Umbravirus-encoded movement protein induces tubule formation on the surface of protoplasts and binds RNA incompletely and non-cooperatively

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Various functions of the cell-to-cell movement protein (MP) of Groundnut rosette virus (GRV) were analysed. The GRV ORF4-encoded protein was shown by immunofluorescence microscopy to generate tubular structures that protrude from the surface of the protoplast. The protein encoded by ORF4 was assessed also for RNA-binding properties. This protein was tagged at its C terminus with six histidine residues, produced in Escherichia coli using an expression vector and purified by affinity chromatography. Gel retardation analysis demonstrated that, in contrast to many other viral MPs, including the 3a MP of Cucumber mosaic virus (CMV), the ORF4-encoded protein bound non-cooperatively to viral ssRNA and formed complexes of low protein:RNA ratios. Competition binding experiments showed that the ORF4-encoded protein bound to both ssRNA and ssDNA without sequence specificity, but did not bind to dsDNA. UV cross-linking and nitrocellulose membrane-retention assays confirmed that both the GRV and the CMV MPs formed complexes with ssRNA and that these complexes showed similar stability in NaCl. Probing the MP–RNA complexes by atomic force microscopy demonstrated that the ORF4-encoded protein bound RNA incompletely, leaving protein-free RNA segments of varying length, while the CMV 3a protein formed highly packed complexes. The significance of the two properties of limited RNA binding and tubule formation of the umbraviral MP is discussed.

Introduction

Movement of viruses within plants occurs in two forms: cell-to-cell spread via the plasmodesmata and long-distance transport via the vascular system (Carrington et al., 1996). Cell-to-cell movement is assumed to involve virus-encoded movement proteins (MPs). Viruses are currently believed to move from cell to cell by two main mechanisms. In the first mechanism, several viruses, including Cucumber mosaic virus, move as virions via tubular structures that pass through and extend from the plasmodesmata (van Lent et al., 1990). Typically, such tubules are formed not only in plant tissues but also protruding from protoplasts (van Lent et al., 1991). It has been demonstrated that MPs induce the formation of such tubules and localize to them (Perbal et al., 1993; Kasteel et al., 1997a, b; Zheng et al., 1997; Jansen et al., 1998; Canto & Palukaitis, 1999).

In the second mechanism, exemplified by Tobacco mosaic virus (TMV), the MP induces an increase in the size-exclusion limit of the plasmodesmata (Ding et al., 1995; Lucas, 1995; Wolf et al., 1989) and possesses non-specific, ssRNA-binding activity to form a transferable complex with viral RNA (Citovsky et al., 1990, 1992; Schoumacher et al., 1992; Thomas & Maule, 1995; Li & Palukaitis, 1996; Donald et al., 1997; Vaquero et al., 1997; Fujita et al., 1998; Jansen et al., 1998). The MP of TMV has been shown also to bind to the cytoskeleton (Heinlein et al., 1995; McLean et al., 1995) and microtubules (Boyko et al., 2000). Although the coat protein (CP) of TMV is essential for
long-distance virus movement, it is not required for cell-to-cell movement (Carrington et al., 1996). Some viruses, such as Cucumber mosaic virus (CMV), use a strategy for cell-to-cell movement similar to that of TMV, increasing the penetration capacity of the plasmodesmata (Ding et al., 1995) and to bind to ssRNA (Li & Palukaitis, 1996), but these viruses require both the MP and CP to move from cell to cell (Suzuki et al., 1991; Boccard & Baulcombe, 1993; Canto et al., 1997). Nevertheless, the formation of virus particles is not required for CMV cell-to-cell movement (Kaplan et al., 1998).

For a growing number of viruses, it has been shown that the MPs possess both the tube-forming and the RNA-binding activities (Perbal et al., 1993; Jansen et al., 1998; Canto & Palukaitis, 1999). This suggests that both ‘virion’ and ‘non-virion’ mechanisms of movement may co-exist and that viruses can use different mechanisms in different hosts or tissues.

Members of the genus Umbravirus, such as Groundnut rosette virus (GRV), neither form virus particles nor code for a CP (Taliansky et al., 2000). Although umbraviruses depend on the assistance of a luteovirus for aphid transmission, the presence or absence of a luteovirus and its CP does not affect the movement of umbraviruses (Taliansky et al., 2000). GRV has two viral proteins that are involved in virus movement. The 27 kDa ORF3-encoded protein was able to facilitate the long-distance movement of TMV in place of the TMV CP (Ryabov et al., 1999a), whereas the overlapping 28 kDa ORF4-encoded product was able to mediate cell-to-cell movement of CMV (Ryabov et al., 1999b) or Potato virus X (PVX) (Ryabov et al., 1998) in place of their corresponding MPs. The GRV ORF4-encoded protein shows significant sequence similarity to the 3a MP of CMV (Taliansky et al., 1996) and, like the CMV 3a (Blackman et al., 1998; Vaquero et al., 1996) and the TMV (Tomenius et al., 1987; Atkins et al., 1991) MPs, localizes to the plasmodesmata (Ryabov et al., 1998).

Recently, atomic force microscopy (AFM) has been exploited to visualize nucleic acids, proteins and some of their complexes (Lyubchenko et al., 1995; Fritz et al., 1997; Hansma et al., 1997; Smith et al., 1997; Drygin et al., 1998; Klinov et al., 1998; Kiselyova et al., 2001). AFM operates by measuring tiny contact forces between the surface of the molecule and the scanning tip to visualize molecules on a nanometre scale under ambient and/or physiological conditions. Recently, Kiselyova et al. (2001) described two types of structures formed by the TMV 30 kDa (30K) MP with ssRNA. At a low (non-saturated) molar ratio of protein:RNA, complexes referred to as ‘beads-on-a-string’ were generated, in which ‘beads’ of MPs were distributed along the RNA. At a high (saturated) molar ratio of protein:RNA, ‘thick string’ complexes were formed, in which MPs were cooperatively and tightly packed around the RNA. Thus, imaging using AFM allows the architecture of MP–RNA complexes to be discerned.

In this work, we demonstrate that, in spite of the lack of umbraviral particles, the GRV ORF4-encoded protein was capable of forming tubular structures on the surface of protoplasts. This had formerly been believed to be typical only for viruses moving from cell to cell as virions. To further characterize the GRV ORF4-encoded protein and to compare it to the CMV 3a MP, both MPs were expressed in Escherichia coli and the isolated proteins were compared for their RNA-binding characteristics. It was shown that, in contrast to the CMV 3a MP, which like many other viral MPs binds ssRNA cooperatively, the ORF4-encoded protein bound ssRNA in a non-cooperative manner and formed complexes of low protein:RNA ratios, independently of its concentration. These observations were supported by images of the MP–RNA complexes obtained by AFM.

Methods

**Isolation and inoculation of protoplasts.** Nicotiana benthamiana protoplasts were prepared as described by Power & Chapman (1985). Approximately 10° protoplasts were inoculated using polyethylene glycol 6000, with extracts obtained by homogenizing 0.5 g of leaf tissue of GRV-infected N. benthamiana plants in 0.5 ml of 0.1 M sodium phosphate buffer, as described previously for Coopemosa virus (Eggen et al., 1989). At 48 h after inoculation, protoplasts were analysed by immunofluorescence microscopy, as described by van Lent et al. (1991), using antibodies produced in rabbits against the recombinant ORF4-encoded protein expressed in E. coli (see below). Controls consisted of samples treated with pre-immune sera. Samples were analysed using a confocal laser scanning microscope (MRC 1000; excitation, 488 nm; emission, 522 nm) (Bio-Rad).

Inoculation of protoplasts with recombinant TMV (TMV-4-GFP; Ryabov et al., 1998) and PVX (PVX-4-GFP; Ryabov et al., 1998), each expressing the GRV ORF4-encoded protein fused to the jellyfish green fluorescent protein (GFP), was conducted by electroporation with 10–20 µg of transcript, as described by Gal-On et al. (1994). In vitro transcription was carried out using the mMESSAGE mMACHINE T7 kit (Ambion) as described by Ryabov et al. (1998).

**Expression and purification of the GRV ORF4 protein in E. coli.** To clone and subsequently express the GRV ORF4 protein with a His-Tag affinity tag, restriction sites upstream (NcoI) and downstream (BclI) of ORF4 were generated by PCR using the oligonucleotide primers 5’ GCATCCATGGCTTCGCAAGTGGC 3’ and 5’ CATTGATCGTCTTGTTCCGGC 3’. The PCR product was digested with BclI/NcoI and cloned into the pQE60 vector using the QA Express Expression System (Qiagen) to give pQEGRV4-His. In this plasmid, ORF4 was linked to the strong bacteriophage T7 promoter at its 5′ terminus and to six histidine codons at its 3′ terminus. E. coli strain SG13009 (pREP4) expressing low levels of protease and containing an inducible T7 polymerase gene was used as the host for protein expression. Induction of protein synthesis from ORF4 by IPTG and purification of the expressed protein were conducted according to the Qiagen protocol. Protein purification involved solubilization of the bacterial pellet in buffer B (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris–HCl, pH 8.0, binding to Ni2+–NTA–agarose, washing with buffers B and C (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris–HCl, pH 6.0) and elution with buffers D (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris–HCl, pH 5.9) and E (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris–HCl, pH 4.5). To allow for protein refolding, the solubilized protein was dialysed sequentially against buffer F (10 mM Tris–HCl, pH 7.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 4 M urea, 1 M urea, or no urea.
RNA-binding experiments. \(^{32}\)P-labelled full-length Fnyc-CMV RNA 3 and GRV RNA corresponding to the 5’-terminal 2556 nucleotides of the GRV genome were generated by \textit{in vitro} transcription of pfny309 (Rizzo & Palukaitis, 1990) and the GRV cDNA clone grpol (Taliansky \textit{et al}., 1996), respectively, in the presence of 5 \(\mu\)Ci \(^{32}\)P[UTP (Amersham) using the mMessage mMachine T7 kit. The labelled transcripts were mixed with purified proteins in 15 \(\mu\)l of binding buffer A (50 mM Tris–HCl, pH 7.0, 50 mM NaCl, 1 mM DTT, 1 mg/ml BSA, 10% glycerol). After incubation on ice for 30 min, the mixture was subjected to electrophoresis in 1% agarose in TAE buffer, as described by Li & Palukaitis (1996). For UV cross-linking experiments, the mixture was irradiated with UV light (twice at 900 mJ) in a Stratalinker (Stratagene), treated with RNase A and analysed by 12% SDS–PAGE. The gels were dried and autoradiographed. Nitrocellulose membrane-binding assays were carried out as described by Li & Palukaitis (1996). To analyse the stability of the MP–RNA complexes, buffer A contained different concentrations of NaCl. In competition binding assays, different amounts (250 and 1250 ng) ofcompetitor RNA or DNA (CMV ssRNA, TMV ssRNA, bacteriophage M13 ssDNA or a dsDNA plasmid fragment (Smal-digested pUC18 DNA)) were added to the mixtures. The mixtures were then filtered through a 45 \(\mu\)m nitrocellulose membrane (Schleicher & Shuell). The membranes were then washed, dried and counted using a liquid scintillation counter (LKB).

Atomic force microscopy. RNA samples were isolated from Fnyc-CMV as described by Ryabov \textit{et al} (1999 \textit{b}). The final RNA concentration was 1 \(\mu\)g/ml. The concentration of the CMV 3a and GRV ORF4-encoded proteins was 10 \(\mu\)g/ml. Protein (10 \(\mu\)g/ml) and RNA (0–1 \(\mu\)g/ml) complexes were prepared on ice for 30 min in a buffer containing 50 mM Tris–HCl, pH 7.0, 1 mM EDTA and 5 mM NaCl.

Freshly cleaved strips of mica were incubated in an atmosphere of 3-aminopropyltriethoxysilane (APTES), as described by Lyubchenko \textit{et al} (1993 \textit{a}, \textit{b}). The amino groups of APTES are bound covalently to a freshly cleaved mica surface, leaving it with properties similar to an anion exchanger. Modified mica (AP–mica) strips were immersed into the samples of RNA, purified proteins or their complexes in a Tris–HCl buffer and incubated at room temperature for 10–15 min. The substrates were then rinsed with deionized water and vacuum-dried at room temperature. Imaging was carried out in the tapping mode on a Nanoscope IIIa (Digital Instruments) using standard AFM cantilevers (Digital Instruments). Images were processed using Nanoscope software and transferred to Adobe PhotoShop for layout. Heights were measured using the Nanoscope software.

Results

Tubule-forming capacity of the GRV ORF4 protein

Typically, the formation of tubules on the surface of infected protoplasts is associated with viruses that require virus particles or at least the CP for cell-to-cell virus movement. To determine whether GRV also induces the formation of tubules, in spite of the lack of its own CP, \textit{N. benthamiana} protoplasts were inoculated with GRV and analysed by immunofluorescence microscopy using antibodies against the recombinant ORF4-encoded protein. There was no immunofluorescent labelling by these antibodies in any structures of uninfected protoplasts (data not shown). At 48 h post-inoculation, an average infection of 40% of inoculated protoplasts was recorded by immunofluorescent analysis, although the intensity of fluorescence varied significantly between protoplasts (Fig. 1A, B). Approximately 50% of protoplasts infected with GRV and stained with the anti-ORF4 MP sera showed fluorescent tubular structures on the surface (Fig. 1A, B).

To determine if the ORF4-encoded protein is responsible for the formation of tubules, we used two plant vectors (TMV and PVX) expressing the ORF4-encoded protein fused to GFP [TMV.4-GFP and PVX.4-GFP (Ryabov \textit{et al}., 1998), respectively]. Electroporation of \textit{N. benthamiana} protoplasts with either of these viruses resulted in the formation of fluorescent tubular structures at 24 h post-inoculation. At 48 h post-inoculation, tubules were more apparent in many protoplasts (Fig. 1C–E), although they were typically shorter than those formed in GRV-infected protoplasts (Fig. 1A, B) and their length showed significant variability. It is worth noting that neither TMV nor PVX expressing free GFP [TMV(30B)-GFP or PVX.GFP-CP (Ryabov \textit{et al}., 1998), respectively] induced
fluorescent tubules. Data for TMV(30B)-GFP are shown in Fig. 1F. Moreover, neither TMV nor PVX expressing the GRV ORF3-encoded protein fused to GFP (TMV.GFP-3/4 or PVX.GFP-3/4 respectively; Ryabov et al., 1998) induced fluorescent tubules (data not shown). These results suggest that the only GRV-encoded product required for tubule formation is the MP encoded by GRV ORF4.

**Comparative analysis of nucleic acid binding of the GRV and CMV MPs**

The GRV ORF4 MP containing a C-terminal (His)$_6$-tag was overexpressed in E. coli (Fig. 2, asterisk in lane 2) and was further purified by Ni$^{2+}$-NTA affinity chromatography. The purified preparation of the protein resulted in a single band by SDS–PAGE (Fig. 2, lane 3). CMV 3a MP tagged with six histidine residues was produced and purified in a similar manner (data not shown) using the pETMP3a-His vector (Li & Palukaitis, 1996). Binding of the His-tagged CMV 3a and GRV ORF4 proteins to ssRNA was assayed by gel retardation mobility. These results are consistent with a previous report on the mode of RNA binding of untagged CMV 3a protein (Li & Palukaitis, 1996).

Fig. 3(B, C) shows clearly that the profile of RNA binding of the GRV ORF4-encoded protein is rather different from that of the CMV 3a protein. In general, the ORF4-encoded protein retarded the mobility of both GRV and CMV RNA probes much less than the 3a protein did (Fig. 3B, C). However, the ORF4-encoded protein also bound to ssRNA in a sequence-independent manner (Fig. 3B, C) and the predominantly labelled RNA molecules migrated more slowly than the protein-free RNA molecules (Fig. 3B, lanes 4–8; Fig. 3C, lanes 2–5). Moreover, RNA binding by the ORF4-encoded protein apparently was not cooperative: an increase in the protein:RNA ratio resulted in an increase in the degree of retardation and a number of complexes with intermediate mobility was formed (Fig. 3B, lanes 4–6). While cooperativity of RNA binding may depend on salt concentration and incubation time, longer incubations and variations in salt concentrations did not result in changes to the pattern of RNA binding of the ORF4-encoded protein (data not shown).

To verify the ability of the ORF4-encoded protein, and not just undetected contaminating E. coli proteins, to bind to ssRNA, UV cross-linking experiments were carried out. The $^{32}$P-labelled GRV or CMV RNA was incubated with the ORF4 protein and the incubation mixture was irradiated with UV light. This treatment can covalently cross-link protein–RNA complexes. Unbound RNA was removed by RNase A digestion and the mixtures were analysed by SDS–PAGE and autoradiography. As shown in Fig. 4 (lanes 1, 2), a band can be seen in the position expected for the His-tagged ORF4-encoded protein (arrow in Fig. 4). The intense band migrating faster than the ORF4-encoded protein may be a degradation product of the labelled RNA. When no RNase was added, the band corresponding to the ORF4-encoded protein disappeared into a smeared background (Fig. 4, lane 3). No ORF4 protein-specific band could be detected if UV irradiation was omitted (data not shown). When BSA was substituted for the ORF4 protein by adding BSA to an equivalent protein preparation from E. coli transformed with pQE60, which lacks the GRV sequences, no band was observed (Fig. 4, lane 4). This shows that RNA binding is due to the ORF4-encoded protein and not to a protein contaminant. Similar results have been obtained for complexes of the CMV 3a protein with CMV (Fig. 4, lanes 5, 7) or GRV (Fig. 4, lane 6) RNA. These data do not contradict the results that show a difference in the behaviour of the
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Fig. 3. Gel retardation electrophoresis assay for RNA binding by MPs. Increasing amounts of the CMV 3a (A) and GRV ORF4-encoded (B, C) proteins were incubated in 15 µl of binding buffer A with 2 ng of 32P-labelled GRV (A, B) or CMV (C) RNA transcripts and the mixtures were electrophoresed in a 1% non-denaturing agarose gel. The amount of the MPs used in the assay is indicated. The positions of free (f) and fully retarded (r) RNAs are indicated.

Fig. 4. UV cross-linking of MPs with labelled RNA. The GRV ORF4-encoded (lanes 1–3) or CMV 3a (lanes 5–7) proteins were incubated with labelled GRV (lanes 1, 6) or CMV (lanes 2, 5) RNA transcripts for 30 min on ice. After UV irradiation, the mixture was either untreated (lanes 3, 7) or incubated with RNase A (lanes 1, 2, 5, 6). The ORF4-encoded protein was substituted by BSA in lane 4. Samples were analysed by SDS–PAGE and autoradiography. Arrows indicate the positions of the Histagged ORF4 and 3a proteins. The positions of the molecular mass markers (M) are indicated.

Fig. 5. Salt stability of RNA–MP complexes. The GRV ORF4-encoded and CMV 3a proteins (450 ng) were incubated with labelled GRV RNA (4 ng) in the presence of the indicated concentrations of NaCl. After incubation, the mixtures were analysed by nitrocellulose membrane filter-binding assays. RNA binding was quantified by determining the radioactivity of the membrane by liquid scintillation counting.

ORF4-encoded and 3a proteins in gel retardation assays. UV cross-linking measures all the complexes, even those in which RNA and protein are in nearly equimolar ratios and, therefore, does not correlate either with cooperativity of binding or with protein:RNA ratio.

The stability of protein–RNA complexes with respect to salt concentration is often used as a criterion to measure the stability of protein–RNA association. Complexes formed between the 32P-labelled GRV RNA transcripts and the 3a or ORF4-encoded proteins were incubated in buffers with different concentrations of salt and the incubation mixtures were filtered through nitrocellulose membranes and washed to remove unbound RNA. As shown in Fig. 5, both MPs bound RNA essentially with similar profiles, indicating that the stability of the complexes was similar for both proteins. Maximal binding was observed at NaCl concentrations of up to 150 mM; at higher salt concentrations, the complexes dissociated. This is consistent with results reported earlier for the CMV 3a protein (Li & Palukaitis, 1996).

It has been demonstrated by Li & Palukaitis (1996) that the CMV 3a protein binds to ss nucleic acids (RNA or DNA) without obvious preference, but does not bind to ds nucleic acids. To determine whether the GRV ORF4-encoded protein
has any sequence or nucleic acid-type binding specificity, competition binding assays were carried out by incubating unlabelled competitor nucleic acids of various types and \(^{32}\)P-labelled GRV RNA transcripts together with the ORF4-encoded protein and analysing the incubation mixture using nitrocellulose filter retention assays (Fig. 6). The ss nucleic acids, such as TMV ssRNA, CMV ssRNA or bacteriophage M13 ssDNA, were able to compete efficiently with the labelled transcript for binding the ORF4-encoded protein, while dsDNA was not (Fig. 6), suggesting that the ORF4-encoded protein, like the CMV 3a protein, binds to ss nucleic acids without any sequence specificity. However, pre-treating the ORF4-encoded protein with proteinase K or heating at 100 °C for 10 min abolished the ability of the protein to bind ssRNA (data not shown).

**Visualization of MP–RNA complexes using AFM**

To characterize the protein–RNA complexes with regard to the spacing of protein molecules, we used AFM to analyse complexes formed in vitro by either the GRV ORF4-encoded or the CMV 3a proteins with ssRNA. These complexes were deposited onto AP-mica, which is a useful substrate for the immobilization of a wide variety of biomolecules imaged and measured by AFM (Lyubchenko et al., 1993a, b). It should be noted that physical measurements made by AFM usually give heights that are slightly reduced by compression and widths that are greatly increased by the effects of tip convolution (Keller, 1991; Fritz et al., 1995; Bustamante & Rivetti, 1996). Since compression effects are much smaller (and can be neglected) than the effects of tip convolution, we present the height measurements of the molecules and their complexes.

In the first series of experiments, MP and RNA components were visualized separately (Fig. 7 A, B). The height of most of the GRV ORF4 MP molecules was about 0.8 nm. Given the uniform size of these molecules, they probably represent monomers of the ORF4 MP (Fig. 7 A). CMV 3a MP molecules have a similar size, but sometimes form clumps of aggregated molecules (data not shown).

The RNA-binding assays described above demonstrated that both GRV ORF4-encoded and CMV 3a proteins bound ssRNA without any sequence specificity. Therefore, for the AFM experiments, we used just one RNA sample, a total RNA preparation isolated from CMV. In general, RNA is difficult to image because it is difficult to adsorb onto mica and is eventually washed away (Smith et al., 1997). However, using high concentrations of RNA and AP-mica as a suitable substrate, we produced some CMV RNA images (Fig. 7 B). The self-affinity of the ssRNA molecules in water (or in solutions with a low concentration of salt) induced ‘loose’ globular structures of CMV RNA (Fig. 7 B). Similar AFM images of ssRNA molecules had been obtained previously by Fritz et al. (1997). The heights of the CMV RNA globules varied significantly, apparently due to the presence of different RNA species (RNA 1–4) and were in the range of approximately 1.2–4.6 nm. The height of the chain-like CMV RNA structure, denatured with formamide, was 0.3–0.5 nm.

Complexes of GRV ORF4-encoded or CMV 3a protein with CMV RNA were prepared at saturated protein:RNA ratios (100 : 1) in binding buffer A containing no glycerol. The GRV MP–RNA complexes (Fig. 7 C–F) were very similar to the ‘beads-on-a-string’ type of complex described by Kiselyova et al. (2001) and consisted of small globules (‘beads’; circles in Fig. 7 D, F) with heights of about 1.1–1.5 nm. These apparently represent individual MP molecules or their dimers bound to RNA non-cooperatively and separated by protein-free RNA segments of varying length (arrows in Fig. 7), with heights similar to those of denatured RNA chains. Sometimes, the GRV MP–RNA complexes also contained the larger globules (asterisk in Fig. 7 F), which might represent extensive unbound RNA sequences (in the form of globular structures), with extending ribonucleoprotein ‘beads-on-a-string’ chains (Fig. 7 E, F).

In contrast, the CMV 3a protein–RNA complexes were immobilized onto the AP-mica surface in the form of net-type aggregates consisting of tangled chains, apparently representing strands of RNA molecules tightly and cooperatively bound by clusters of CMV MP molecules (Fig. 7 G), without any apparent protein-free RNA segments. The heights of the chains were 1.1–1.6 nm, which are comparable with those of the GRV MP–RNA ‘beads’. The tangling of the chains of the CMV MP–RNA complexes possibly occurred due to strong interactions between protein molecules belonging to different chains. This may explain the aggregated state of the CMV MP–RNA complexes (Fig. 7 G) and their failure to enter even an agarose gel.
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Fig. 7. AFM images of the MPs and viral RNA before (A, B) and after (C–G) the formation of complexes on AP-mica in air at room temperature: GRV ORF4-encoded protein (A), CMV RNA in globular form (B), GRV MP–CMV RNA complex (C)–(F) and CHV MP–CMV RNA complex (G). Complexes were formed at saturated protein:RNA ratios (100:1). (D, F) Circles represent protein beads. (D, F) Arrows indicate protein-free RNA segments. (F) The asterisk indicates a large globule that presumably represents an unbound by MP RNA structure. (A, B, C, E, G) Horizontal bars, 250 nm; vertical bars, 10 nm. (D, F) Horizontal bars, 50 nm; vertical bars, 20 nm. (C, G) Three-dimensional images of the protein–RNA complexes.

Discussion

In the present study, the biochemical and functional properties of the GRV MP were investigated. GRV MP, like MPs of some other plant viruses, such as CMV (Canto & Palukaitis, 1999), Alfalfa mosaic virus (AMV) and Brome mosaic virus (Kasteel et al., 1997a; Jansen et al., 1998), displayed both RNA-binding and tubule-forming activities that are usually related to two different mechanisms of cell-to-cell movement. This may suggest that both mechanisms of movement can be used by these viruses in some circumstances in different hosts or tissues.

Induction of tubules on the surface of protoplasts

Like other umbraviruses, GRV does not form conventional virus particles. In this respect, it was rather unexpected to find that the umbraviral ORF4-encoded protein formed tubular structures on the surface of protoplasts; this is a typical characteristic of viruses that move from cell to cell in the form of virions. Although data concerning the formation of tubules in intact tissues of plants infected by GRV have not been obtained, tubules associated with the cell wall plasmodesmata were detected in plants infected with another umbravirus, Carrot mottle virus (Murant et al., 1973). Of course these results cannot rule out completely that tubule formation induced by umbraviruses is just an atavistic remnant remaining from an earlier hypothetical evolutionary stage when umbraviruses did code for a functionally active CP (Ryabov et al., 1999a, b). However, it is also possible that the tubules are still used by umbraviruses for cell-to-cell movement, at least in some hosts or tissues or under some physiological circumstances. If so, we could speculate that GRV (and other umbraviruses) can move through tubules in a form other than a virion (e.g. as MP–RNA complexes). It is worth noting that the plant-infecting bunyavirus Tomato spotted wilt virus is also able to induce the formation of tubules in both protoplasts and intact plant tissues, but virus particles are not observed in these tubules (Storms et al., 1995; Kikkert et al., 1997). Possibly, Tomato spotted wilt virus can also move through the tubules as a complex of viral RNA with its MP (the NSm protein).

Another possible function for these tubules relates to the observation that umbraviruses are often associated with luteoviruses in nature and can be encapsidated by the CP of the
latter (reviewed by Taliansky et al., 2000). Luteoviruses alone are normally restricted to the phloem, but in the presence of umbraviruses, they can spread into mesophyll cells (Mayo et al., 2000). The ORF4-mediated tubular structures might be used by both umbraviruses encapsidated by the luteoviral CP and luteoviruses themselves to spread from cell to cell during mixed infections.

**RNA-binding properties**

Results of the gel retardation analysis and competition binding assays presented here demonstrate that the GRV ORF4-encoded MP binds to both ssRNA and ssDNA without any obvious sequence specificity, but does not bind to dsDNA. UV cross-linking assays and analysis of the stability of MP–RNA complexes at different salt concentrations indicated that the GRV ORF4-encoded protein binds RNA as stably as does the CMV 3a protein. Results of AFM analysis complemented these conclusions and allowed direct visualization of the complexes. The architecture of the GRV MP–RNA complexes obtained as a result of non-cooperative protein–RNA binding, even at high protein:RNA ratios, resembled another type of ‘non-cooperative’ complex: the ‘beads-on-a-string’ structure described by Kiselyova et al. (2001) for TMV MP–RNA complexes obtained at low protein:RNA ratios. On the other hand, CMV MP bound to RNA cooperatively and formed net-type structures. It is worth noting that another MP–RNA complex, the TMV MP–RNA complex, also formed as a result of cooperative binding to RNA at high protein:RNA ratios and exhibited a ‘thick string’ appearance (Kiselyova et al., 2001), quite different from the CMV MP–RNA net-type complexes. It was suggested that ‘thick string’ complexes formed by TMV MP and RNA are able to move from cell to cell as they are (Kiselyova et al., 2001). However, it is difficult to imagine how cooperative CMV MP–RNA aggregates containing tangled chains are able to pass through the plasmodesmata. In the case of CMV, another factor may be required to unwind the tangled aggregates. Since the CMV CP is required for cell-to-cell movement (Suzuki et al., 1991; Boccard & Baulcombe, 1993; Canto et al., 1997), it may play such a role.

Previously, we have shown that GRV MP localizes to the plasmodesmata (Ryabov et al., 1998). Thus, with respect to RNA binding and localization to the plasmodesmata, this protein is similar to the MPs of other plant viruses, including CMV, which can move from cell to cell through modified plasmodesmata as a complex containing viral RNA and MP rather than in the form of a virion. However, in contrast to many other viral MPs, such as the CMV 3a MP, that bind RNA non-cooperatively and to near saturation, the GRV MP binds to RNA non-cooperatively and only to a limited extent, even at high protein:RNA ratios, forming ‘beads-on-a-string’ structures. To the best of our knowledge, none of the known native viral MPs possesses such characteristics of RNA binding. The AMV 3a MP can bind RNA non-cooperatively, but at high protein:RNA ratios it forms fully retarded complexes (Schoumacher et al., 1992). Giesman-Cookmeyer & Lommel (1993) described several mutated forms of the MP of Red clover necrotic mosaic virus that bound RNA only to a limited extent in vitro, but were still able to transport the virus from cell to cell in vivo. Assuming that RNA-binding activity in vitro reflects the formation of transferable RNA–MP complexes in vivo, they suggested that relatively little RNA binding is actually required for the cell-to-cell movement of Red clover necrotic mosaic virus. A high level of cooperative binding may be required under certain conditions or in some hosts or tissues. The formation of cooperative complexes containing fully and tightly packed RNA molecules has at least one obvious advantage, a high level of RNA protection, which may be particularly important under certain conditions. On the other hand, this advantage can also turn into a disadvantage, as it can make more difficult the process of RNA release from the ‘dense’ complex for expression (translation) after transport from infected cells to healthy ones. For example, it has been demonstrated that TMV RNA, fully and cooperatively bound by TMV CP, cannot be translated in vitro and is infectious in protoplasts (Karpova et al., 1997). Thus, these results suggested that a specific mechanism should operate to convert untranslatable, cooperatively bound TMV MP–RNA complexes into a translatable form. In the case of TMV, such a conversion is probably regulated by MP phosphorylation (Karpova et al., 1999).

The specific mode of limited RNA binding by the GRV MP may not need a specific regulatory mechanism for releasing RNA. Interaction of the exposed unbound RNA sequences with ribosomes might result in the ‘uncoating’ of the RNA from the complex, thereby providing access for translation. Thus, taking into account the dimensions of the GRV MP–RNA ‘beads-on-a-string’ complex, we suggest that it may be able to move from cell to cell either through the plasmodesmata or through the ORF4-mediated tubules. In the latter case, the lack of cooperative binding would prevent absorption into tubules containing the GRV ORF4 protein.

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