RAPID COMMUNICATION

Phosphorylation of Tobacco Mosaic Virus Movement Protein Abolishes Its Translation Repressing Ability

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Previously we showed that the ribonucleoprotein complexes (RNPs) of the TMV 30-kDa movement protein (MP) with TMV RNA are nontranslatable in vitro and noninfectious to protoplasts, but are infectious to intact plants. It has been suggested that MP–TMV RNA complexes could be converted into the translatable and replicatable form in planta in the course of passage through plasmodesmata (Karpova et al., 1997, Virology 230, 11–21). The role of TMV MP phosphorylation was investigated in terms of its capacity to modulate the translation-repressing ability of the MP. Phosphorylation of the TMV MP, either before or after RNP complex formation, caused a conversion of nontranslatable MP–RNA complexes into a form that was translatable in vitro and infectious to protoplasts and plants.

Key Words: TMV; movement protein; phosphorylation; regulation of translation.

Introduction. The cell-to-cell movement of tobamoviruses is mediated by the 30-kDa movement protein (MP, P30) encoded by TMV. The 30-kDa MP is targeted to plasmodesmata (PD) in infected cells as well as in transgenic plants expressing the MP gene (reviewed in 10, 6, 11). The viral MP and genomic RNA are thought to form an extended, linear RNP complex that is targeted to and translocated through PD (3, 4). MP–RNA complexes therefore represent a particular pool of viral RNA molecules that are destined for cell-to-cell transport and excluded from replication (2). Recently, (His)_{6}-MPs of tobamoviruses were found to be efficient nonspecific translational repressors (9). In vitro translation of viral RNA in MP–TMV RNA complexes was blocked and they were not infectious in isolated protoplasts. However, MP–TMV RNA complexes were infectious in planta, suggesting that these complexes that could not replicate in the primary infected cells were converted into a translatable form in the course of passage through PD. This result has led to speculation (9) that MP–RNA complexes could be converted into a translatable form as a result of MP phosphorylation by cellular protein kinases. The MP of TMV was shown to be specifically phosphorylated in vitro by cell wall-associated protein kinase (PK) of tobacco leaves (5). In the present work we show that in vitro phosphorylation of TMV U1 MP before or after RNP complex formation resulted in their conversion into a translatable form infectious to protoplasts and plants.

Results. In the first series of experiments, we examined the ability of protein kinase C (PKC) to phosphorylate preparations of bacterially produced TMV MP in parallel experiments with cell wall-associated (CW) kinase(s) of two plant species, Nicotiana tabacum and Brassica napus. For phosphorylation experiments, P30 was incubated with the PKC- or CW-enriched fraction from mature tobacco leaves in the presence of [γ-32P]ATP and subjected to PAGE followed by staining with Coomassie blue and autoradiography. Figure 1B shows that the PKC- and the CW-enriched fractions from N. tabacum and B. napus are able to phosphorylate P30 preparations. It is noteworthy that incubation of the CW fraction of N. tabacum with [γ-32P]ATP without MP resulted in the production of a discrete band (Fig. 1B, lane 2) of about 30 kDa that was different from phosphorylated P30 (MPP) (Fig. 1B, lane 4). This band corresponds to a cellular phosphorylated CW-associated protein with higher electrophoretic mobility than TMV MP. Such a protein was not revealed when the B. napus CW fraction was incubated with [γ-32P]ATP (Fig. 1B, lanes 3 and 6). This observation allowed us to distinguish with confidence the MPP from phosphorylated cellular protein (cf. lanes 2, 4, and 6 in Fig. 1B). No additional phosphorylation could be detected when P30 preparations phosphorylated at the first step with unlabeled (‘cold’) ATP by a CW-associated kinase(s) were incubated thereafter with [γ-32P]ATP and PKC (Fig. 1D, lane 4). Similarly, P30 pro-
tein phosphorylated by PKC could not be phosphorylated by a CW-associated activity upon incubation with labeled ATP (Fig. 1D, lane 6). Consequently, the specificities of TMV P30 phosphorylation by a CW-associated PK activity and by PKC were similar.

To examine the role of MP phosphorylation in its translation inhibiting activity, preparations of (His)$_6$-MP of TMV U1 were phosphorylated in vitro by a PKC- or by a CW-enriched fraction of mature tobacco leaves; in the latter case the MPP was reisolated from the incubation mixture by metal affinity chromatography. MPP preparations then were examined for the ability to inhibit TMV RNA translation in the two types of experiments: (i) the MP and TMV RNA were added separately to the cell-free translation system and (ii) incomplete MP–RNA complexes preformed at subsaturating concentrations of the MP were added after MP and TMV RNA preincubation. Nonphosphorylated P30 was used as a control.

Figure 2 shows that in contrast to nonphosphorylated MP (lanes 3 and 4), no inhibition of TMV RNA translation was caused by TMV MPP preparations at a MPP:RNA ratio as high as 300:1 (lanes 5 and 6). Analogous results were obtained when MP phosphorylated by the CW-enriched fraction of tobacco leaves was incubated with TMV RNA before translation (data not shown). It should be emphasized that the translation-inhibiting activity of MP was abolished not only when it was phosphorylated before RNP formation but also when the protein was phosphorylated within MP–TMV RNA complexes preformed before incubation with PKC (lane 7). Analogous results were obtained in rabbit reticulocyte lysates (RRL) (Fig. 1) and wheat germ extracts (WGE) (data not shown).
Formation of complexes between TMV RNA and MPP was tested by (i) double-filter nitrocellulose filter binding (NFB) assays and (ii) gel retardation electrophoresis. In a control experiment with nonphosphorylated MP, all the RNA was retained on the nitrocellulose membrane at a molar MP:RNA ratio of 100:1 (see also Ref. 9). The NFB assays showed that about 75% of the RNA was bound to MPP at a ratio of 100:1; however, virtually all RNA (98.5%) was bound to MPP at a ratio of 150–200:1. When the binding of MP and MPP to TMV RNA was assayed by gel retardation electrophoresis (Fig. 3), all the RNA was completely retarded after incubation of TMV MP with viral RNA at a molar ratio of 100:1 (lane 1). Figure 3 also shows that the ability of TMV MP to retard TMV RNA was somewhat reduced by phosphorylation. Only part of the TMV RNA was retarded at MPP:TMV RNA molar ratios of 100:1 and 150:1 (lanes 3 and 4), although all the RNA could be retarded at ratios between 200:1 and 300:1 and higher (lanes 5–6). These results are in line with those obtained by NFB assays. It is worthy of notice that the MPP–RNA complexes formed at a ratio as high as 300:1 were translatable in vitro (Fig. 2, lanes 5 and 6).

To characterize the salt stability of MP–RNA and MPP–RNA complexes, the 32P-labeled RNA transcripts were incubated with unmodified and phosphorylated P30 in buffers with different NaCl concentrations, and incubation mixtures were analyzed by NFB assays. No significant difference in salt stability was revealed between the two types of RNP complexes: the MP and MPP bind RNA maximally between 50 and 100 mM NaCl. At 400 mM NaCl, only 20% of the RNA was bound to MP and MPP and at 800 mM NaCl, neither of the two proteins could bind RNA. Furthermore, the TMV RNA was completely unprotected from RNase attack in MPP–RNA complexes. No RNA was revealed by agarose electrophoresis after RNase treatment of MPP–RNA, whereas a heterogeneous RNA isolated from RNase-treated MP–RNA complexes produced a diffuse band of about 500 nucleotides (data not shown). The infectivities of complexes formed by TMV RNA with unmodified MP and MP phosphorylated in vitro by PKC were compared (Table 1). In agreement with (9), no TMV accumulated in protoplasts inoculated with the MP–TMV RNA complexes. By contrast, MPP–TMV RNA complexes were infectious even at a MPP:RNA ratio of 300:1. These results indicate that phosphorylation of TMV MP causes a conversion of bound RNA that was nontranslatable and noninfectious to protoplasts into a form that was translatable in vitro and infectious to protoplasts. It is noteworthy that both types of complexes readily infected intact Nicotiana glutinosa plants (see also Ref. 9).

**Discussion.** The 30-kDa MP of TMV is phosphorylated when expressed in insect cells (1) and in TMV-infected protoplasts (13). A plant CW-associated PK activity has been shown to specifically phosphorylate the residues near the C-termini of TMV MP in vitro (5). Haley et al. (7) have revealed multiple serine phosphorylation sites on the 30-kDa MP produced in TMV-infected protoplasts. It is possible that more than one PK is involved in TMV MP phosphorylation. More recently (12), the phloem-specific 17-kDa MP of potato leafroll virus was shown to be phosphorylated in vitro by potato membrane-associated PK (related to animal serine/threonine PKC). The effect of TMV MP phosphorylation on the biological activity is obscure. One suggestion is that it serves as a mecha-

**FIG. 2.** Phosphorylation of TMV MP abolishes its ability to inhibit RNA translation in RRL. (Lane 1) no RNA added; (lane 2) TMV RNA; (lane 3) nonphosphorylated MP and TMV RNA at a molar ratio of 100:1 were added separately to RRL; (lane 4) nonphosphorylated MP was incubated with RNA at a molar ratio of 100:1 before addition to RRL; (lane 5) PKC-phosphorylated 30-kDa MP and TMV RNA were added at a molar ratio of 300:1 separately to RRL; (lane 6) MP phosphorylated by PKC was incubated with TMV RNA at a molar ratio of 300:1 to preform the MPP–RNA complexes before their addition to RRL; (lane 7) MP–RNA complexes preformed at a molar ratio of 100:1 were incubated with PKC before translation; (lane 8) TMV RNA and PKC were added to RRL without 30-kDa MP. Concentration of TMV RNA in RRL was 75 μg/ml; molecular weight markers are shown on the right. The predominant band and the faint top band correspond to the 126- and 183-kDa TMV-coded proteins, respectively.

**FIG. 3.** Retardation gel electrophoresis assay for TMV RNA binding by MP and MPP. (Lane 1) MP and TMV RNA preincubated at a molar ratio of 100:1; (lane 2) free TMV RNA; MP and TMV RNA preincubated at molar ratios of 100:1 (lane 3), 150:1 (lane 4), 200:1 (lane 6), and 300:1 (lane 6). Each lane was loaded with 4 μg RNA taken as free RNA (lane 2) or after preincubation with the protein.
MP–TMV RNA complexes form translational repressors of exogeneously added RNAs: The TMV RNA was 8×(His)6-tagged tobamovirus MPs act as nonspecific translocated through PD (5) to form extended RNP complexes that should be translocated in tobacco plants (5). We recently reported that fusion (His)6-tagged tobamovirus MPs act as nonspecific transcriptional repressors of exogenously added RNAs: the MP–TMV RNA complexes formed in vitro were nontranslatable and noninfectious to isolated protoplasts; however, they were infectious to intact plants. It was reasonable to suggest that a certain cellular mechanism operating in PD is responsible for conversion of nontranslatable RNP into a translatable form in the course of their translocation through PD. In line with this assumption we found that phosphorylation of TMV MP by tobacco CW-associated PK or by animal PKC abolished the ability of MP to inhibit TMV RNA translation in RRL (Fig. 2) and WGE (not shown). The MPP–TMV RNA complexes containing phosphorylated 30-kDa MP were translatable and infectious to protoplasts (Table 1) and plants. Phosphorylation of P30 resulted only in moderate reduction of RNA-binding ability of MPP (Fig. 3, and the results of NFB assays). There were no significant differences in the levels of salt stability between the complexes formed by MP and MPP with TMV RNA; i.e., the RNA-binding affinities of MP and MPP were similar.

However, TMV RNA was completely unprotected from RNase attack in MPP–RNA and was partially protected in MP–RNA complexes. The inability of MPP to repress the translation and high level of TMV RNA sensitivity to RNase in MPP–RNA complexes may be attributed to a conformational change in MPP molecules altering the RNA-binding affinities of MP and MPP were similar.

The PK(s) associated with CW and/or cytoplasmic PK(s) may phosphorylate the TMV MP molecules that constitute the MP–RNA complexes targeted to PD. The functional significance of phosphorylation for MP activity is not clear. Phosphorylation of MPP molecules may occur before or after a putative MP–RNA complex formation. In any case, phosphorylation of MP molecules can be regarded as a mechanism enabling the MP–RNA complexes to be translation competent, irrespective of whether the phosphorylation occurs before or after these complexes form.

Materials and Methods. Expression in Escherichia coli and purification of TMV U1 (His)6-MP were carried out as described in (8). Preparation of plant CW-enriched fractions was performed by the procedure of (5). In vitro phosphorylation of bacterially expressed MP was done in the reaction mixture containing 20 μl of purified 30-kDa MP (0.2 μg/μl); 7 μl of 5× activation buffer (Promega) (1.6 mg/ml phosphatidylserine, 0.16 mg/ml diacetyl-glycerol, 100 mM Tris–HCl, pH 7.5, 50 mM MgCl2); 7 μl of 5× coactivation buffer (Promega) (1.25 mM EGTA, 2 mM CaCl2, 0.5 mg/ml BSA); 0.5 μl of [γ-32P]ATP (5000 Ci/ml, 400 MBq/ml); and 0.5 μl of PKC (Promega). The reaction was incubated at room temperature for 15 min and then it was terminated by adding 15 μl of 3× Laemmli sample buffer, which contained 8 M urea. The phosphoproteins were further resolved by SDS–PAGE, gels were stained with Coomassie brilliant blue R-250, and radiolabeled proteins were visualized by autoradiography. Alternatively, CW-enriched fractions (~50–100 μg of protein in 25 μl of buffer, as described in (5)) were added to the reaction mixture instead of PKC. Following incubation, hydrophobic cell walls proteins were removed from the reaction mixture by centrifugation (10 min, 20000g at 25°C) and Laemmli sample buffer was added to the supernatant as described above. If necessary, the reaction was carried out in the presence of cold ATP (final concentration 1 mM). To determine the RNase sensitivity of TMV RNA in MP–RNA complexes TMV RNA and MP were preincubated in 10 mM Tris–HCl, pH 7.0, on ice for 40–60 min at different molar ratios: MP:RNA at the ratio of 100:1 and MPP:RNA at the ratio of 300:1. Then the complexes were incubated for 25 min at room temperature with 0.5 μg of RNase A or 3.5 units of RNase T1 per 1 μg of RNA. RNA was isolated with phenol and analyzed including cell-free translation in RRL and WGE, in vitro transcription, double-filter nitrocellulose filter binding assays, protoplast isolation and electroporation, and plant

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<tr>
<th>Inoculum</th>
<th>Molar MP:RNA ratio upon preincubation</th>
<th>Amount of TMV accumulated in mesophyll protoplasts (ng/5×10^6 protoplasts)</th>
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<tbody>
<tr>
<td>TMV RNA</td>
<td>730</td>
<td>Experiment 1: 730</td>
</tr>
<tr>
<td>TMV MP–RNA complex</td>
<td>1200</td>
<td>Experiment 2: 1200</td>
</tr>
<tr>
<td>MPP–RNA complex</td>
<td>650</td>
<td>Experiment 3: 650</td>
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* Protoplasts were electroporated with TMV RNA or TMV RNA preincubated with unmodified MP and MP phosphorylated by PKC. The amount of TMV RNA was 8 μg in 100 μl of inoculum. Concentration of TMV was determined by DAS-ELISA.
inoculation with TMV U1 RNA or RNP complexes were carried out as described previously (9).

For retardation gel electrophoresis, MP–TMV RNA complexes were subjected to electrophoresis in 1% agarose gels.

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REFERENCES