV moving from cell to cell by inducing host cell membrane fusion.

We have shown that the RDV P2, a plant viral protein, has a distinct role in membrane fusion. Unlike the fusion proteins of enveloped viruses that require proteolytic cleavage to expose the fusion peptide, the fusion peptide of RDV P2 is already present in the N terminus of the native protein. This not only makes the RDV P2 a simpler model to further study the role of fusion proteins in membrane fusion, but also raises the question of whether the RDV P2 represents just one example of a class of fusion proteins yet to be identified from plant viruses. Because many animal viruses and most plant viruses are nonenveloped, the identification of a plant viral protein with membrane fusion activity paves the way for further mechanistic studies of viral entry into host cells that are of general significance. Detailed structural and functional analyses of fusion proteins from nonenveloped and enveloped viruses may shed light on the evolution of these important proteins and viruses. The fusion protein may also become a valuable tool in biotechnology, such as drug delivery and insect control.

Materials and Methods

Cells, Viruses, and Antibodies. Sf9 cells were grown in 10% FBS-supplemented TNMFH medium (Invitrogen). Recombinant baculoviruses were obtained as described (34, 35).

NC-24 cells, originally established from embryonic fragments that had been dissected from eggs of *Nephotettix cincticeps*, vector insect of RDV, were maintained in monolayer culture at 25°C in a growth medium prepared as described (36). The O strain of RDV was purified from infected rice plants without using CCl4 as described (17). Fractions from a sucrose gradient containing purified RDV particles in a solution of 0.1 M histidine that included 0.01 M MgCl2 (pH 6.2) were pooled and stored at −70°C.

Antisera raised in rabbits against intact viral particles (intact IgG) (37), P2, or P8 (38) were used in this study. The antibodies against viral particles were conjugated directly to FITC as described (39). Rabbit polyclonal anti-VSVG was a kind gift from I. M. Jones (Reading University, Reading, U.K.).

PCR Primer Sequences. The oligonucleotides used in this study are listed in Table 1.

Constructs for Expressing RDV Proteins. Standard procedures were used for PCR and plasmid DNA manipulation (40). For construction of recombinant baculovirus transfer vectors, the coding regions of the P2 and P8 genes of RDV were amplified by PCR

Table 1. Primers used in this study

Primers	Primer sequences
S2 5′	5′-ATTAGGCCATTATGGCCATATGGCTTATCCTAATGAGT-3′
S2 3′	5′-AATTGGCCGAGGCGGCCCAATGCATCATAGATAGA-3′
S2 50 5′	5′-ATTAGGCCATTATGGCCATGATCAATTGATTATTGCT-3′
S2 150 5′	5′-AGCAGGCCATTATGGCCATGTTAACATGAAAAATGTC-3′
S2 100 5′	5′-AGCAGGCCATTATGGCCATGTTTTAGACTTAACTGGT-3′
S2 750 3′	5′-AATTGGCCGAGGCGGCCATATGAATTAACGCCGGTG-3′
S2 stop	5′-AATTGGCCGAGGCGGCCTCAAACATCTAGTTTCTCATC-3′
S8 5′	5′-CGATGGCCATTATGGCCTGTCACGCCAGATGTGGTTAGA-3′
S8 3′	5′-GCTAGGCCGAGGCGGCCATTTGGTCTATAGTATCT-3′

Underlining indicates the restriction sites.

using pBV220-S2 and pBV220-S8 as templates (38). The primers were designed to introduce SfiI sites at both ends of the PCR products. After restriction digestion with SfiI, the PCR products were ligated into the SfiI site of the transfer vector pAcVSVG/SfiI. The same procedure was used to generate constructs in which the predicted domains of P2 were deleted separately. Recombinant plasmids were identified by restriction digestion and verified by sequence analysis. The transfer plasmids were then used to generate recombinant baculoviruses as described.

Western Blotting Assay. Sf9 cells were infected with recombinant baculoviruses at a MOI of 5. Cells were harvested 48–60 h after infection and lysed as described (41). The lysates were resolved by electrophoresis in SDS-polyacrylamide gels (8%), and proteins were transferred to Hybond-P membrances (Amersham Biosciences). The membrane was blocked overnight with blocking buffer (1% BSA/0.3% Triton X-100/0.05% Tween 20 in PBS) and incubated with 0.2% (vol/vol) antiserum against RDV P2 or P8 for 1 h. After washing, the membrane was probed with goat anti-rabbit alkaline phosphatase conjugate for 1 h. Signals were developed with nitroblue tetrazoliun and 5-bromo-4-chloro-3-indolyl phosphate (15).

Cell Fusion Assay. Monolayer cultures of Sf9 cells were infected at MOI of 0.1–2.5. At 24, 36, or 60 h after infection, the cells were washed in TNMFH medium and then incubated for 1 h with a mAb against gp64 at 1:1,000 dilution. After washing with the medium, the cells were washed and then incubated for 2 min in low-pH buffer (PBS, pH 5.0). To return the cells to the normal pH 6.2, the cells were washed twice with and then incubated in the TNMFH medium at 28°C. Syncytium formation was observed and photographed under a light microscope (14).

Immunofluorescence Microscopy. The recombinant baculoviruses expressing the chimeric proteins were used to infect a monolayer of Sf9 cells at a MOI of 2.5 on 22- to 22-mm glass coverslips. After 42 h, the infected cells were washed with PBS three times (5 min) and then incubated in PBS containing 5% BSA for 20 min at 37°C. The cells were then incubated with primary antibodies diluted in PBS containing 1% BSA for 1 h at room temperature. After three washes in PBS, the cells were incubated with secondary antibodies diluted in PBS containing 1% BSA for 1 h at 37°C. The cells were finally incubated with 300 nM DAPI (Molecular Probes), in PBS for 5 min to counterstain the nuclei, briefly washed in PBS, and mounted on glass slides with 50% glycerol. Samples were examined under an inverted microscope (Leica) and photographed with a digital camera (Nikon) or under a Leica confocal microscope with the associated software.

RDV-Infected VCMs Cell Fusion Assay. VCMs (1 day after seeding at low density) were inoculated at a low MOI of 0.001 with RDV. After a 2-h adsorption period at 25°C, the inoculum was removed. After 5 days incubation at 25°C, VCMs were fixed by 2% paraformaldehyde, stained with viral particle-specific IgG conjugated to FITC (39), and visualized by fluorescence microscopy. Mock-inoculated healthy cells were processed in the same manner. Photomicrographs of representative florescent foci showing infected cell morphology or syncytium formation were obtained.

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