Complex molecular architecture of beet yellows virus particles

Valera V. Peremysovy†, Igor A. Andre ev‡, Alexey I. Prokhn evsky*, George H. Duncan‡, Michael E. Taliansk y*, and Valerian V. Dolja*‡

*Department of Botany and Plant Pathology and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR 97331; ‡Gene Expression Programme, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom; and §Electronic Engineering and Physics Division, University of Dundee, Dundee DD1 4NH, United Kingdom

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Viruses and retroviruses (9, 10). The unexpectedly complex structure of closterovirus virions has important mechanistic and functional implications that may also apply to other virus families.

Our data for virion assembly and function in virus transport are discussed in relation to other plant viruses with helical capsids.

Abbreviations: CP, capsid protein; TMV, tobacco mosaic virus; BYV, beet yellows virus; CPm, minor capsid protein; p64, 64-kDa protein; Hsp70h, Hsp70 homolog; p20, 20-kDa protein; AFM, atomic force microscopy; EM, electron microscopy.

Virus particles (virions) provide the functions of genome packaging, protection, and transfer between host organisms, tissues, and cells. All virions possess a protein shell that surrounds the viral genome. In addition, enveloped viruses acquire a lipoprotein “overcoat” by budding through the cell membrane. Two types of shell architecture exist:icosahedral and helical. A textbook example of helical architecture is provided by the rigid, rod-shaped virions of tobacco mosaic virus (TMV) that are formed from a single RNA molecule and ~2,000 copies of a capsid protein (CP), with the RNA winding in a helical path that follows the arrangement of the CP subunits (1, 2). Variations on this theme are seen in many families of rod-shaped (3) and filamentous (4) plant RNA viruses. Based on amino acid sequence analyses, the CPs that assemble into helical capsids of plant viruses belong to two distinct protein families that might have diverged from a single ancestor (5, 6).

In the TMV, virions self-assemble under appropriate conditions, because all required information is contained within the virion components (7); that is, the RNA possesses an origin of assembly or packaging signal, and the folding of the CP molecule facilitates interactions with RNA and other CP molecules. Although “automation” is used by virtually all viruses for assembly of the virions or parts thereof, the complex architecture of some virions requires participation of scaffolding or chaperone proteins that normally are not incorporated into the mature virion. Some of these proteins are virus-encoded (8), whereas others are borrowed from the cell molecular chaperone machinery. Among the latter, members of the Hsp70 family of proteins are recruited for virion formation or transport of virion components by diverse RNA and DNA viruses and retroviruses (9, 10).

Except for TMV, little is known about the assembly mechanisms of helical plant viruses. It has been suggested that, unlike TMV, whose packaging signal is located at a distance of ~1,000 nucleotides from the RNA 3′ terminus, virions of the family Potyviridae and the genus Potexvirus start their assembly near the RNA 5′ terminus (11, 12). However, attempts to achieve efficient in vitro self-assembly of these filamentous virus particles were largely unsuccessful (7), suggesting that host factors may be required to aid assembly.

Virus species from the family Closteroviridae have long filamentous virus particles that encapsidate the 15- to 20-kb positive-strand RNA genome (13, 14). The 15.5-kb genome of beet yellows virus (BYV), codes for at least 10 proteins (Fig. 1A). Recent studies demonstrated that BYV virions incorporate at least five of these proteins (15-18). The major CP (17, 22–24), indicating that the formation of the “tailed” virions is a prerequisite for intercellular trafficking. In contrast, p20 is dispensable for virion assembly and cell-to-cell movement, but is necessary for BYV transport through the plant vascular system (18). A CPm, Hsp70h, and p64 ortholog were also found in the viroin of the tomato asperatus (26). Both these viruses belong to the family Closteroviridae and are distantly related to BYV.

Perhaps the most remarkable component of BYV virions is the virus-encoded homolog of cellular Hsp70 molecular chaperones (Hsp70h) (16, 20). So far, only closteroviruses have been found to harbor a Hsp70 gene, although papovaviruses and a baculovirus code for Hsp70 chaperones called J-domain proteins (10, 21). In addition to functions in DNA replication and tumorogenesis, the papovavirus J-domain protein was implicated in assembly of the icosahedral virions. The fifth virion protein of BYV is a 20-kDa protein (p20) that interacts with Hsp70h (18). BYV CP, CPm, p64, and Hsp70h are each required for virion assembly and subsequent cell-to-cell movement (17, 22–24), indicating that the formation of the “tailed” virions is a prerequisite for intercellular trafficking. In contrast, p20 is dispensable for virion assembly and cell-to-cell movement, but is necessary for BYV transport through the plant vascular system (18). A CPm, Hsp70h, and p64 ortholog were also found in the viroin of the tomato asperatus (26). Both these viruses belong to the family Closteroviridae and are distantly related to BYV.

Despite the rapid progress in closterovirus research, the exact architecture of the virion has not been established. Here we determine the molecular composition of the virion tails and reveal their complex morphology by using atomic force microscopy (AFM). Possible implications of our data for virion assembly and function in virus transport are discussed in relation to other plant viruses with helical capsids.
Protein (p21). (protein (p21), major capsid protein (CP), 20-kDa protein (p20), and 21-kDa protein (p6), Hsp70-homolog (Hsp70h), 64-kDa protein (p64), minor capsid ferase (MET), RNA helicase (HEL), and RNA polymerase (POL) domains, 6-kDa for leader proteinase (L-Pro), replication proteins that possess methyltrans-

Materials and Methods

Isolation and Immunoblot Analyses of the Virion Tails. Wild-type virions of the BYV-California isolate were purified from Nicotiana benthamiana plants as detailed (16). Mutation Nop20, which inactivated the start codon of the p20 (p20) was introduced into the binary vector p35S-BYV-GFP harboring a full-length cDNA copy of the BYV genome tagged by insertion of the gene encoding the green fluorescent protein (BYV-GFP) (18). The resulting mutant was inoculated to N. benthamiana plants by the agroinfection protocol; because of the limited systemic spread of the p20-deficient virus, virions were purified from the green fluorescent areas (18). Wild-type or mutant virions were resuspended in buffer 1 (20 mM sodium phosphate buffer, pH 7.2/1 mM EDTA/0.1% Triton X-100) and treated with an Ultrasonic Processor XL (Heat Systems/Ultrasonics), at a setting of 20% power output, in 0.5-mL aliquots with eight 15-s bursts. The fragmented virions were then loaded on the top of 20–60% sucrose gradients prepared in buffer 1 and centrifuged at 100,000 × g for 16 h in a Beckman SW40 rotor at 4°C. Gradients were separated into 15 fractions; those fractions containing the peak of CP were pooled and diluted in buffer 1; virion fragments were recovered by ultracentrifugation for 6 h at 150,000 × g. Immunoblot analysis was performed according to standard procedures (16). Membranes were incubated with polyclonal antibodies to CP (1:10,000 dilution), CPm (1:2,000 dilution; ref. 23), Hsp70h (1:4,000 dilution; ref. 28), p64 (1:1,000 dilution; ref. 17), or p20 (1:1,000; ref. 18), followed by goat anti-rabbit IgG-horseradish peroxidase conjugate (1:5,000 dilution, Bio-Rad) and visualized by chemiluminescence (ECL Kit, Amersham Pharmacia).

Northern Hybridization Analyses. Total RNA was extracted from virion tail pellets or from intact BYV particles by using TR Izol (Invitrogen) according to the manufacturer’s protocol, and the hybridization analysis was carried out as described (27). To generate [32P]UTP-labeled, negative-sense RNA probes, 5’ and 3’ regions of the BYV genome were cloned downstream of the T7 RNA polymerase promoter, linearized to yield cDNA templates of 534 and 590 nucleotides, respectively, and transcribed in vitro. Prehybridization and hybridization steps were carried out at 65°C in NorthernMax Prehyb/Hyb buffer (Ambion, Austin, TX). To determine the size distribution of the RNAs derived from the fragmented virions, a specially made set of RNA size markers was designed. These positive-sense, 5’-c-terminal BYV RNA fragments were derived in the same way as the RNA probes described above, except that the SP6 RNA polymerase promoter was introduced upstream of the 5’ terminus of the BYV cDNA. In vitro transcription was used to generate a mixture of the RNAs corresponding to 5’-terminal 140, 270, 490, 840, 1,580, and 2,150 nucleotides of BYV RNA. These RNA fragments were detected by hybridization with the 5’ end, negative-sense RNA probe.

Atomic Force and Electron Microscopy. BYV virions or their fragments were diluted to ~5 ng/µl in 0.02 M phosphate buffer (pH 7.3), and 5–10 µl were placed onto freshly cleaved mica strips for 5–15 min. The strips were rinsed with deionized water and vacuum-dried at room temperature. Imaging of particles was done in the tapping mode (29–31) in air at a frequency of 300–380 kHz on a NanoScope IIIa multimode scanning probe microscope (Digital Instruments, Santa Barbara, CA) using standard AFM silicon-nitride cantilevers with a length of 123 µm (Nanosensors, Neuchatel, Switzerland). Images, including 3D representations were processed with NANOSCOPE software and transferred to PHOTOSHOP (Adobe Systems, Mountain View, CA) for layout. Sample heights and lengths were measured automatically with the NANOSCOPE software. Structural models of BYV tails were developed by averaging the dimensions (heights and lengths) of the tail segments in cross sections (see Fig. 5, which is published as supporting information on the PNAS web site). Data were mean ± standard errors for 100 virus particles observed in three independent experiments. For electron microscopy, fragments of the BYV virions were negatively stained with 2% uranyl acetate and photographed with a Philips CM 10 electron microscope (Philips Electronic Instruments, Eindhoven, The Netherlands).

Results

Molecular Composition of Virion Tails. Previous work demonstrated that the BYV Hsp70h and p64 proteins are required for assembly of the virion tails, but not the bodies (17, 23), suggesting that each of these proteins is an integral tail component. It was also suggested that the tails are assembled in the proximity of the RNA 5’ terminus, although the length of the RNA fragment encapsidated by the tails was not determined (29). To ascertain the molecular composition of the tails, the virions were sonicated and the resulting random fragments were separated in sucrose gradients according to their buoyant density. Immunoblot analysis revealed that most of the CP remained at the top of the gradient (fractions 1–3) with a minor peak in fractions 6–8 (Fig. 1B). In contrast, when
of the 3′capsidate a 5′that these 3′6 Thus, enrichment of the CPm content relative to CP in fractions BYV genome present in fractions 6–8. Even more importantly, the signal from 3′5032/H20841AFM Reveals Complex Morphology of the Tails. The unusual mor-
plexes (30–32). Recent work demonstrated the ability of AFM to resolve fine details of virion architecture without the need for staining (33, 34). AFM imaging of intact BYV virions in air readily revealed their polar nature (Fig. 3A). Tails were apparent as ~90-nm-long virion segments that were darker than the virion bodies. In AFM, a darker appearance indicates a lesser height or, in the case of a filament, a lesser diameter. Reconstructed 3D images of the virion tails consistently showed a peculiar three-
segment morphology with a tapered-tip segment (Fig. 3B). A similar topography of segmented BYV tails was revealed by AFM in a liquid environment (data not shown). In contrast, the opposite end of the virion appeared to be blunt and lacked any fine structural features (Fig. 3C). Both particle ends of another filamentous virus, potato virus X, or of the rod-shaped TMV analyzed by AFM as controls, were blunt and did not differ in their appearance (Fig. 6, which is published as supporting information on the PNAS web site).

The virion tails isolated after sonication exhibited same three-segment morphology as those seen in the intact virions (Fig. 3D). In addition, two-segment tail fragments and small virion fragments of undefined morphology were also occasionally seen in these preparations (not shown).

Statistical evaluation of the tail measurements in cross sections (as shown in Fig. 5) allowed us to develop a model of the tail structure shown in Fig. 3E (Upper). This model indicates that the tail has two ~35-nm-long segments with a diameter of ~8 nm, and a tip segment that is ~25 nm long and has a diameter of ~6 nm. In contrast, the ~12-nm diameter of the virion body is virtually constant along its entire length. This model likely reflects differences in geometry and/or physical properties between the segments and their connecting areas. It is well known that horizontal dimensions of objects are always overes-
imated in AFM because of the effect of the geometry of the
cantilever (30–32). As a result, the objects appear broader and longer than their real dimensions. This appearance could explain some differences in the lengths of virion tails measured by using either EM or AFM. Alternatively, it is possible that AFM reveals the smallest tip tail segment, whereas EM does not. This outcome could be because the tail is less efficient at excluding the uranyl acetate during negative staining; unlike EM, AFM does not require staining.

The p20 Is Required for Proper Tail Morphology. As mentioned in the introduction, all the virion components of BYV except for p20 are required for assembly of cell-to-cell movement-competent virions. Because of this, inactivation of either the CP, CPm, p64, or Hsp70h limits virus accumulation in the inoculated plants to single cells, thus precluding isolation of the high-quality virion preparations required for AFM. However, we were able to isolate p20-deficient virions by using the BYV mutant Nop20 (27) in which the p20 start codon was inactivated. AFM analysis of these virions revealed that most possess two-segmented tails (Fig. 4A). No virions containing all three tail segments were found in these preparations. As expected, immunoblot analysis of the mutant virions revealed normal amounts of the CP, CPm, Hsp70h, and p64, but no p20 (Fig. 4B). These data confirm our previous results with other p20 mutants (18) and extend the analysis to include p64. In addition, these data suggest that although p20 is not required for incorporation of other proteins into virions, it either forms the tip segment of the virion tail or is necessary for its assembly.

Discussion

Here we describe the unusually complex molecular composition and morphology of the BYV virion. We demonstrate that these filamentous virions are polar with a peculiar, tail-like formation that encapsidates 650 bases of the 5’ terminal of the genomic RNA. The wild-type virion tails contain at least four proteins, including CPm, which is the principal tail component, and three additional constituents, Hsp70h, p64, and p20. The AFM analyses revealed that the tail is thinner than the rest of the virion and that it is subdivided into three segments. Very similar tail morphology was observed for a BYV relative, grapevine leafroll-associated virus 2 (35) (Fig. 7, which is published as supporting information on the PNAS web site), indicating that the tail structure is conserved at least among two members of the genus Closterovirus. Because the p20-deficient virions contain all other virion proteins but lack the pointed-tip segment, we propose that this segment is formed by p20 (Fig. 3E). The fifth virion protein, the major CP, is a principal constituent of the long virion body that encapsidates 96% of the BYV genome. The body morphology appears to be uniform along its entire length and is similar to that described for many diverse filamentous plant viruses outside the Closteroviridae family.

We have recently developed an Agrobacterium-based, in planta model system for investigation of BYV assembly. Using this system, we isolated transport-deficient virions that were assembled in the presence of nonfunctional CPm (D. V. Alzhanova and V.V.D., unpublished data). These virions lacked not only CPm, but also Hsp70h and p64, suggesting that these proteins are coordinately incorporated into tails but not bodies, thus supporting conclusions reached in this study.

How do the multicomponent BYV virions assemble? The fact that CPm and the other tail proteins encapsidate the 5’-terminal
portion of the RNA strongly suggests that this region harbors a packaging signal, which governs tail formation. Our preliminary results with the trans-encapsidation of a BYV minireplicon by ectopically expressed CPm indicate that this protein alone can initiate virion assembly (A. J. Napuli and V.V.D., unpublished data). Moreover, a recent report on another closterovirus, citrus tristeza virus, indicated that the CPm recognizes a packaging signal. It is tempting to speculate that the assembly of the virion body from CP involves a second packaging signal. Alternatively, the CP could assemble by attachment of the subunits to the surface at one end of a preformed tail. The roles of Hsp70h and p64 in tail formation are less clear. It seems that, collectively, these proteins may act as molecular rulers for determining the tail’s length or as connectors between tail and body and/or individual tail segments.

Perhaps the most remarkable aspect of this study is the demonstration that Hsp70h is an integral component of the virion tail. Normally, Hsp70 and other protein chaperones are not incorporated into the final products of chaperone-mediated processes (36). Thus, closteroviral Hsp70h violates a chaperone dogma.

What are the possible functional implications of the complex architecture of BYV virions? It was demonstrated that, in the absence of functional CPm, CP is capable of encapsidating the entire BYV genome (23), suggesting that the tail has not evolved merely for RNA protection. Because the Hsp70h, p64, and CPm are each required for BYV cell-to-cell movement, it was proposed that the tail represents a viral transport device (17, 23). This interpretation seems to be even more likely in relation to the tip segment. The only function assigned to the tip-forming p20 is long-distance transport of the virions by the plant phloem (18). Therefore, it seems plausible that the tip segment mediates transport of the virions by plasmodesmata interconnecting companion cells and sieve elements and/or stabilizes the virion tails during their passage through the hostile phloem environment (37). The latter possibility is supported by the lesser stability of the p20-deficient virions compared with that of the wild-type virions (I.A.A. and M.E.T., unpublished data).

Note Added in Proof. After submission of this manuscript, Satyanarayana et al. (47) demonstrated that, in the absence of CP, CPm of citrus tristeza closterovirus recognizes the packaging signal near the 5’ terminus of viral RNA. Moreover, in the presence of Hsp70h and p61 (ortholog of BYV p64), CPm encapsidates ~630 5’-terminal nucleotides of the viral RNA. These results are in agreement with our analyses of the tails derived from intact BYV particles.

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What could be the reason for the attachment of a complex transport device to the virions? One possibility is that the closterovirus virions are exceptionally long, and simply cannot be trafficked from cell to cell without an extra mechanism powered by the ATPase activity of Hsp70h. Plasmodesmal localization of Hsp70h (38) is also suggestive of a specific role played by the thin tails in the entry of virions into the narrow channels of plasmodesmata. A complementary possibility is a need for a directional, 5’ to 3’ transport of the viral RNA. Because the large BYV genome needs to be protected from degradation by the host machinery used in RNA silencing (39, 40), particle disassembly on arrival in the adjacent cells should be tightly regulated and coupled to primary translation. One of the tail functions could be control of virion stability and disassembly.

The evidence suggests that directional transport and virus particle disassembly could also be used by other elongated viruses. Such functions could be performed by the potyviral VPg, which is attached to the 5’ terminus of the viral genome and is exposed at the virion’s end (41). VPg has been implicated in potyvirus transport (42, 43). Another possible example is provided by the potexviral movement protein TGBp1, which attaches to virions and mediates their destabilization and disassembly in a 5’ to 3’ direction (44, 45). Given that closteroviruses, potexviruses, and potyviruses belong to distinct evolutionary lineages of positive-strand RNA viruses (46), it seems conceivable that directional intercellular transport could be a general tendency among viruses sharing filamentous virus particle morphology.