RNA-binding properties of the 63 kDa protein encoded by the triple gene block of poa semilatent hordeivirus

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The 63 kDa ‘63K’ movement protein encoded by the triple gene block of poa semilatent virus (PSLV) comprises the C-terminal NTPase/helicase domain and the N-terminal extension domain, which contains two positively charged sequence motifs, A and B. In this study, the in vitro RNA-binding properties of PSLV 63K and its mutants were analysed. Membrane-immobilized 63K and N-63K (isolated N-terminal extension domain) bound RNA at high NaCl concentrations. In contrast, C-63K (isolated NTPase/helicase domain) was able to bind RNA only at NaCl concentrations of up to 50 mM. In gel-shift assays, C-63K bound RNA to form complexes that were unable to enter an agarose gel, whereas complexes formed by N-63K could enter the gel. Full-length 63K formed both types of complexes. Visualization of the RNA–protein complexes formed by 63K, N-63K and C-63K by atomic force microscopy demonstrated that each complex had a different shape. Collectively, these data indicate that 63K has two distinct RNA-binding activities associated with the NTPase/helicase domain and the N-terminal extension domain. Mutations in either of the positively charged sequence motifs A and B had little effect on the RNA binding of the N-terminal extension domain, whereas mutations in both motifs together inhibited RNA binding. Hybrid viruses with mutations in motifs A and B were able to infect inoculated leaves of Nicotiana benthamiana plants, but were unable to move systemically to uninoculated leaves, suggesting that the RNA-binding activity of the N-terminal extension domain of PSLV 63K is associated with virus long-distance movement.

Introduction

Transport of viruses within infected plants requires virus-encoded non-structural movement proteins (MPs) and, in some cases, other movement-related proteins, such as coat proteins (CPs) (Carrington et al., 1996; Ding, 1998; Lazarowitz & Beachy, 1999; Tzfira et al., 2000). Although transport systems in distant groups of plant viruses can vary significantly in the number and type of protein involved, at least one of the movement-related polypeptides generally has nucleic acid-binding activities (Carrington et al., 1996; Lazarowitz & Beachy, 1999; Morozov & Solovyev, 1999; Tzfira et al., 2000). It has been suggested previously that cell-to-cell transport of RNA viruses involves ribonucleoproteins (RNP) that are formed by virus-encoded MPs and viral genomic RNA (Atabekov & Dorokhov, 1984; Dorokhov et al., 1984). This hypothesis is now supported by extensive evidence (Brakke et al., 1988; Citovsky & Zambryski, 1991; Carrington et al., 1996; Donald et al., 1997). The MPs that form the transport RNP could (i) select the population of genomic RNA molecules to be transported to neighbouring cells; (ii) target the RNP to the plasmodesmata; and (iii) chaperone viral RNA to enable its translocation through the plasmodesmata micro-channels (Citovsky & Zambryski, 1991, 1993; Lazarowitz & Beachy, 1999; Lucas, 1999; Tzfira et al., 2000). Therefore, the RNA-binding properties of the MPs are of key importance for virus cell-to-cell movement.
In hordeiviruses, movement-related RNPs are formed by the TGBp1 protein (Brakke et al., 1988; Donald et al., 1997), which is encoded by the first of three overlapping genes constituting the so-called ‘triple gene block’ (TGB). The TGB is an evolutionarily conserved gene module found in a number of genera of rod-shaped and filamentous plant viruses (Morozov et al., 1989; Solovyev et al., 1996; Morozov & Solovyev, 1999); all three TGB-encoded proteins are required for virus cell-to-cell movement (Pettu & Jackson, 1990; Beck et al., 1991; Gilmer et al., 1992; Herzog et al., 1998). Sequences of the TGBp1 proteins contain an NTPase/helicase domain with seven distinctive sequence motifs (Gorbalenya et al., 1989; Gorbalenya & Koonin, 1989). Accordingly, the TGBp1 proteins have NTPase and RNA-binding activities that are required for virus cell-to-cell movement (Rouleau et al., 1994; Bleykasten et al., 1996; Kalinina et al., 1996; Donald et al., 1997; Lough et al., 1998; Wung et al., 1999; Erhardt et al., 2000). Potexvirus TGBp1 is able to increase the size-exclusion limit of the plasmodesmata and moves through the plasmodesmata into adjacent cells (Angell et al., 1996; Lough et al., 1998, 2000; Yang et al., 2000). In addition, the TGBp1 of potato virus X (PVX) is capable of binding to PVX virions and inducing changes in their conformation in vitro (Atabekov et al., 2000). PVX TGBp1 is also involved in the suppression of virus-induced gene silencing (Voinnet et al., 2000).

The TGBp1 proteins of members of the proposed family Potexviridae (genera Potexivirus, Carlavirus, Allexivirus and Potexvirus) range from 24 to 26 kDa and the NTPase/helicase domain comprises their entire sequence (Morozov & Solovyev, 1999). The TGBp1 proteins in members of the genus Hordeivirus are substantially larger, ranging from 50 to 63 kDa, and the NTPase/helicase domain occupies only the C-terminal half of the protein (Solovyev et al., 1996). The N-terminal half, called the ‘extension domain’, is characterized by clusters of positively charged amino acid residues that are possibly involved in RNA binding (Solovyev et al., 1996; Donald et al., 1997).

In this paper, we describe the RNA-binding properties of poa semilatent hordeivirus (PSLV) TGBp1 protein and analyse the contribution of different protein regions to RNA binding. In addition, we demonstrate the involvement of the positively charged sequence blocks located within the N-terminal extension domain in RNA-binding and virus systemic movement.

**Methods**

**Construction of recombinant clones.** In order to express the 63 kDa ‘63K’ TGBp1 protein of PSLV in Escherichia coli, we used a bacterial strain harbouring an expression vector based on the pQE plasmid (Qiagen), which was modified to express the PSLV 63K protein, as described previously by Solovyev et al. (1999). The resulting protein contained a six residue histidine-tagged N-terminal sequence (MRGSHHHHHHGSPRA). The following 63K deletion mutants were constructed: C-63K, the C-terminal NTPase/helicase domain expressed alone; N-63K, the N-terminal extension domain expressed alone; 63K-ΔV-VI, a C-terminally truncated protein lacking the helicase motifs V–VI; 63K-ΔIV-VI, a C-terminally truncated protein lacking the helicase motifs IV–VI; 63K-ΔIII-VI, a C-terminally truncated protein lacking the helicase motifs III–VI; and 63K-AN, an N-terminally truncated mutant in which the extension domain and the NTPase/helicase domain comprising motifs I, II, III, and V were deleted (Fig. 1). To express different regions of the 63K protein, the appropriate domains of the gene were amplified by PCR and cloned into the pQE vector. Specific oligonucleotide primers were used to introduce mutations into the positively charged sequence motifs A and B. The resulting clones were verified by sequencing. The Nhel site located between motifs A and B was used to combine mutations in both motifs into one clone. The barley stripe mosaic virus (BSMV) mutant P§64, which contains the PSLV TGB, has been described previously (Solovyev et al., 1999). To introduce the mutations generated in motifs A and B into P§64, Nco–StuI DNA fragments from the clones N-63KmutA, N-63KmutB and N-63KmutAB were ligated into the similarly digested P§64 plasmid.

**Expression and purification of histidine-tagged TGBp1.** E. coli strain M15 transformed with the recombinant vectors was grown at 37 °C in liquid culture to an OD_{600} of 0.6–0.9. Protein expression was induced with 1 mM IPTG and the cultures were incubated for 2–4 h at 37 °C. Purification of recombinant proteins was performed according to the manufacturer’s instructions for denaturing Ni^{2+}–NTA chromatography (Qiagen).

**North-Western and gel-shift assays.** RNA-binding assays with membrane-bound proteins were performed as described by Kalinina et al. (1996) using RNA probes labelled with [α-32P]UTP and synthesized by T7 RNA polymerase transcription of an Miiil-linearized BSMV RNAβ cDNA clone (Pettu et al., 1988). In the modified gel-shift assay (Zhao et al., 1996), tobacco mosaic virus (TMV) RNA was incubated with various concentrations of protein for 15 min on ice in 30 µl of gel-shift buffer (10 mM Tris–HCl, pH 8.0, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT) containing 50, 150, 300 or 500 mM NaCl. Samples were analysed in ethidium bromide-containing 1% agarose gels in 1× Tris–acetate buffer (Sambrook et al., 1989).

**Infection of plants and analysis of virus movement.** Prior to transcription, P§64 and its mutants, P§64mutA, P§64mutB and P§64mutAB, were linearized with AccIβ; BSMV RNAα and RNAβ full-length cDNA clones were linearized with Miiiβ. In vitro transcripts were generated as described previously (Pettu et al., 1989). Nicotiana benthamiana plants were inoculated with RNA transcripts in GKP buffer (50 mM glycerine, 30 mM K₂HPO₄, pH 9.2, 1% bentonite, 1% celite) (Pettu et al., 1989). The BSMV CP was detected by Western blotting with specific antiserum (kindly provided by V. K. Novikov), as described previously (Solovyev et al., 1999).

**Atomic force microscopy (AFM).** Samples containing RNA and protein at a molar ratio of 1:100 (63K) or 1:200 (N-63K or C-63K) were incubated for 10 min in gel-shift buffer supplemented with 5 mM MgCl₂ and 1 mM EDTA and placed on the surface of modified highly oriented pyrolytic graphite (HOPG) or freshly cleaved mica. HOPG was glow-discharged in air (residual pressure 0.15–0.2 Torr, discharge current 2–5 mA, duration of discharge 20–40 s), as described previously (Dubochet et al., 1971). To adsorb RNA–protein complexes, the diffusional droplet method was used. After completing adsorption, the mica sheet or the HOPG was dried in a flow of argon. Samples were imaged with a NanoScope II (Digital Instruments) operating at constant force in the height mode using silicon nitride tips with an apex curvature radius of 50 nm. To minimize relative humidity, heated nitrogen was streamed over the scanning area (Prokhorov et al., 1999). The scanning rate was 7 Hz with scanning angles of 180 and 90°. Images were filtered using the flatten filter of the NanoScope II software.
RNA-binding properties of PSLV 63K protein

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Fig. 1. (a) Schematic representation of the PSLV 63K protein. A and B indicate positively charged amino acid stretches within the N-terminal extension domain. The NTPase/helicase domain is shown in light grey, with conserved sequence motifs (dark grey) numbered I–VI.
(b) The positions of the 63K deletion mutants N-63K (residues 1–290), C-63K (residues 296–576), 63K-ΔIV-VI (residues 1–461), 63K-ΔIV-VI (residues 1–406), 63K-ΔIII-VI (residues 1–424) and 63K-ΔN (position 405–576) are indicated relative to the 63K protein.
(c) Site-directed mutations in the N-terminal extension domain of 63K are shown.

Results

RNA binding of the PSLV 63K protein and its mutants in RNA–protein blot assays

The RNA-binding potential of PSLV 63K was initially tested by North-Western blot assay. After electrophoresis, the purified protein was transferred onto a membrane and renatured. A labelled RNA probe was then added to the membrane at different NaCl concentrations. As RNA probes, we initially used four RNA transcripts of different specificity, corresponding to full-length PSLV RNAβ, the 5′-terminal region of BSMV RNAβ, a region of the PVX genome that included the 25K MP gene and the green fluorescent protein gene. As these probes were bound by 63K with similar efficiency (data not shown), we concluded that the PSLV 63K protein interacts with RNA in a non-sequence-specific manner, as was also found for the closely related 58K TGBp1 protein of BSMV (Donald et al., 1997). Therefore, in subsequent experiments, we used only an RNA probe corresponding to the 5′-terminal region of BSMV RNAβ, a region of the PVX genome that included the 25K MP gene and the green fluorescent protein gene. As these probes were bound by 63K with similar efficiency (data not shown), we concluded that the PSLV 63K protein interacts with RNA in a non-sequence-specific manner, as was also found for the closely related 58K TGBp1 protein of BSMV (Donald et al., 1997). Therefore, in subsequent experiments, we used only an RNA probe corresponding to the 5′-terminal region of BSMV RNAβ (residues 1–774). The 63K protein bound RNA in the North-Western assay at NaCl concentrations of up to 500 mM, but not at NaCl concentrations greater than 600 mM (Fig. 2; data not shown), thus exhibiting a binding profile similar to that of BSMV 58K (Donald et al., 1997).

To map RNA-binding regions within the 63K protein, we tested a set of deletion mutants in an RNA–protein blot assay. N-63K and C-63K, which represent the two separate halves of the protein (Fig. 1b), were both able to bind RNA at low salt concentrations (Fig. 2), in agreement with observations that the BSMV 58K protein has multiple RNA-binding sites (Donald et al., 1997). However, C-63K exhibited RNA-binding activity only at 50 mM NaCl or without salt (Fig. 2). In contrast to C-63K, N-63K bound RNA at concentrations of up to 500 mM NaCl (Fig. 2). The C-terminally truncated 63K mutants, 63K-ΔV-VI, 63K-ΔIV-VI and 63K-ΔIII-VI, all retained RNA-binding activity at 300 mM NaCl or greater (Fig. 2). These data suggested that the RNA-binding activity of the PSLV 63K protein is associated with the N-terminal extension domain and a region of the NTPase/helicase domain, including motifs I, IA and II (Fig. 1). In agreement with this suggestion, mutant 63K-ΔN, which contains only motifs III–VI (Fig. 1), was unable to bind RNA, even at 0 mM NaCl (Fig. 2). Taken together, these data indicated that the PSLV 63K protein had at least two RNA-binding activities: one is associated with the N-terminal extension domain and is characterized by salt-resistant RNA binding and the other, characterized by salt-sensitive RNA binding, is associated with the NTPase/helicase domain and, in particular, with the region comprising sequence motifs I, IA and II.

Gel-shift analysis of RNA binding of the 63K protein and its mutants

To characterize RNA–protein interactions in solution, a gel-shift assay was used. In these experiments, proteins were incubated with unlabelled TMV RNA and then subjected to electrophoresis in an ethidium bromide-containing agarose gel. In such experiments, the mutant C-63K formed RNA–protein complexes that were unable to enter the gel (Fig. 3). A similar retardation pattern was found for the PVX 25K MP (Fig. 3), indicating that the observed profile is characteristic of the NTPase/helicase domain of the TGBp1 proteins.

The mutant N-63K formed complexes whose mobility gradually decreased as the protein:RNA ratio increased (Fig. 3). Even at the highest N-63K concentration tested
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Fig. 2. North-Western blot analysis of the 63K protein and its deletion mutants. Purified recombinant proteins (1–5 µg) were separated by SDS–PAGE and electroblotted onto nitrocellulose membranes. Membrane-bound proteins were denatured in 6 M urea and 0–1% Tween 20 and renatured in 20 mM Tris–HCl, pH 7–5, 0–2 g/l BSA, 0–2 g/l Ficoll and 0–2 g/l polyvinylpyrrolidone. Membranes were incubated in the same buffer at different salt concentrations (0, 50, 150, 300 and 500 mM) with 32P-labelled RNA transcripts (1–2 × 106 d.p.m./ml) at room temperature. Membranes were then washed, dried and autoradiographed. Protein staining with Ponceau S is shown on the upper left panel. Lane 1, 63K; lane 2, 63K-∆V-VI; lane 3, 63K-∆IV-VI; lane 4, 63K-∆III-VI; lane 5, N-63K; lane 6, C-63K; and lane 7, 63K-∆N. Positions of proteins in the gels are indicated.

Fig. 3. Gel-shift assay of RNA-binding activity of N-63K, C-63K, PVX 25K and p50 (the rabbit major core informosome protein). Increasing amounts of proteins were incubated with 1 µg of TMV RNA and electrophoresed in 1% non-denaturing agarose gel. Protein:RNA molar ratios are indicated for each lane. RNA, probe without added protein.

Fig. 4. Gel-shift assay of RNA-binding activity of 63K and 63K-∆III-VI. Increasing amounts of proteins were incubated with 1 µg of TMV RNA and electrophoresed in 1% non-denaturing agarose gel. Protein:RNA molar ratios are indicated for each lane. RNA, probe without added protein.

(protein:RNA ratio of 185:1), the N-63K–RNA complex still migrated into the gel (Fig. 3). On the other hand, RNA retardation was detectable at protein:RNA ratios as low as 6:1 and 12:1 (Fig. 3). A similar retardation profile was also found for the RNA-binding protein p50, which is the major core informosome protein associated with eukaryotic cell mRNA (Evdokimova et al., 1995) (Fig. 3).

The full-length 63K protein formed both fully retarded and intermediate complexes that migrated into the agarose gel (Fig. 4). Even at protein:RNA ratios as low as 15:1, both types of complexes were observed (Fig. 4). With increasing protein:RNA ratios, increasing proportions of RNA were involved in fully retarded complexes, so that at protein:RNA
Visualization of protein–RNA complexes by AFM

The NTPase activity exhibited by the N-63K and C-63K mutants. These findings indicate that, as in the North-Western experiments suggested that the C-terminal region of the 63K protein is not involved in RNA binding; we tested truncated mutants in the gel-shift assay. Similar to the full-length 63K protein, both 63K-ΔIV-VI (data not shown) and 63K-ΔIII-VI (Fig. 4) formed two types of complexes. These findings indicate that, as in the North-Western assay, RNA binding of 63K in solution is determined by the N-terminal extension domain and the region containing the NTPase/helicase motifs I and II. However, in experiments with 63K-ΔIII-VI, all added RNA was converted to fully retarded complexes at a protein:RNA ratio of 170:1, whereas a similar effect was observed for 63K at the ratio of 60:1 (Fig. 4). These data demonstrate that transition to fully retarded complexes required three times as much mutant protein as native protein, indicating that the 63K-ΔIII-VI deletion may affect protein conformation and, consequently, cooperative interaction of protein molecules. Indeed, at a protein:RNA ratio of 45:1, most of the RNA was in the form of intermediate complexes with 63K-ΔIII-VI, rather than in retarded complexes, as observed when the full-length protein was used (Fig. 4). Moreover, application of a Hill transformation to the RNA-binding data (Daros & Carrington, 1997; Marcos et al., 1999) showed a Hill coefficient of 2.0 and 1.08 (mean value of five experiments) with 63K and 63K-ΔIII-VI, respectively (data not shown). If the Hill coefficient is above 1, these data can be taken as an indication of positive cooperativity. Thus, quantifying the cooperative interactions in protein–RNA binding confirms a significant loss of cooperativity in the case of 63K-ΔIII-VI.

In another series of experiments, incubation of protein with RNA prior to agarose gel analysis was carried out in the presence of higher NaCl concentrations (150, 300 and 500 mM NaCl). It was found that the complexes formed by 63K, N-63K and C-63K at 50 mM NaCl were also stable at 500 mM NaCl (data not shown). These data indicate that electrostatic interactions may not be the only type of interaction involved in the formation of protein–RNA complexes in solution.

Visualisation of protein–RNA complexes by AFM

To further characterize binding of 63K, N-63K and C-63K to RNA and to visualize the possible structural differences of the RNPs, we imaged TMV RNA–protein complexes by AFM (Binnig et al., 1986). In preliminary experiments, we examined TMV RNA and free proteins imaged by AFM, as described by Prokhorov et al. (1999). TMV RNA exhibited a considerable heterogeneity of shapes. The observed condensed forms are due presumably to the formation of single-stranded RNA secondary structure (maximum height 6±3 nm, thread height 2.4±0.4 nm) (Fig. 5a) (Klinov et al., 1998). Images of the 63K protein in the absence of RNA revealed two populations of particles with heights of 1.7±0.3 nm and 2.8±0.6 nm (Fig. 5b), which may represent monomers and dimers of 63K. Fig. 5(c–e) shows typical complexes formed by TMV RNA and 63K, N-63K and C-63K, respectively, at protein:RNA ratios of 100:1 (63K) and 200:1 (N-63K and C-63K). The 63K RNPs appear as large globular complexes (average height 11±2±0 nm) with a halo of thin threads (height 2.0±0.4 nm) protruding from them. It could be speculated that the appearance of globular structures was the result of the multimerization of RNA-bound 63K at the protein:RNA ratio (100:1) used in these experiments. It should be noted that in the gel-shift assay, only fully retarded complexes could be found at this ratio. Imaging of N-63K–RNA complexes showed partially condensed threads (average height 2.4±0.4 nm) with a tendency to form toroid-like structures (maximum height 4.4±0.4 nm) (Fig. 5d). Some non-condensed threads exhibited a knobby appearance (Fig. 5d) that was similar to the ‘beads-on-a-string’ appearance found in AFM images of the RNP structures formed by TMV MP at low protein:RNA ratios (Kiselyova et al., 2001). The C-63K RNPs were represented by non-branched curved structures (Fig. 5e) with an average height of 4.8±0.8 nm and a maximum height of 9±2±1 nm.

Although the fine structure of the observed complexes remains to be established, it can be concluded that, in vitro, 63K, N-63K and C-63K can form RNPs that have distinct shapes when observed by AFM. The different behaviour of these proteins in gel-shift assays may be related to the differences of RNP structures visualized by AFM.

Mapping of N-terminal domain regions responsible for RNA binding

We have shown previously that the sequences of the N-terminal extension domain in hordeivirus TGBp1 proteins contain clusters of positively charged amino acid residues that could contribute to RNA-binding activity (Solovyev et al., 1996). In the N-terminal extension domain of PSLV 63K, two clusters of this kind were found and are referred to, hereafter, as motifs A and B. Motif A (residues 114–125) is 12 residues in length and contains nine lysine and arginine residues; motif B (residues 175–189) is 15 residues in length and contains eight lysine residues. A role for these motifs in RNA interaction is consistent with the data presented in this paper, as the 63K RNA-binding activity responsible for salt-stable nucleic acid binding is located in the N-terminal extension domains.

To analyse the RNA-binding properties of the N-terminal extension domain, we mutated motifs A and B in N-63K (Fig. 1). The following mutants were constructed: N-63KmutA (KAKSKRKKKNNK → KASNGALKNAA in motif A), N-63KmutB (KKATKKESSKQTKDK → AEATTNASAQQTE-DK in motif B) and N-63KmutAB (both substitutions) (Fig. 1c). Mutant proteins were expressed in E. coli, purified to near...
Fig. 5. AFM imaging of RNA–protein complexes. (a) TMV RNA alone, (b) 63K protein alone and complexes of TMV RNA with (c) 63K, (d) N-63K and (e) C-63K are shown. (a, b) Bar, 100 nm. (c–e) Bar, 50 nm. (c) Arrows indicate threads protruding from globular complexes. (d) Arrows in the enlarged region (bar, 10 nm) demonstrate knobby structures.

Fig. 6. North-Western analysis of RNA binding of N-63K and N-63KmutA, N-63KmutB and N-63KmutAB. Proteins (1–5 µg) were resolved in 15% gels, transferred onto a membrane and probed with a labelled RNA transcript at 150 and 300 mM NaCl. Protein staining with Ponceau S is shown on the top panel.

Homogeneity and their RNA-binding potential was tested by North-Western hybridization (Fig. 6).

At NaCl concentrations of 50 and 150 mM, the RNA-binding activity of N-63KmutA and N-63KmutB was similar to that of N-63K, whereas the double mutant N-63KmutAB was unable to bind RNA (Fig. 6; data not shown). At 300 mM NaCl, only N-63KmutB and N-63K exhibited RNA-binding activity, demonstrating that the positively charged motif A makes the major contribution to salt-resistant RNA binding of the N-terminal extension domain. These data also suggest that motifs A and B could participate independently in RNA binding in vitro. It is possible that motifs A and B represent the only sites of the N-terminal extension domain responsible for interactions with nucleic acids. The existence of at least two distinct protein regions involved in RNA binding has already been reported for TMV MP (Citovsky et al., 1992) and movement-related proteins of potyviruses (Fernandez & Garcia, 1996; Urcuqui-Inchima et al., 2000).

RNA-binding sites in the N-terminal domain of 63K are necessary for virus long-distance movement

To analyse the contribution of the positively charged sequence motifs A and B to the functions of 63K in virus cell-to-cell movement, we used infectious transcripts (Petty et al., 1988, 1989) of the hybrid hordeivirus P684, as described previously (Solovyev et al., 1999). P684 contains RNAα and RNAγ of BSMV and BSMV RNAβ in which the TGB has been replaced with its counterpart from PSLV. The mutations made in motifs A and B were introduced into P684 to give P684mutA, P684mutB and P684mutAB, which were inoculated into the leaves of N. benthamiana plants.
Table 1. Analysis of *N. benthamiana* plants inoculated with hordeivirus P684 and its mutants

Wild-type hordeivirus P684 and P684 mutants, in which the positively charged motifs A and B located within the N-terminal extension domain were disabled, were inoculated into *N. benthamiana* plants. The number of infected/uninoculated leaves, as detected by Western blotting, is indicated.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Inoculated leaves</th>
<th>Systemic infection</th>
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<tbody>
<tr>
<td>P684</td>
<td>3/11</td>
<td>+</td>
</tr>
<tr>
<td>P684mutA</td>
<td>3/10</td>
<td>–</td>
</tr>
<tr>
<td>P684mutB</td>
<td>6/10</td>
<td>–</td>
</tr>
<tr>
<td>P684mutAB</td>
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Both inoculated and uninoculated upper leaves of *N. benthamiana* were tested in Western blots with BSMV antiserum. The data of two independent experiments presented in Table 1 demonstrate that mutation of either or both of the positively charged motifs A and B had little effect on cell-to-cell movement of the P684 hybrid virus. The percentage of infected leaves and the amount of viral CP detected by Western blotting in the infected plant tissue were similar for P684 and the mutants (Table 1; data not shown). However, Western blot analysis of the upper, uninoculated leaves demonstrated that the mutants were unable to infect *N. benthamiana* systemically (Table 1). Thus, these data demonstrate that motifs A and B are important not only for RNA binding in vitro, but also for the systemic spread of the virus in plants.

**Discussion**

In this paper, we have analysed the RNA-binding properties of the TGBp1 protein of PSLV. RNP complexes formed by 63K and various deletion mutants (Fig. 1b) were tested by North-Western blots, gel-shift assays and AFM. In the North-Western assay, full-length 63K and the separated N-terminal domain of 63K (N-63K) were able to bind RNA at NaCl concentrations of up to 500 and 300 mM, respectively (Fig. 2). The level of salt resistance of RNA binding displayed by the 63K protein was higher than that of a number of plant viral MPs and comparable to that of the TMV 30K MP (Li & Palukaitis, 1996; Wobbe et al., 1998). In contrast, the NTPase/helicase domain (C-63K) bound RNA only at NaCl concentrations of up to 50 mM (Fig. 2). Similar salt-unstable RNA binding has been found previously for the PVX 25K TGBp1 protein, which lacks the N-terminal extension domain (Kalinina et al., 1996). Therefore, both the NTPase/helicase and the N-terminal extension domains can bind RNA. This is in agreement with observations described previously about the 58K TGBp1 of BSMV, where multiple RNA-binding sites were found in both the N-terminal and the C-terminal regions of the protein (Donald et al., 1997). As deletions in the C-terminal region of 63K (63K-ΔV-VI, 63K-ΔIV-VI and 63K-ΔIII-VI) had little effect on RNA interaction (Fig. 2), it follows that RNA binding of 63K is specified by its N-terminal extension domain and a region that includes the I, IA and II motifs of the NTPase/helicase domain. The importance of the latter region for RNA binding was demonstrated further by the comparison of 63K-AN and C-63K (Fig. 1b): unlike 63K-AN, C-63K could still bind RNA, although only at low salt concentrations (Fig. 2).

These data are consistent with the observations that in vitro RNA binding of the potexvirus TGBp1 helicases required N-terminal sequences upstream of and including motifs I and II (Morozov et al., 1999; Wung et al., 1999). Taking into account the data of these authors, it should be emphasized that the term ‘salt-unstable RNA binding’ is rather provisional. Deletion of motifs III–VI of the PVX 25K NTPase/helicase significantly increased the salt stability of RNA binding (Morozov et al., 1999), demonstrating that the helicase domain might exist in conformation(s) favourable for salt-stable RNA binding.

In gel-shift experiments, 63K formed two types of RNA–protein complexes: fully retarded (unable to enter the agarose gel) and partially retarded (complexes that migrated into the gel but with decreasing mobility with increasing ratios of protein:RNA). Gel-shift analysis of C-63K and N-63K revealed that the fully retarded complexes could be formed by the NTPase/helicase domain of 63K, whereas RNA-binding activity of the N-terminal extension domain could be responsible for the formation of partially retarded complexes (Fig. 3). Importantly, PVX 25K, like C-63K, formed only fully retarded complexes (Fig. 3), demonstrating that the observed profile is characteristic of the NTPase/helicase domain of the TGBp1 proteins. It should be noted that such a retardation pattern for proteins of other viruses has been taken to be indicative of cooperativity (Citovsky et al., 1992; Jansen et al., 1998; Osman et al., 1992; Tsai et al., 1999). Indeed, application of a Hill transformation to RNA-binding data (see Daros & Carrington, 1997; Marcos et al., 1999) showed a Hill coefficient of 3:3, 4:5 and 2:4 (mean value of five independent experiments) with C-63K, PVX 25K and TMV 30K MP, respectively (data not shown). These data allow us to quantify cooperativity of RNP formation by the latter proteins and can be regarded as strong evidence for positive cooperativity.

Analysis of the stability of protein–RNA interaction in salt provided further evidence that C-63K could bind RNA in a cooperative manner. As RNA binding of C-63K was salt-sensitive in the North-Western assays, whereas the complexes formed by C-63K were resistant to high salt concentrations in gel retardation assays, it can be hypothesized that formation of RNP’s by C-63K in solution involves cooperative interactions that are impossible in North-Western assays when the protein is fixed onto a solid phase. A similar effect was observed for the PVX 25K protein (see above; Morozov et al., 1999). It should...
be noted, however, that the formation of partially retarded complexes (as in the case of N-63K) does not necessarily rule out cooperative RNA–protein binding, since it may reflect cooperative binding of protein molecules in a non-contiguous manner along the RNA molecule (Zhou et al., 1996).

North-Western and gel-shift assays suggest that the 63K protein can exist in two different conformations that allow the formation of two different types of complexes (Fig. 4). Interestingly, p50, which was found to form intermediate complexes in the gel-shift assay (Fig. 3), is known to form two types of mRNPs with different functional characteristics. This protein is present in translatable mRNPs at low protein:RNA ratios, whereas at high ratios, p50 forms non-translatable informosomes (Evdokimova et al., 1998). The different gel mobility of complexes formed by the N-terminal extension and NTPase/helicase domains of 63K could reflect these structural differences. This hypothesis is reinforced by AFM observations that demonstrate a dissimilar shape of the RNPs formed by N-63K and C-63K (Fig. 5). Importantly, full-length 63K formed complexes different from the RNPs of either N-63K or C-63K, suggesting that both RNA-binding activities in the full-length protein contribute to complex formation or 63K can participate in protein–protein interactions different from those of the separated protein domains.

To identify region(s) responsible for the RNA-binding properties of PSLV 63K N-terminal extension domain, we mutated two arginine/lysine-rich sequences that have been proposed previously to contribute to RNA binding (Solovyev et al., 1996). Analysis of the mutant proteins (Fig. 1c) by North-Western blotting demonstrated that both positively charged stretches (motifs A and B) participated independently in RNA binding in vitro (Fig. 6) and may represent the only regions of the N-terminal extension responsible for interactions with nucleic acids. This is in agreement with recent data showing that in vitro RNA binding of some plant virus MPs and other non-virion proteins could be determined by arginine/lysine-rich regions of between 12 and 20 residues (Bleykasten et al., 1996; Marcos et al., 1999; Tsai et al., 1999). In BSMV 58K, deletions spanning positively charged regions in the N-terminal extension domain also dramatically affected RNA binding in vitro (Donald et al., 1997).

As the RNA-binding properties of the hordeivirus TGBp1 proteins are apparently associated with their ability to form movement-competent RNPs, we attempted to distinguish functions of the independent 63K RNA-binding activities in virus movement. The biological importance of RNA binding of the NTPase/helicase domain is difficult to demonstrate experimentally, as mutations in the conserved motifs inhibit virus movement (Donald et al., 1997; Erhardt et al., 2000; Lough et al., 1998). Therefore, we analysed the function(s) of motifs A and B in the N-terminal extension domain. Surprisingly, mutations in either or both of the motifs had little effect on virus cell-to-cell movement in *N. benthamiana* (Table 1). Similarly, mutation of an arginine/lysine-rich region in the N-terminal extension domain of beet necrotic yellow vein virus TGBp1 resulted in a loss of RNA-binding activity, but did not affect virus cell-to-cell movement (Bleykasten et al., 1996; Erhardt et al., 2000). On the other hand, none of the PSLV 63K mutants in motifs A and B was able to potentiate systemic virus movement (Table 1), indicating that concerted RNA-binding activity of the two motifs in the N-terminal domain is associated with long-distance movement. Recent studies on non-TGB MPs also revealed that amino acid changes affecting RNA binding in vitro may influence the systemic spread of the virus, but not cell-to-cell movement (Wobbe et al., 1998). These findings are in agreement with our recent conclusion drawn from experiments with a series of hybrid hordeiviruses, in that the N-terminal domain of the hordeivirus TGBp1 protein is involved in virus systemic spread (Solovyev et al., 1999).

In conclusion, 63K has two in vitro RNA-binding activities: one is associated with the extension domain and is important for long-distance movement and the other is associated with the NTPase/helicase domain and is important (by analogy with PVX) for the formation of cell-to-cell movement-competent RNPs (Lough et al., 1998; Yang et al., 2000). One can speculate that the 63K protein in *vivo* might form two types of viral RNPs specific for cell-to-cell movement and long-distance transport. Such a system has been described recently for two groundnut rosette umbravirus RNA-binding proteins, where the ORF4-encoded protein is involved in cell-to-cell movement and the ORF3-encoded protein is responsible for long-distance transport (Ryabov et al., 1998, 1999; Taliansky et al., 1996). It should be noted, however, that the N-terminal extension domain of the hordeivirus TGBp1, unlike the umbravirus ORF3 protein, is involved in cell-to-cell movement as well (Donald et al., 1997).

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**References**


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