Cooperative binding of the red clover necrotic mosaic virus movement protein to single-stranded nucleic acids

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The movement protein of red clover necrotic mosaic dianthovirus was produced in *Escherichia coli* using an expression vector. Gel retardation analysis and u.v. cross-linking studies showed that the movement protein bound cooperatively to ssRNA and ssDNA, but not to dsDNA. Binding competition experiments

established that the movement protein bound to ssRNA and ssDNA with similar affinities and that the binding was not sequence-specific in the experimental conditions employed. A truncated movement protein lacking the C-terminal 88 amino acids was also shown to bind to ssRNA.

Introduction

Systemic infection of a plant by a virus requires two forms of spread from the cells initially infected: short-distance movement from cell to cell probably via plasmodesmata and long-distance movement via the vascular system (reviewed by Hull, 1989; Robards & Lucas, 1990; Atabekov & Taliansky, 1990; Maule, 1991). Virus cell-to-cell movement is not a passive process but requires virus-encoded movement proteins. The movement protein of tobacco mosaic tobamovirus (TMV) is located in the plasmodesmata of virus-infected plants (Tomenius *et al.*, 1987) or plants transgenic for the movement protein (Atkins *et al.*, 1991). In such plants the molecular size exclusion limit of the plasmodesmata is increased (Wolf *et al.*, 1989; Deom *et al.*, 1990). The discovery that the TMV movement protein binds cooperatively to single-stranded nucleic acids (Citovsky *et al.*, 1990) led to the suggestion that it has two functions, increasing the permeability of plasmodesmata and forming an unfolded RNA–movement protein complex that is able to move through the modified plasmodesmata. The subsequent finding that the putative movement protein (the gene I protein) of cauliflower mosaic caulimovirus (CaMV) also binds to single-stranded nucleic acids led to speculation that movement of single-stranded nucleic acid–movement protein complexes through plasmodesmata may be a general mechanism for spread of plant viruses from cell to cell (Citovsky *et al.*, 1991). However, before this generalization can be proved, studies on the movement proteins of a range of diverse plant viruses will be needed.

Red clover necrotic mosaic dianthovirus (RCNMV) has a genome of two positive-sense ssRNA components,

RNA 1 (3.9 kb) and RNA 2 (1.4 kb) (Lommel *et al.*, 1988; Xiong & Lommel, 1989). The RCNMV movement protein is encoded by RNA 2 (Lommel *et al.*, 1988; Osman *et al.*, 1991*a*) and no significant amino acid sequence homology with the movement protein of TMV or the gene I protein of CaMV could be detected using a modified FASTA programme (Pearson & Lipman, 1988). In the present paper, we have investigated the ability of the RCNMV movement protein to bind to nucleic acids.

Methods

Expression and purification of the RCNMV movement protein. The recombinant plasmid pTM34-210, containing a full-length cDNA copy of RNA 2 of RCNMV isolate TpM-34 flanked by a phage T7 promoter cloned into pUC19, has been described previously (Osman *et al.*, 1991*b*). An *Nde*I site was introduced at the translational initiation codon of the movement protein gene by *in vitro* mutagenesis (Kunkel *et al.*, 1987) using the oligonucleotide AGGTAGGTTTCATATGGC-TATTC. A DNA fragment containing the movement protein coding region and the 3' untranslated region was then excised using *Nde*I and *Bam*HI and cloned into the corresponding sites of the T7 RNA polymerase vector pET3a (Rosenberg *et al.*, 1987) to give pETMP. The *Nde*I restriction site of pET3a contains the translational initiation codon so that the pETMP plasmid expresses the RCNMV movement protein without alterations to the coding sequence. The plasmid pETMP was then transformed into *Escherichia coli* strain BL21(DE-3)pLysE (Studier & Moffatt, 1986). Induction of movement protein synthesis and purification of the movement protein were as described by Citovsky *et al.* (1990) for the TMV movement protein, except that after induction the cell pellet was lysed by sonication (MSE Soniprep 150; amplitude 10 μ m; 4 min). Similar methods were used to produce and purify a truncated movement protein starting with the recombinant plasmid pTM341-210 (Osman *et al.*, 1991*b*).

Preparation of RNA probes. Labelled RNA probes, corresponding to different lengths of RCNMV RNA 2, were prepared by *in vitro* transcription of pTM34-201 with T7 RNA polymerase in the presence of [32 P]UTP as described previously (Osman *et al.*, 1991*b*). Probes of 387 nucleotides (nt), 1082 nt and 1448 nt (full-length RNA 2) were obtained by linearization of the plasmid with *Tth*1111, *Bst*BI or *Pst*II, respectively, prior to transcription. After transcription, DNA and protein were removed by digestion with RNase-free DNase, phenol extraction and ethanol precipitation.

Preparation of DNA probes, retardation gel electrophoresis, nucleic acid-protein cross-linking by u.v. irradiation and binding competition. These experiments were carried out essentially as described by Citovsky *et al.* (1990).

Results

Expression and purification of the RCNMV movement protein

The RCNMV movement protein was expressed in *E. coli* cells carrying plasmid pETMP which contains the coding region of the movement protein linked to a strong bacteriophage T7 promoter. Induction of expression of the T7 RNA polymerase resulted in the synthesis of a protein with the expected size of the movement protein (M_r 36K) (Fig. 1, lane 3). The movement protein formed insoluble aggregates which could be separated from the soluble bacterial proteins by centrifugation. However, the movement protein could be solubilized in 4 M-urea and remained soluble after removal of the urea. Thus, the RCNMV movement protein behaved similarly to both the TMV movement protein and the CaMV gene I protein when these were expressed in *E. coli* using similar vectors (Citovsky *et al.*, 1990, 1991). The purified RCNMV movement protein gave a single band in SDS-PAGE (Fig. 1, lane 4). Similar methods were used to express and purify a truncated movement protein (M_r 30K) using an RNA 2 cDNA clone from RCNMV mutant TpM-341 (Osman *et al.*, 1991*b*) (Fig. 1, lane 5).

The RCNMV movement protein binds to single-stranded nucleic acids

To study the potential of the RCNMV movement protein to bind to ssRNA, a 32 P-labelled ssRNA corresponding to the 5' 387 nt of RCNMV RNA 2 was synthesized by *in vitro* transcription. After incubation of this RNA with the movement protein, the RNA was cross-linked to the protein by u.v. irradiation and unbound RNA was removed by digestion with RNase. Analysis of the protein by SDS-PAGE and autoradiography revealed a band with an apparent M_r of 38K i.e. in the position expected for the movement protein cross-linked to a small number of nucleotides (Fig. 2*a*). A strong band was detected when the incubation was

Fig. 1. Analysis of the expression and purification of the RCNMV movement protein by SDS-PAGE. Lane 1, marker proteins; lane 2, protein from uninduced *E. coli* (pETMP); lane 3, protein from induced *E. coli* (pETMP); lane 4, purified RCNMV movement protein; lane 5, purified truncated RCNMV movement protein. The gel was stained with Coomassie blue R250. The M_r values of marker proteins (BSA, 66K; ovalbumin, 45K; glyceraldehyde 3-phosphate dehydrogenase, 36K; carbonic anhydrase, 29K) are shown on the side of the gel.

carried out in 0.2 M-NaCl or 0.4 M-NaCl (Fig. 2*a*, lanes 1 and 2), a weaker band was found at 0.6 M-NaCl (Fig. 2*a*, lane 3) and no band could be detected at 0.8 M-NaCl or 1.0 M-NaCl (Fig. 2*a*, lanes 4 and 5). This indicates that the movement protein-RNA complex was stable at 0.2 M-NaCl and 0.4 M-NaCl, was partially dissociated at 0.6 M-NaCl and completely dissociated at 0.8 M-NaCl and 1.0 M-NaCl. No band could be detected when the incubation was carried out in the presence of 1000-fold excess unlabelled ssRNA (Fig. 2*a*, lane 7), after incubation with proteinase K (Fig. 2*a*, lane 6) or when the u.v. irradiation step was omitted (Fig. 2*a*, lane 8). The same method was used to demonstrate binding of the movement protein to an ssRNA corresponding to the 5' 1082 nt of RCNMV RNA 2 and to full-length RCNMV RNA 2 (1448 nt) (Fig. 2*b*, lanes 3 and 4).

To ensure that the observed binding was not due to an undetected *E. coli* protein in the movement protein preparation, two controls were carried out. In the first, an equivalent protein preparation from *E. coli* transformed with pET3a lacking the RCNMV sequences was made. No bands could be detected in SDS-PAGE after Coomassie blue staining of the free protein or after carrying out the u.v. cross-linking assay with the 32 P-labelled 387 nt RNA in 0.2 M-NaCl. In the second, binding to a truncated movement protein, which lacks the C-terminal 88 amino acids of the full-length movement protein and has an M_r of 30K (Osman *et al.*, 1991*b*), was demonstrated. In the u.v. cross-linking assay, a band of apparent M_r 32K was formed with this truncated movement protein (Fig. 2*b*, lanes 1 and 2). It is

Fig. 2. Analysis of the binding of the RCNMV movement protein to ssRNA by u.v. cross-linking and SDS-PAGE. (a) Effect of NaCl concentration. The RCNMV movement protein (25 ng) was incubated for 30 min at 4 °C with 1.0 ng of 32 P-labelled 387 nucleotide ssRNA probe and analysed by the u.v. cross-linking assay. Lane 1, 0.2 M-NaCl; lane 2, 0.4 M-NaCl; lane 3, 0.6 M-NaCl; lane 4, 0.8 M-NaCl; lane 5, 1.0 M-NaCl; lane 6, incubation in 0.2 M-NaCl followed by treatment with proteinase K (1 mg/ml) at 37 °C for 30 min; lane 7, incubation in 0.2 M-NaCl in the presence of a 1000-fold excess of unlabelled RCNMV RNA; lane 8, incubation in 0.2 M-NaCl, but omission of the u.v. irradiation step. (b) Binding to longer ssRNAs. Full-length (lanes 3 and 4) or truncated (lanes 1 and 2) RCNMV movement protein (25 ng) was incubated for 30 min at 4 °C in 0.2 M-NaCl with 1.0 ng of 32 P-labelled 1082 nt RNA (lanes 1 and 3) or RCNMV RNA 2 (lanes 2 and 4). Marker proteins are as in Fig. 1, with the addition of phosphorylase b (M_r 97K).

Fig. 3. Gel retardation analysis of the binding of the RCNMV movement protein to DNA. Lane 1, dsDNA probe incubated with the RCNMV movement protein; lane 2, dsDNA probe; lane 3, ssDNA probe incubated with the RCNMV movement protein, followed by incubation with proteinase K (1 mg/ml) for 30 min at 37 °C; lane 4, ssDNA probe incubated with the RCNMV movement protein; lane 5, ssDNA probe incubated with the RCNMV movement protein in the presence of a 1000-fold excess of unlabelled ssDNA; lane 6, ssDNA probe. Incubations were at 4 °C for 30 min in 0.2 M-NaCl with 25 ng of movement protein and 1.0 ng of 32 P end-labelled probe.

clear that the electrophoretic mobilities of the covalently cross-linked protein-nucleotide complexes (Fig. 2b) correlate with those of the free full-length and truncated movement proteins (Fig. 1), showing that the binding is the activity of the movement proteins and not that of a protein contaminant.

To ensure that the assay conditions did not promote indiscriminate binding of proteins to single-stranded nucleic acids, binding of the *E. coli* maltose-binding protein (Duplay *et al.*, 1984) to the above ssRNAs in 0.2 M-NaCl was studied by the u.v. cross-linking method. No binding could be demonstrated (data not shown).

The ability of the RCNMV movement protein to bind to ssDNA was shown by gel retardation analysis. A 298 bp *Hae*III restriction fragment of pUC18 (Yanisch-Perron *et al.*, 1985) was end-labelled with 32 P, denatured and incubated with the RCNMV movement protein in 0.2 M-NaCl. Electrophoresis in a polyacrylamide gel, followed by autoradiography, showed a single band with a mobility (Fig. 2, lane 4) considerably slower than that of the free ssDNA (Fig. 3, lane 6). When the incubation was carried out in the presence of a 1000-fold excess of unlabelled ssDNA, or when the complex was treated with proteinase K, a band corresponding to only the free ssDNA was detected (Fig. 3, lanes 5 and 3). No retardation was observed when the movement protein was incubated with the 298 bp dsRNA probe (Fig. 3, compare lanes 1 and 2).

Fig. 4. Cooperative binding of the RCNMV movement protein to ssRNA and dsDNA. Increasing amounts of the RCNMV movement protein were incubated with (a) ^{32}P -labelled 387 nt ssRNA probe (50 ng) or (b) ssDNA probe (50 ng) as in Fig. 3 and analysed by gel retardation analysis. Amounts of protein (μg) were (a) lane 1, 0; lane 2, 0.01; lane 3, 0.02; lane 4, 0.05; lane 5, 0.1; lane 6, 0.2; lane 7, 0.5; lane 8, 1.0; lane 9, 2.0; lane 10, 4.0 or (b) lane 1, 0; lane 2, 0.5; lane 3, 1.0; lane 4, 1.5; lane 5, 2.0.

Binding of the RCNMV movement protein to single-stranded nucleic acids is cooperative

When the ^{32}P -labelled 387 nt ssRNA was incubated with increasing amounts of the RCNMV movement protein, followed by PAGE, a band of free RNA and a band of retarded RNA were detected at the lower protein:RNA ratios (Fig. 4a, lanes 2 to 7). However, at higher protein:RNA ratios, only the band of retarded RNA was detected (Fig. 4a, lanes 8 to 10). The absence of any bands intermediate between the free and retarded RNA indicates that the binding of the movement protein to this RNA is cooperative. Similar results were obtained with the 1082 nt RNA and full-length RNA 2 (results not shown), and with the 298 nt ssDNA (Fig. 4b).

Competition of binding of the RCNMV movement protein to different nucleic acids

The u.v. cross-linking method was used to compare the abilities of unlabelled RCNMV RNA (RNA 1, 3889 nt; RNA 2, 1448 nt), TMV RNA (strain L, 6384 nt; Ohno *et al.*, 1984) and M13 mp18 ssDNA (7250 nt, Yanisch-Perron *et al.*, 1985) to compete with labelled RCNMV RNA 2 for binding to the RCNMV movement protein. The results showed that both unlabelled RCNMV RNA and TMV RNA efficiently compete with the labelled RCNMV RNA 2. For both competing RNAs, the intensities of these bands were reduced concurrently with increasing amounts of unlabelled to labelled RNA

Fig. 5. Competition of binding of the RCNMV and TMV movement proteins to ssRNA analysed by u.v. cross-linking and SDS-PAGE. The RCNMV movement protein (25 ng) was incubated for 30 min at 4°C with 1.5 ng of ^{32}P -labelled RCNMV RNA 2 in the presence of increasing amounts of unlabelled TMV-L RNA. The competitor:probe ratios were lane 1, 0; lane 2, 100; lane 3, 500; lane 4, 1000; lane 5, 2000; lane 6, 4000. Marker proteins are as in Fig. 1 and 2.

and the bands were no longer detected at a ratio of unlabelled to labelled RNA of 2000:1 (shown for TMV RNA in Fig. 5). Essentially similar results were also obtained using M13 ssDNA as a competitor (results not

shown). Although the data were not quantified, visual inspection of the autoradiographs failed to reveal any pronounced differences in movement protein binding affinities between RCNMV RNA, TMV RNA and M13 ssDNA.

Discussion

The results obtained show that the RCNMV movement protein binds cooperatively to ssRNA and ssDNA, does not bind to dsDNA and is not sequence-specific. In these respects, it is similar to the TMV movement protein and the CaMV gene I protein (Citovsky *et al.*, 1990, 1991). However, the RCNMV movement protein resembled the TMV movement protein more closely in its affinity for binding to ssRNA and in its ability to bind to ssRNA and ssDNA with similar affinities.

The recent finding that the RCNMV movement protein is located in a cell wall fraction (Osman & Buck, 1991), combined with the demonstration of its ability to bind cooperatively to ssRNA, is consistent with the model of Citovsky *et al.* (1990, 1991) for movement of viral single-stranded nucleic acid-movement protein complexes through plasmodesmata, although it does not prove it, nor exclude the possibilities of other mechanisms for virus cell-to-cell movement. Nevertheless, the conservation of the ability of the proteins of three diverse plant viruses to bind to single-stranded nucleic acids is interesting, particularly because the RCNMV movement protein is not related perceptibly in primary amino acid sequence to the TMV movement protein or the CaMV gene I protein. It is possible, as suggested by Melcher (1990), that the functions of plant virus movement proteins depend more on their three-dimensional structure than on their overall sequence similarity.

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