

Cylindrical inclusion protein of potato virus A is associated with a subpopulation of particles isolated from infected plants

Rasa Gabrenaite-Verkhovskaya,¹ Igor A. Andreev,² Natalia O. Kalinina,³ Lesley Torrance,² Michael E. Taliany² and Kristiina Mäkinen¹

Correspondence

Kristiina Mäkinen
kristiina.makinen@helsinki.fi

¹Department of Applied Chemistry and Microbiology, FIN-00014 University of Helsinki, Finland

²Plant Pathology Programme, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

³A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119992, Russia

Potato virus A (PVA) particles were purified by centrifugation through a 30% sucrose cushion and the pellet (P1) was resuspended and sedimented through a 5–40% sucrose gradient. The gradient separation resulted in two different virus particle populations: a virus fraction (F) that formed a band in the gradient and one that formed a pellet (P2) at the bottom of the gradient. All three preparations contained infectious particles that retained their integrity when visualized by electron microscopy (EM). Western blotting of the P1 particles revealed that the viral RNA helicase, cylindrical inclusion protein (CI), co-purified with virus particles. This result was confirmed with co-immunoprecipitation experiments. CI was detected in P2 particle preparations, whereas F particles were devoid of detectable amounts of CI. ATPase activity was detected in all three preparations with the greatest amount in P2. Results from immunogold-labelling EM experiments suggested that a fraction of the CI present in the preparations was localized to one end of the virion. Atomic force microscopy (AFM) studies showed that P1 and P2 contained intact particles, some of which had a protruding tip structure at one end, whilst F virions were less stable and mostly appeared as beaded structures under the conditions of AFM. The RNA of the particles in F was translated five to ten times more efficiently than RNA from P2 particles when these preparations were subjected to translation in wheat-germ extracts. The results are discussed in the context of a model for CI-mediated functions.

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INTRODUCTION

Potato virus A (PVA) belongs to the genus *Potyvirus* in the family *Potyviridae*. A distinct feature of this virus family is the ability to induce formation of laminate or pinwheel-shaped cytoplasmic inclusion bodies, which consist of accumulated cylindrical inclusion protein (CI; reviewed by Edwardson, 1992; Hammond, 1992). The first indication of possible involvement of CI in virus movement came when CIs of several potyviruses were shown to form cone-shaped structures anchored to the cell wall or plasma membrane in close proximity to plasmodesmata (Lawson & Hearon, 1971; Langenberg, 1986; Rodríguez-Cerezo *et al.*, 1997; Roberts *et al.*, 1998). Genetic evidence that potyviral CI plays an essential role in virus movement was obtained when it was shown that several replication-competent tobacco etch virus CI mutants possessed cell-to-cell or long-distance movement defects in tobacco plants (Carrington *et al.*, 1998). Observations from infected cells led to the suggestion that CI may be functional only for a short period of time, in the first five to seven cells of the

infection front (Roberts *et al.*, 1998). The ability of CI to form pinwheel structures and to assist in virus movement depends on its ability to self-interact, and the self-interaction domain has been located in the N-terminal part of the protein (Lopez *et al.*, 2001). Mutations abolishing CI self-interaction inhibit potyvirus cell-to-cell movement (Gómez de Cedrón *et al.*, 2006) and it has been proposed that these mutations may affect CI structural features required for virus transport to neighbouring cells.

Efficient potyvirus transport through plasmodesmata requires specific interactions between CI and the coat protein (CP). CPs of three different potyviruses were found to interact only with their homologous CI proteins in mixed infections (Langenberg, 1993). In the yeast two-hybrid system, PVA CI interacts strongly with HC-Pro (Guo *et al.*, 2001). According to the current models of potyvirus infection (Carrington *et al.*, 1998; Roberts *et al.*, 1998), CI in an infected cell attaches to plasmodesmal apertures forming a cone-shaped structure, and newly formed virions or ribonucleoprotein complexes associate

with a central channel in the structure. CI then mediates the passage of virus into the next cell by feeding virions or transport complexes through the CI structures and plasmodesmata to the adjacent cell. This occurs only during the phase of active virus replication in the cell, after which the inclusion bodies disassociate from the cell wall, accumulate in the cytoplasm and begin to degenerate (Roberts *et al.*, 1998). This model is supported by the fact that plasmodesmata-associated CI structures have been shown to contain CP and viral RNA (Rodríguez-Cerezo *et al.*, 1997; Roberts *et al.*, 1998). In pea seed-borne mosaic virus infection, the CP forms fibrils similar to virus particles in the central core of the pinwheel structures and plasmodesmal cavities (Roberts *et al.*, 1998).

Recent studies on filamentous plant virus particles using atomic force microscopy (AFM) have revealed the presence of virion tails at one end of the particles of closteroviruses (Peremyslov *et al.*, 2004; Alzhanova *et al.*, 2007). Similar protruding tip structures have also been found in potyviruses (Torrance *et al.*, 2006). Although no tip structure was observed in potexvirus particles, the movement protein, triple gene block protein 1 (TGBp1), was shown to be attached to one of the extremities of potato virus X (PVX) filamentous virions (Atabekov *et al.*, 2000) and of *in vitro*-assembled, single-tailed, ternary 'CP-RNA-TGBp1' complexes (Karpova *et al.*, 2006). These virion terminal structures consist of movement-related viral proteins harbouring enzymic activities such as ATPase (Peremyslov *et al.*, 1999) and helicase (Kalinina *et al.*, 2002). Potyviral CI has also been shown to have RNA helicase and ATPase activities (Lain *et al.*, 1990, 1991; Eagles *et al.*, 1994). As the presence of RNA helicase activity has been demonstrated in the terminal structures of filamentous closteroviruses and potexviruses, we decided to study whether CI interacts with PVA virion structures. A virus purification protocol that allowed separation of two different PVA populations from infected plants was developed and the presence of PVA CI in these virus samples was studied. Both electron microscopy (EM) and AFM techniques, in addition to biochemical analysis, were used to compare the properties of these virus populations.

METHODS

Plants and viruses. PVA strain B11 (Puurand *et al.*, 1994) was propagated in tobacco plants (*Nicotiana tabacum* cv. SR1). One gram of homogenized PVA-infected leaf material diluted with 4 ml distilled water was used as an inoculum. Tobacco plants were mechanically inoculated by rubbing the inoculum onto the lower leaves with carborundum as an abrasive. PVA infection was detected by immunoblotting with anti-CP antiserum from rabbit and with a SpotCheck LF PVA Rapid Detection kit (Neogen). Systemically infected leaves were used for virus purification by centrifugation through a 30% sucrose cushion as described previously (Browning *et al.*, 1995) to produce a viral pellet (P1). PVA particles were further purified through a 5–40% (w/v) sucrose gradient for 1 h at 80 000 g at 4 °C. The band fraction (F) was collected, diluted 1:2 in 100 mM HEPES (pH 8.0) and concentrated for 1 h at 100 000 g at 4 °C. The pellet (P2) was resuspended in 100 mM HEPES (pH 8.0).

Recombinant protein expression and purification. His₆-tagged proteins CP, VPg and CI were expressed from pQE-30 expression vectors (Merits *et al.*, 1998) in *Escherichia coli* strain M15(pREP4) cells for 4 h with IPTG induction and purified using Ni-NTA agarose (Qiagen) according to standard protocols under denaturing and native conditions. Proteins purified under denaturing conditions were refolded by rapid dialysis against water at room temperature (~22 °C).

SDS-PAGE and Western blotting. Protein and virus samples were separated by 12% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) and Safe Coomassie (Bio-Rad Laboratories), or blotted by electrophoretic transfer to a PVDF membrane (Immobilon-P; Millipore). Quantification of the band intensities in the Coomassie-stained SDS-PAGE was done using the volume array tool of Quantity One 1-D Analysis Software (Bio-Rad). Western blot membranes were blocked for 15 min in 2% dried milk in PBS and incubated for 60 min with either rabbit polyclonal anti-CP antiserum or affinity-purified rabbit polyclonal anti-CI IgG (both diluted 1:2000 in PBS). Proteins were detected with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich) diluted 1:5000 in PBS.

Immunoprecipitation. PVA particles were immunoprecipitated with protein A-coated magnetic beads (diameter 2.8 mm, Dynabeads; Dynal Biotech ASA), following the manufacturer's protocol. Briefly, protein A-coated magnetic beads were washed with 100 mM sodium phosphate buffer (pH 8.1), incubated with affinity-purified polyclonal anti-VPg IgG for 10 min at room temperature and again washed with 100 mM sodium phosphate buffer (pH 8.1). For cross-linking of the antibodies to protein A, beads were washed twice with 200 mM triethanolamine (pH 8.2) and incubated in 20 mM dimethyl pimelimidate dihydrochloride in 200 mM triethanolamine for 30 min at 20 °C by rotating the tube. The beads were then incubated in 50 mM Tris/HCl (pH 7.5) for 15 min at room temperature and washed three times with PBS buffer. The cross-linked beads were incubated with purified PVA particles for 60 min at room temperature and washed three times with PBS buffer. After washing, beads were resuspended in 15 µl PBS and used for analysis by SDS-PAGE and Western blotting.

EM. For immunogold-labelling experiments, PVA particles were labelled essentially as described previously (Puustinen *et al.*, 2002), except that grids with bound virus were incubated with diluted anti-CI antibodies and with pre-immune serum.

ATPase assays. An ATPase assay with natively purified recombinant CI protein and purified PVA particles was performed using an EnzChek phosphate assay kit (Molecular Probes) according to the manufacturer's protocol. One reaction was performed in 100 µl reaction mixture containing 5 µl of the supplied 20× buffer [1 M Tris/HCl (pH 7.5), 20 mM MgCl₂], 20 µl MESG substrate (2-amino-6-mercapto-7-methylpurine ribose), 1 µl purine nucleoside phosphorylase, 10 mM DTT and additional MgCl₂ to give a final concentration of 3.5 mM. ATPase activity was measured over time by monitoring UV light absorbance at 355 nm.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis. A sample of purified PVA particles (~35 µg) was separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. The gel piece containing the band of interest was destained with 200 µl 50% 0.25 M ammonium bicarbonate (AMBIC)/50% acetonitrile (ACN) for 15 min and then treated with 200 µl 100% ACN. The gel was dried in a HetoVac vacuum centrifuge for 5 min and rehydrated in 100 µl 20 mM DTT in 0.1 M AMBIC for 30 min at 56 °C. The liquid was removed and the gel piece was treated with ACN as above, alkylated with 100 µl

55 mM iodoacetamide in 0.1 M AMBIC for 15 min at room temperature in the dark, washed with 100 μ l 0.1 M AMBIC and treated again with ACN as above. The gel piece was rehydrated with 10 μ l 0.02 μ g trypsin (Promega) μ l⁻¹ in digestion buffer (90% 0.1 M AMBIC/10% ACN) for 10 min at room temperature. Digestion buffer (10 μ l) was added and the sample was incubated at 37 °C for 16 h. To recover the peptides from the digest, the gel piece was placed in a clean Eppendorf tube and 10 μ l 25 mM AMBIC was added and incubated for 15 min at room temperature. The supernatant was recovered and the procedure was performed twice with 10 μ l 5% formic acid, after which the sample was desalted using a Millipore μ C18 ZipTip and the peptides were eluted with 60% ACN/0.1% trifluoroacetic acid/ α -cyano-4-hydroxycinnamic acid matrix onto the MALDI-TOF probe. The peptides were subjected to MALDI-TOF analysis using an Ultraflex TOF/TOF instrument (Bruker Daltonik). Protein identification was performed using the Mascot peptide mass fingerprint program (<http://www.matrixscience.com>).

AFM. Different samples of PVA virions (P1, F or P2) were diluted to ~5 ng μ l⁻¹ in 0.02 M phosphate buffer (pH 7.3) and 5–10 μ l was 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 carried out in the tapping mode as described by Torrance *et al.* (2006) in air at a frequency of 300–380 kHz on a NanoScope IIIa multimode scanning probe microscope (Digital Instruments) using standard AFM silicon nitride cantilevers with a length of 123 μ m (Nanosensors). Images were processed with NanoScope software and transferred to Photoshop (Adobe Systems) for layout.

Translation. The gene for *Renilla* luciferase (RLuc) was cloned to substitute for the green fluorescent protein gene in the infectious cDNA clone of PVA (Ivanov *et al.*, 2003; Kelloniemi *et al.*, 2006). The resulting infectious cDNA copy of PVA::*rluc* RNA was used to infect tobacco plants and for subsequent virus purification. Purified PVA particles corresponding to 100 ng encapsidated PVA::*rluc* RNA and 100 ng *rluc*-containing viral RNA were translated in a Wheat Germ Extract kit (Promega) according to the manufacturer's instructions. Translation efficiency was monitored by measuring the production of the RLuc activity in the samples using a *Renilla* Luciferase kit (Promega). Luciferase activity was measured at room temperature for 5 s with a 1 s initial delay in a Luminoskan TL Plus (Thermo Labsystems).

RESULTS

PVA particles obtained from the P1 pellet after purification through a 30% sucrose cushion (Fig. 1a) were analysed by Western blotting using CP- and CI-specific purified polyclonal antibodies. CP and CI were readily detected in the blots (Fig. 1b, upper panel), suggesting that CI copurified with PVA particles. MALDI-TOF mass spectrometric analysis was performed to characterize the peptide content of the CI-containing band that was visible in the Coomassie-stained SDS-PAGE (Fig. 1b, middle panel). Of the 29 peptide sequences obtained, 21 were identified that matched sequences in PVA CI, three in CP, two in HC-Pro and two in NIa/VPg (Table 1). As CI can form pinwheel-shaped cytoplasmic inclusion bodies, we wanted to find out whether CI was bound to virions or whether the inclusions simply sedimented together with the PVA particles. We therefore purified PVA particles further through a 5–40% sucrose gradient (Fig. 1a). After centrifugation, we

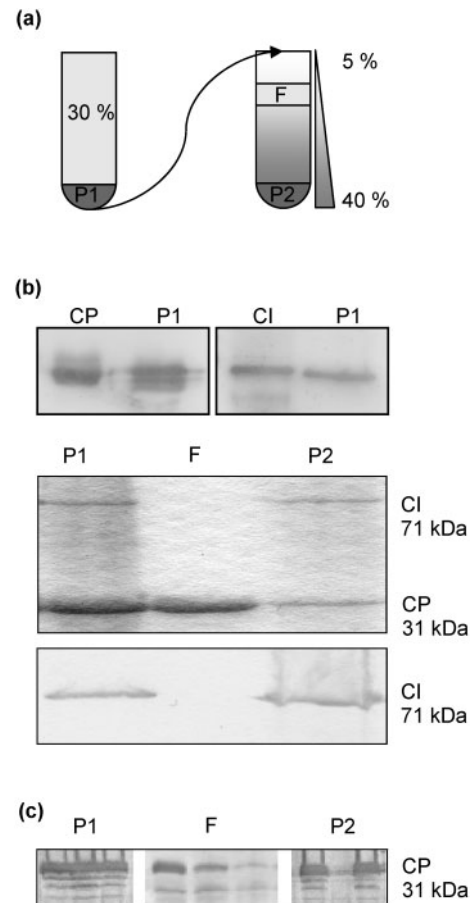


Fig. 1. Analysis of purified PVA particle populations. (a) Schematic representation of virus purification procedure: after sedimentation through a 30% sucrose cushion, the virus pellet (P1) was further purified through a 5–40% sucrose gradient. Virus particles accumulated in a gradient fraction (F) that was visible under a light beam and in a pellet (P2). (b) Upper panel: Western blot analysis of P1 particles with anti-CI and anti-CP antibodies. CP and CI represent the recombinant proteins used as positive controls. Middle panel: Coomassie-stained SDS-PAGE showing the presence of 31 and 71 kDa protein bands in the samples. MALDI-TOF analysis was carried out on the 71 kDa protein bands in P1 and P2. The lower panel represents a Western blot incubated with purified rabbit polyclonal anti-CI IgG, followed by mouse peroxidase-conjugated anti-rabbit IgG. The 71 kDa protein reacted in Western blots with anti-CI antibodies. (c) Western blot analysis of leaf samples from *N. tabacum* plants infected with P1, F and P2 particles at 14 days post-inoculation. The blots were incubated with anti-CP IgG. Three tobacco plants per virus sample were analysed and CP was detected in all plants, which were infected systemically.

collected the virus fraction (F) that formed a visible band in the gradient when viewed with a light beam and the pellet (P2, see Fig. 1a). PVA CP was detected both in F and P2 samples (Fig. 1b, middle panel), whereas CI was found only in the P2 sample (Fig. 1b, middle and lower panels).

Table 1. List of peptide sequences detected by MALDI-TOF peptide fingerprinting

Sequences were obtained from samples of CI-containing bands excised from SDS-PAGE gels of virus particles.

Amino acid sequence	Protein match
VSICYGER	CI
CVPIWMTGK	CI
EQLEGESIEK	HC-Pro
TILIIDQLLER	CI
NLLHEHNYQGK	CI
NHTAGNIEILQR	CI
VEDQVSFQDFVK	CI
EWWDVQLNTNR	CI
LLTHNSQIPDDVR	CI
HFSALAEAYIEMR	CP
HFSALAEAYIEMR oxidation (M)	CP
EVEFSTQYPVEIR	CI
LHENVWAIVEKFK	CI
VAYTLETDIHSVQR	CI
NNGSLTVRSQMGVFK oxidation (M)	Nla
YDGTMHPAIHNLKK	CI
YAFDFYEITATTPIR	CP
EIPDKLHENVWAIVEK	CI
GTVLLLEPTRPLAENVTK	CI
KGTVLLLEPTRPLAENVTK	CI
TMVQFELPIFYMAHLVR	CI
TMHVIDSYGSLNTGYHVLK oxidation (M)	HC-Pro
LLTHNSQIPDDVVRPFLTK	CI
ANAPTIAHQIAHDLHTDIMLR	CI
NHRFVNMYGFDPSDYTFIR oxidation (M)	VPg
NSNTNMFGLDGNVTTSEEDTER	CP
MLSTYDFIIIDEFHVHDSNAIALR	CI
GMAVFGSTPIHVMTTGAFALHYLANLTK	CI
EVEFSTQYPVEIRVEDQVSFQDFVK	CI

This result suggested that CI was not associated with all of the particles and that particles devoid of detectable amounts of CI could be separated by sucrose gradient centrifugation from those associated with CI. The relative amounts of particles in the F sample varied among different purifications. Particles from all three samples (P1, F and P2) infected *N. tabacum* plants as shown by Western blot analysis with anti-CP antibodies (Fig. 1c), suggesting that after sucrose gradient purification particles from both F and P2 samples were largely intact.

We have previously shown that PVA virions can be immunoprecipitated with anti-VPg antibodies (Puustinen *et al.*, 2002). To further rule out the possibility that CI co-purified with PVA virions simply because of similar sedimentation properties, we immunoprecipitated PVA particles with rabbit anti-VPg IgG covalently bound to magnetic Dynabeads. This enabled us to study the association of CI with the virus particles without using centrifugation. CI was detected as a co-immunoprecipitated protein (Fig. 2a), indicating that it is physically attached to PVA virions. The next question was whether CI

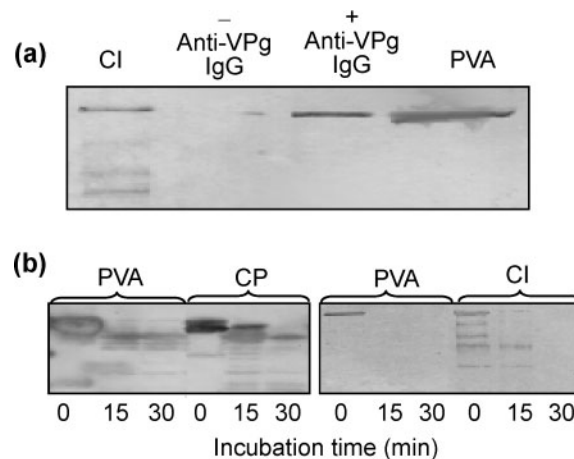


Fig. 2. (a) Detection of CI as a protein co-immunoprecipitating with PVA particles. Immunoprecipitation of P1 virus particles was carried out with anti-VPg IgG covalently bound to magnetic beads. The presence of CI was detected with anti-CI IgG. Recombinant PVA CI was used as a positive control (CI) and beads without anti-VPg IgG (-Anti-VPg IgG) were used as a negative control. CI was detected both in the original P1 sample (PVA) and in the resulting immunoprecipitated sample (+Anti-VPg IgG). (b) Western blot analysis of P1 particles after trypsin treatment. Both recombinant CI and CP as well as P1 PVA particles were subjected to trypsin treatment. Samples were taken after 0, 15 and 30 min incubation. The blots indicate protein degradation after analysis with anti-CP (left panel) and anti-CI (right panel) IgGs.

was exposed on the surface of the virion or whether it was partially buried in the virion structure. To determine this, we treated the purified P1 particles with trypsin (trypsin-treated preparations of recombinant CP and CI were used as controls). Previously, it has been shown that only the N- and C-terminal regions are exposed on the surface of CP and that they are removed by trypsin treatment (Shukla *et al.*, 1988). Western blots of the trypsin-treated samples confirmed this result, as PVA CP had a trypsin-resistant core (Fig. 2b). We found that recombinant CI was degraded completely in 30 min and CI associated with PVA in 15 min. This result suggested that CI was not protected by the PVA virion structure.

To check whether CI in purified PVA preparations retained its enzymic activity, we measured ATPase activity in the P1, F and P2 samples. As a control, ATPase activity was also measured using purified recombinant CI. Phosphatase activity was detected in all virus samples as well as in the recombinant CI in the presence of Mg^{2+} and ATP, whereas negligible activity was observed if no ATP was added (Fig. 3a and b). The increasing absorbance at 355 nm indicating phosphatase activity was measured at 99 time points and plotted against time for each of the samples. Enzyme activity assays were performed with comparable amounts of total protein in the P1, F and P2 samples. However, we could not compare the recombinant CI and

the virus-derived CI activities directly because it was not possible to determine accurately the virus-derived CI concentration. Therefore, the relative differences were calculated from three duplicate experiments only for the P1, F and P2 samples (Fig. 3c), each of which contained equal amounts of total protein. Phosphatase activity in sample F was about twofold less than in P1. Sample P2 gave the greatest phosphatase activity, which was 1.55 ± 0.09 times higher than in P1. Coomassie-stained SDS-PAGE analysed by intensity quantification of the CI- and CP-containing bands indicated that the CI:CP ratio in P2 was on average 1.6 times higher than in P1 as calculated from three independent purifications (data not shown), which correlated with the increase in phosphatase activity of the P2 sample. The molar ratios calculated from these three purifications revealed that there was on average 8.4% CI compared with CP in P1 and 13% in P2.

No visible differences were observed between the negatively stained particles in the P1, F and P2 samples when examined by EM (Fig. 4a). To investigate the localization of CI in relation to the PVA particles, purified PVA virions were incubated with purified polyclonal anti-CI antibodies followed by protein A-gold particles. Gold labelling was carried out either in the absence of antibodies or after incubation of particles with pre-immune serum as negative controls. Gold labelling at one end of the particle was observed in approximately 1% of particles in the P1 sample (mean of counts from several hundred particles; Fig. 4b). This end-labelling was observed only when anti-CI antibody was used. No end-labelling was detected in virus particles from sample F (data not shown). In addition, one or two gold particles were occasionally seen along the sides of the particles and an example of this is given in Fig. 4(b). However, this type of labelling occurred both in the presence and absence of anti-CI-antibody (data not shown) and was not regarded as specific. An EM study of P2 samples indicated that the proportion of virions that were end-labelled was similar to that in the P1 samples.

To determine the fine details of virus particle architecture in the different PVA populations (P1, F and P2), we employed AFM as a high-resolution technique. AFM has previously allowed us to identify protruding tips associated with approximately 10% of purified PVA particles (Torrance *et al.*, 2006). AFM imaging of the P1 sample readily revealed a similar proportion of virions containing such tips. The majority of virions in the P1 sample, regardless of the presence or absence of the tip structures, were represented by smooth filamentous particles with a modal length of ~ 730 nm (Fig. 5a, panels I and II). However, remarkably, approximately 5% of them had a beaded appearance along the whole length of the particle (Fig. 5a, panel III). Importantly, the length distribution of the beaded particles did not differ from that of the intact P1 particles. These results suggested the loss of CP subunits along the entire length of the virions, which took place after their adsorption on the AFM mica substrate. It is worth noting that no beaded structures were detected after

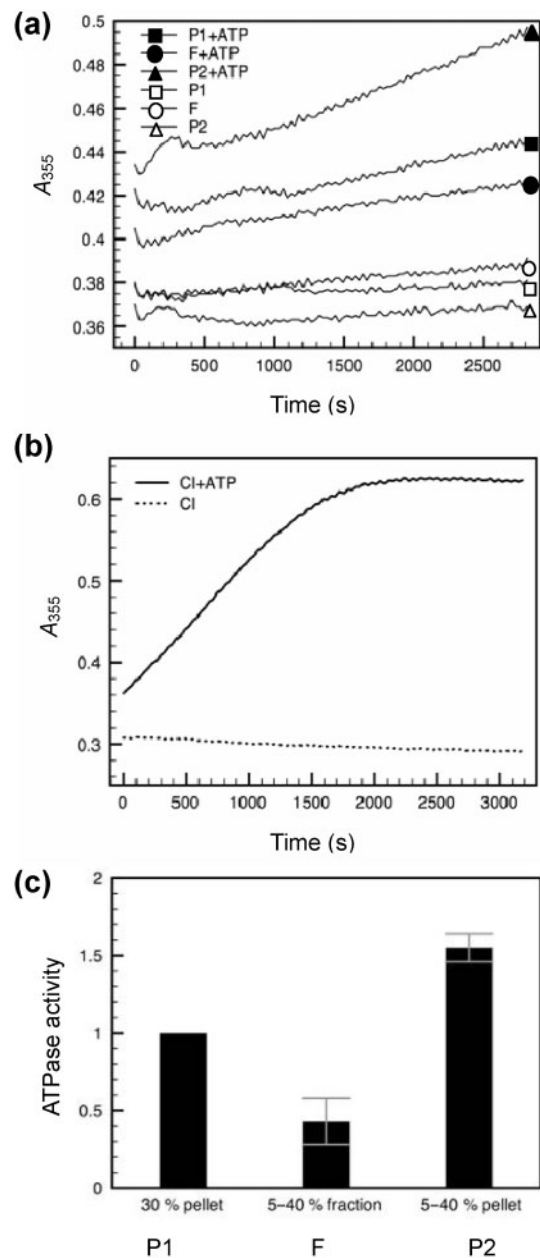


Fig. 3. ATPase activity associated with PVA particles. (a) A single representative ATPase assay experiment for the P1, F and P2 virus samples. ATPase activities were measured both with and without added ATP. In each sample, 50 μ g total protein was used. ATPase activity was measured indirectly by following the increase in A_{355} (99 time points) against time. (b) A single representative ATPase assay experiment for recombinant CI measured in the presence and absence of added ATP. Each reaction used 20 μ g CI. (c) ATPase activity associated with purified PVA particles. The activities of the F and P2 samples were compared with that of P1. The slope for each reaction progress curve representing a single reaction was determined. The mean value of the reaction slopes calculated from three independent experiments with P1 samples was used as a reference and its value was set to a relative value of 1. The normalized means \pm SD for the F and P2 samples calculated from three separate experiments are shown.

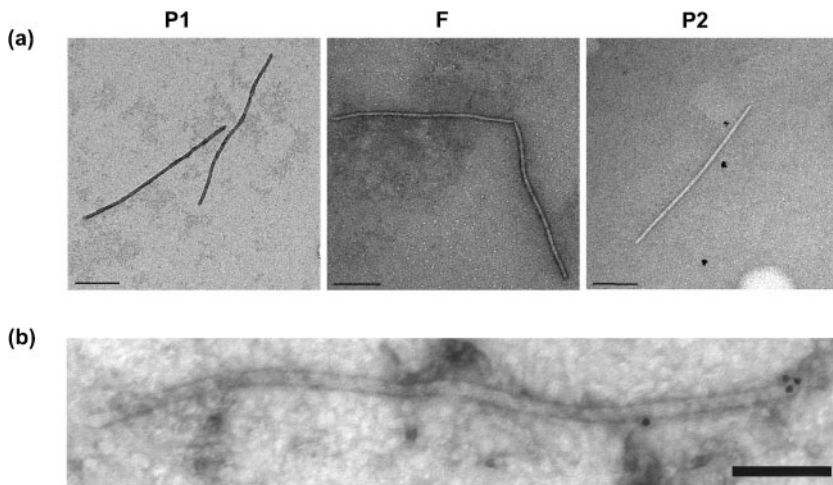


Fig. 4. (a) Negative-staining EM of purified virus P1, F and P2 samples. Grids were stained with 3% aqueous uranyl acetate solution for 4 min at room temperature. Bars, 0.2 μm . (b) Immunogold labelling of P1 particles incubated with anti-CI antibodies. Bar, 0.1 μm . A typical example is shown of gold particles at one extremity, which were visible in approximately 1% of particles. Occasionally, a single gold particle was apparent elsewhere; in this example, a gold particle was detected along the side of the particle.

negative staining and EM examination of virus particles (representative EM images are shown in Fig. 4). These observations suggested that the beaded particles might be less stable than other virions, and that although the instability was exaggerated even by the relatively mild conditions of AFM (e.g. due to the hydrophobic nature of the mica on which virions are adsorbed and then scanned

and/or due to dehydration), they were still visible. AFM analysis of the F sample revealed that the majority of the F virions (about 80%) had a beaded structure (Fig. 5b). Due to the beaded structure, it was not possible to clearly determine whether any of these particles contained tips. The virus particles in this sample did not contain detectable CI, which could mean that detachment of CI

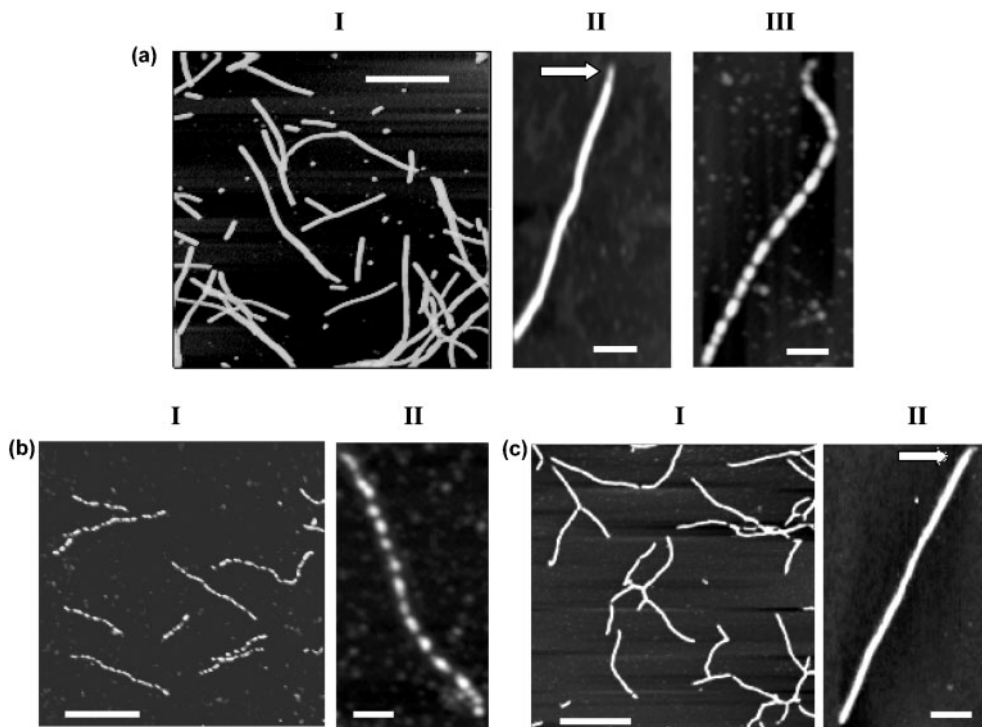


Fig. 5. AFM analysis of different samples of PVA particles: P1 (a), F (b) and P2 (c), shown at low (panels I) and high (panels II and III) magnifications. (a) Typical PVA P1 particles (panels I), a particle containing a protruding tip at the virion extremity indicated by an arrow (panel II) and a P1 PVA particle with a beaded structure (panel III). (b) Typical PVA F particles containing no CI and showing beaded structures. (c) Typical PVA P2 particles containing a protruding tip indicated by an arrow in panel II. Bars, 500 nm (panel I); 100 nm (panels II and III).

and particle destabilization may be linked to each other. In good agreement with this suggestion is the fact that the particles in the P2 sample, which contained a high proportion of CI, had smooth and stable filamentous bodies without any apparent beaded structure (Fig. 5c). In spite of the higher CI:CP ratio in the P2 sample, AFM did not show any differences in the frequency of tip structures in P1 and P2 samples.

AFM studies on PVX have shown that TGBp1 is able to bind to one of the extremities of the PVX particles, mediating linear destabilization of PVX particles and leading to formation of beaded particles under AFM conditions similar to those described here (Kiselyova *et al.*, 2003). This linear remodelling converted untranslatable virus particles into a translatable form (Rodionova *et al.*, 2003). To compare the translatability of purified PVA particles within P1, F and P2 samples, *in vitro* translation was performed with the purified PVA particles containing the *rluc* gene between the NIb and CP genes in their RNA genomes, and with purified *rluc*-containing viral RNA in wheat-germ extracts. The translation efficiency was determined by measuring RLuc activity in the reaction mix. In general, translation of viral RNA resulted in very low luciferase activity. A translation reaction with approximately 100 ng viral RNA gave 1000 times less RLuc activity than the same amount of monocistronic *rluc* mRNA, and was just ten times over the reaction background level (data not shown). Despite that, we observed clear differences among the translation reactions of the P1, F and P2 samples (Fig. 6). P1 and P2 samples were translated with approximately the same efficiency, but the translation reaction of sample F was five to ten times more efficient than that of the P1 and P2 samples. Translation of purified viral *rluc* RNA gave only slightly higher RLuc activity than translation of the P1 virus sample but clearly less than translation of the F sample. Addition of purified recombinant CI, which was shown to exhibit ATPase activity, to the F sample slightly increased the amount of translation (Fig. 6).

DISCUSSION

Cone-shaped CI structures anchored to the cell wall or plasma membrane in close proximity to plasmodesmata have been found in potyvirus-infected cells (Rodríguez-Cerezo *et al.*, 1997; Roberts *et al.*, 1998). It has been suggested that potyvirus genome replication and cell-to-cell movement may be coupled through coordinated functions of the CI protein (Carrington *et al.*, 1998) and that the CI subunits involved directly in RNA synthesis may interact with the nascent genome, transport complex or virion. It has been suggested that the associated CI proteins may direct intracellular translocation of the virion or transport complex to the plasmodesmata-associated structures through protein-protein interactions between the transport complex-associated CI and the plasmodesmata-associated CI subunits. On the basis of the data obtained in this study, we propose that PVA CI is

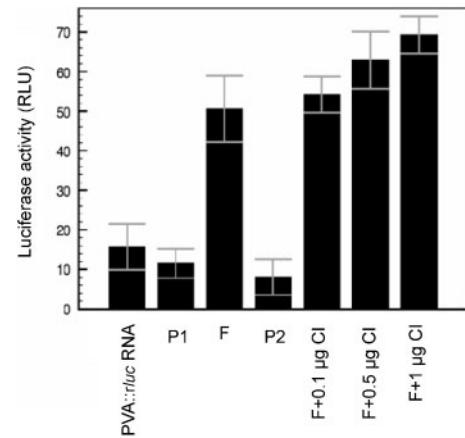


Fig. 6. Translation efficiencies of purified *Renilla* luciferase-tagged PVA (PVA::rluc) virus particles and purified viral RNA. The amount of RNA in the particle preparations was calculated and 100 ng RNA was used in each translation reaction. The figure represents the means \pm SD from six translation reactions for purified RNA, 13 for each of the samples of virus particles and six reactions for samples with added recombinant CI protein. The reaction background was subtracted from the values presented.

co-purified with the virus particles due to a direct physical link between CI and the virus particle. The large amount of CI in proportion to CP in the P1 and P2 preparations together with the remarkable difference in the sedimentation pattern between the particles in the F and P2 samples suggest that some CI-formed structures were co-purifying with virions. We propose that CI-mediated aggregation of virus particles may be the force driving a fraction of particles through the sucrose gradient into the P2 pellet, whereas some of the particles detached from CI structures remained in the gradient. All of the proteins identified in the CI-containing Coomassie-stained SDS-PAGE band by MALDI-TOF are essential for virus movement. It is not clear why they co-migrated together with CI, even after boiling and in the presence of a reducing agent during SDS-PAGE. Possibly, the sensitivity of MALDI-TOF analysis was great enough to detect those few molecules of the other proteins that remained associated with CI, which would support the premise that these proteins are involved in the movement-associated CI structures. The amount of CP found in the F sample varied among purifications, indicating that the ratio of F and P2 particles is a dynamic parameter and may depend on the viral cell-to-cell transport activity within the host plant at the time when the virus isolation was initiated. Alternatively, varying amounts of the P2 viruses may have detached from the CI structures due to the mechanical forces that the particles faced during the purification scheme, which may have converted the P2-type particles to F-type particles.

An important question is how the link between CI and the virus could be formed. There are reports of potyviral

CI-CP interactions *in vivo* (Rodríguez-Cerezo *et al.*, 1997; Riedel *et al.*, 1998; Guo *et al.*, 2001), but to our knowledge no interaction between CI and VPg has been reported. PVA CI seems to interact strongly with HC-Pro (Guo *et al.*, 2001). A similar interaction was shown for CI and HC-Pro of wheat streak mosaic virus, a member of the genus *Tritimovirus*, family *Potyviridae* (Choi *et al.*, 2000). These observations point to two possibilities: either the virion-associated CI could be in contact with HC-Pro or it could interact directly with CP. In AFM experiments, HC-Pro was detected solely in the tip structures of PVY particles labelled with anti-HC-Pro antibodies, whereas VPg was also detected with anti-VPg antibodies in particles containing no apparent tip structure (Torrance *et al.*, 2006). Our immunogold-labelling EM data indicated that CI was associated with one end of approximately 1% of the particles. HC-Pro has been detected at the ends of only 2% of PVA and PVY virions by immunogold labelling (Torrance *et al.*, 2006). If, as stated above, the tip structures are relatively fragile, this might explain the difference in detection of CI structures between EM and Western blots. Nevertheless, the EM studies support the premise that CI may associate with HC-Pro in the PVA tip structure and that transport complex-associated CI could be an important constituent of the tip.

A few years ago, the complex molecular architecture of closteroviruses was reported (Peremyslov *et al.*, 2004). The potyvirus tips in the AFM figures resemble those from beet yellows virus (BYV), except that the BYV tail is tripartite, whereas only two parts have been detected in the potyvirus tips (Torrance *et al.*, 2006). One of the four proteins in the BYV tail structure is the virus-encoded p65 homologue of the cellular Hsp70 molecular chaperone, which has ATPase activity (Kalinina *et al.*, 1996; Agranovsky *et al.*, 1997). Recently, it was demonstrated that the actin cytoskeleton interacts with the viral Hsp70 homologue and targets it to plasmodesmata (Prokhnevsky *et al.*, 2005), and it was shown that the ATPase activity of the Hsp70 homologue is required for BYV cell-to-cell movement (Peremyslov *et al.*, 1999). Although it is known that CI associates with plasmodesmata, no association between the potyviral CI and cell cytoskeleton has been shown. Neither is it clear whether the ATPase activity of potyviral CI is required for cell-to-cell movement, as not all CI mutations that abolished plum pox virus cell-to-cell movement affect the ATPase activity (Gómez de Cedrón *et al.*, 2006). We detected ATPase activity in all of the purified preparations of virus particles. The ATPase activity was four times weaker in preparations of F sample virions that did not contain detectable CI (by Western blotting); weak ATPase activity in the F sample might be explained by the presence of a few active CI molecules remaining attached to the F sample particles, as detection of an enzymic activity may be more sensitive than the antibody-based detection of PVA CI. It has also been shown that PVA CP has ATPase activity (Rakitina *et al.*, 2005), which may have contributed to the low level detected.

Vaccinia virus DexH/D group RNA helicase/ATPase NPH-II can displace proteins from an RNA substrate (Jankowsky *et al.*, 2001). Protein displacement is independent of RNA duplex unwinding (Fairman *et al.*, 2004). Potyviral CI has also been shown to belong to the DexH/D group of helicases (Fernández *et al.*, 1995, 1997; Fernández & García, 1996) and CI was reported to unwind RNA duplexes in the 3'→5' direction in plum pox virus (Lain *et al.*, 1990). A similar RNA helicase activity was described for the PVX movement protein TGBp1, but in this case RNA unwinding was bidirectional (Kalinina *et al.*, 2002). Binding of TGBp1 to PVX particles converts the particles from a non-translatable into a translatable form (Atabekov *et al.*, 2000). A very similar result was obtained by PVX CP phosphorylation (Atabekov *et al.*, 2001). The binding of TGBp1 helicase to the PVX virions induced virus particle disassembly and formation of the beads-on-a-string structure (Kiselyova *et al.*, 2003). Thus, in the PVX system, binding of TGBp1 to virions initiates particle destabilization and prepares them for translation. In contrast, our results suggest that attachment of CI to PVA particles is required to retain the particle integrity. Particles in the F samples, devoid of CI and according to the AFM results less stable than the P2 particles, were more translatable. As the addition of CI did not reduce the translatability of the F particles, it is probable that the presence of CI is not directly responsible for the low translatability of P1 and P2 particles. The factors that prepare PVA particles for translation require further investigation; however, our data allow us to propose that detachment of CI structures from particles during transport through the plasmodesmata to adjacent cells may be required. Due to the likely location of CI at the virion end corresponding to the 5' end of the genome and its ATPase and RNA helicase activities, CI may provide a molecular motor function both to disassemble and to translocate the viral genome through the plasmodesmatal pore. Our results imply that the concept of virion-associated movement devices first described for closteroviruses (Peremyslov *et al.*, 2004) may be applicable to a broader range of evolutionarily diverse filamentous viruses.

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