Binding of monoclonal antibodies to the movement protein (MP) of Tobacco mosaic virus: influence of subcellular MP localization and phosphorylation

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Monoclonal antibodies (mAbs) to recombinant movement protein (MPREC) of Tobacco mosaic virus (TMV) were used to reveal the dependence of MP epitope accessibility to mAbs on subcellular MP localization and post-translational MP phosphorylation. Leaves of Nicotiana benthamiana or N. tabacum were inoculated mechanically with TMV or agroinjected with an MP expression vector. At different time post-inoculation, ER membrane- and cell wall-enriched fractions (ER-MP and CW-MP, respectively) were isolated and analysed. The N-terminal region (residues 1–30) as well as regions 186–222 and 223–257 of MP from the CW and ER fractions were accessible for interaction with mAbs. By contrast, the MP regions including residues 76–89 and 98–129 were not accessible. The C-terminal TMV MP region (residues 258–268) was inaccessible to mAbs not only in CW-MP, but also in ER-MP fractions. Evidence is presented that phosphorylation of the majority of TMV MP C-terminal sites occurred on ER membranes at an early stage of virus infection, i.e. not after, but before reaching the cell wall. C-terminal phosphorylation of purified MPREC abolished recognition of C-proximal residues 258–268 by specific mAbs, which could be restored by MP dephosphorylation. Likewise, accessibility to mAbs of the C-terminal MP epitope in ER-MP and CW-MP leaf fractions was restored by dephosphorylation. Substitution of three or four C-terminal Ser/Thr residues with non-phosphorylatable Ala also resulted in abolition of interaction of mAbs with MP.

INTRODUCTION

The movement protein (MP) of Tobacco mosaic virus (TMV) facilitates movement of the virus genome within and between cells (for reviews, see Citovsky, 1999; Heinlein & Epel, 2004; Lucas, 2006). Polyfunctional TMV MP plays a key role in virus infection. It increases the size exclusion limit of plasmodesmata (PD) (Tomenius et al., 1987; Deom et al., 1987; Wolf et al., 1989), serves as an anchor of the viral genomic RNA to the ER (Mas & Beachy, 1999) and plays an important role in intracellular transport of replication complexes (Heinlein et al., 1998; Reichel & Beachy, 1998; Kawakami et al., 2004). Unlike other TMV proteins, the MP is produced transiently in the early stages of virus infection (Watanabe et al., 1984; Lehto et al., 1990). Within the infected cell, MP is synthesized at the leading edge and degraded at the trailing edge of the infection site (Padgett et al., 1996). Polyubiquitination of MP and subsequent degradation by the 26S proteasome pathway occurs after the early stages of infection (Reichel & Beachy, 2000). However, the mechanisms responsible for the regulation of transient MP expression are not clear.

Post-translational modifications, in particular phosphorylation/dephosphorylation, are important in regulation of gene expression and protein activity. It is known that, during virus infection, TMV MP undergoes phosphorylation by a cellular protein kinase(s). It has been shown that multiple phosphorylation events in internal regions of TMV MP occur in TMV-infected protoplasts (Haley et al., 1995). On the other hand, only three C-terminal residues of TMV MP, Ser258, Thr261 and Ser265, were phosphorylated in vivo in PD of MP-transgenic plants (Waigmann et al., 2000), and a cell-wall-associated protein kinase(s) was capable of phosphorylation of TMV MP in vitro at the same three sites (Citovsky et al., 1993). However, the subcellular compartments of MP phosphorylation as well as the sites and functional roles of TMV MP phosphorylation remain obscure.

It has been shown that the C-terminal region of TMV MP is not essential for virus spread in Nicotiana tabacum. Deletion of 33 amino acids from the C terminus of the MP did not affect cell-to-cell movement (Gafny et al., 1992). However, the ability of MP to gate PD was abolished when
the C-terminal phosphorylation sites were mimicked by negatively charged amino acid substitutions. The spread of an appropriate TMV mutant was reduced in tobacco plants (Waigmann et al., 2000). Non-phosphorylatable mutants (del 43, sb3A) were movement-competent in N. tabacum and N. benthamiana (Waigmann et al., 2000; Trutnyeva et al., 2005). Therefore, C-terminal phosphorylation was suggested to represent a host-dependent regulatory mechanism for inactivating the MP gating function in order to minimize detrimental effects of MP on host metabolism (Citovsky et al., 1993; Waigmann et al., 2000).

It cannot be ruled out that phosphorylation of MP may control certain steps of the virus life cycle, acting as a molecular device to regulate virus spread and replication/translation events (Lee & Lucas, 2001; Rhee et al., 1990). Furthermore, it has been shown that TMV MP binds RNA in vitro, resulting in the formation of unfolded and elongated RNA–protein complexes (vRNP) (Citovsky et al., 1990, 1992; Kiselyova et al., 2001). It was speculated that these complexes might represent a particular pool of viral RNA molecules that were destined for translocation to neighboring cells and excluded from the process of replication/translation (Citovsky et al., 1990, 1992). RNP formed in vitro from TMV RNA and recombinant baculovirally produced TMV MP (MPREC) was shown to be untranslatable in vitro and in isolated protoplasts, but not in planta, suggesting that the vRNP underwent modification upon passage through PD (Karpova et al., 1997). Phosphorylation of TMV MP by a protein kinase C (PKC) or a cell-wall-associated protein kinase(s) removed the translation-inhibiting effect of MP and converted RNA of vRNP into a translatable form (Karpova et al., 1999).

In TMV-infected plants, the MP accumulates in PD (Oparka et al., 1997), transiently associates with the ER membrane (Heinlein et al., 1998; Reichel & Beachy, 1998) and colocalizes with the cytoskeleton (Heinlein et al., 1995; McLean et al., 1995), suggesting roles of these components in intracellular movement of MP and the viral RNA genome. Several studies (Wright et al., 2007; Guenoune-Gelbart et al., 2008; Epel, 2009) support a model in which targeting of MP to PD during infection is mediated by the actin/ER network in the absence of an intact microtubule (MT) cytoskeleton. Some reports suggest that the MT cytoskeleton is involved in the degradation pathway of MP (Gillespie et al., 2002; Mas & Beachy, 1999; Reichel & Beachy, 1998). On the other hand, as has been reported by Ashby et al. (2006), MT-associated MP is not ubiquitinated, and the ER/actin network may act together with MT in intracellular trafficking of MP-containing RNA transport particles (Sambade et al., 2008).

The functional domains of TMV MP have been investigated by Berna et al. (1991), Gafny et al. (1992), Citovsky et al. (1992), Waigmann et al. (1994) and Kahn et al. (1998). The hypothesis that MP contains two transmembrane domains (Berna, 1995) was supported by studies using recombinant MP (Brill et al., 2000, 2004). Results of circular dichroism spectroscopy, trypsin treatment and mass spectrometry led to the proposal of a topological model in which TMV MP had two putative α-helical transmembrane domains and a protease-sensitive C terminus. Considerably less is known about the conformation of TMV MP in vivo. In particular, it is not known whether the conformation of TMV MP changes depending on its subcellular location. In this study, we reveal distinctions in accessibility to monoclonal antibodies (mAbs) of TMV MP located in different intracellular sites: the ER membrane and cell wall (presumably in PD). The influence of TMV MP C-terminal epitope phosphorylation on mAb binding was also examined.

RESULTS AND DISCUSSION

Description of the system

The mAbs used in this work were specific to different MP domains of recombinant TMV MP (Sukhacheva et al., 2005). Results of MP TMV mapping are summarized in Fig. 1. Western blotting assays were used to reveal the effect of subcellular MP localization and post-translational modification of MP by phosphorylation on accessibility of TMV MP epitopes to different mAbs.

N. benthamiana or N. tabacum leaves were inoculated mechanically with TMV or agroinjected with the MP expression vector. At different times post-inoculation, ER- and CW-enriched fractions were isolated. A series of

![Fig. 1. Schematic representation of TMV MPREc and MP regions that bind corresponding mAbs. Numbers indicate amino acids located on the borders of the corresponding regions (shaded boxes). Designations of mAbs are below the figure. Cytoplasmic, transmembrane and ER lumenal domains of the TMV MP are presented according to Brill et al. (2000). Functional domains of TMV MP are given according to Citovsky et al. (1992) and Waigmann et al. (1994). Domains A (residues 112–185) and B (residues 185–268) correspond to single-strand nucleic acid-binding regions. Domain C (residues 65–86) is required for correct protein folding. Domain D is the phosphorylated region and domain E (residues 126–224) is involved in increasing the PD size exclusion limit.](image-url)
preliminary experiments showed that the procedure used for subcellular fractionation allowed the isolation of the CW fraction without significant contamination by ER elements (Fig. 2a), and the ER fraction was not contaminated by CWs (Fig. 2b). In our experiments, BiP was used as the ER marker protein. A considerable amount of TMV MP was readily detected in the CW fraction at 5 days post-infection (p.i.) and in the ER fraction from 1 to 3 days p.i. (Fig. 2b).

Binding of mAbs to epitopes in TMV MP associated with CW and ER fractions

The ER-MP or CW-MP fractions from virus-infected leaves were incubated with mAbs to TMV MP. As an integral membrane protein (Moore et al., 1992; Reichel & Beachy, 1998), TMV MP was tightly associated with the pellet of ER- or CW-enriched fractions, and could be readily separated from unbound (free) mAbs by repeated centrifugation and resuspension. Washed ER-MP and CW-MP fractions containing TMV MP–mAb complexes were then subjected to SDS-PAGE to reveal the mAbs bound to the MP. mAbs were detected on PVDF membranes after incubation with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies. Under the denaturing conditions of SDS-PAGE, the mAbs bound to the MP were dissociated into H- and L-chains, i.e. the presence of IgG chains in Fig. 3 provided evidence for the accessibility of appropriate MP epitopes to corresponding mAbs. The results presented in Fig. 3 illustrate the accessibility to different mAbs of MP in ER-MP and CW-MP fractions from TMV-infected leaves of N. benthamiana. The same results were obtained with ER and CW fractions from TMV-infected leaves of N. tabacum and from agroinjected leaves of N. benthamiana transiently expressing the MP (not shown). The results shown in Fig. 3(a, b) indicate that the N-terminal region (residues 1–30) and regions 186–222 and 223–257 of MP were accessible for interaction with appropriate mAbs. By contrast, the MP regions including residues 76–89 and 98–129 were inaccessible to mAbs (Fig. 3a, b). These data are in agreement with the topology model of MP in ER membranes proposed by Brill et al. (2000). According to this model, the N terminus (residues 1–30) is exposed to the cytoplasm and, therefore, is accessible to mAbs. The next region of MP (residues 78–89) forms part of a predicted α-helix absorbed in the membrane and therefore should be inaccessible for interaction with mAbs. The model of Brill et al. (2000) proposes that the region 98–129 is hidden in the lumen of the ER and, therefore, may be unavailable for interaction with mAbs, whereas the region 186–268 is exposed to the cytoplasm and may interact with mAbs.

Contrary to our expectations, the C-terminal region (residues 258–268) was unable to bind the mAbs used (Fig. 3a, b). Inaccessibility of the C-terminal TMV MP region to mAbs in ER-MP and CW-MP fractions may be explained by the fact that the MP conformation was somewhat different from that predicted by the topological model of Brill et al. (2000) and/or that the inaccessibility of the C-terminal TMV MP region to mAbs could be the result of modification (presumably by phosphorylation).

Binding of mAbs to epitopes in TMV MP extracted from CW and ER fractions

In the next series of experiments, MP was extracted from ER membranes and CW fractions and examined by immunoblotting with mAbs. As expected, mAbs 3B10 and 7C7 specific for residues 98–129 readily interacted with MP isolated from cell structures (Fig. 4). Previously, mAbs 8D12 and 7D9 were shown to be specific for the C-terminal region of TMV MP (Fig. 1), whereas mAbs 8D12 and 7D9 did not recognize a C-terminal MP deletion mutant (residues 257–268 deleted) (Sukhacheva et al., 2005). However, the C-terminal region of MP extracted from ER and CW fractions from TMV-infected leaves of N. benthamiana was not accessible to mAb 7D9 (Fig. 4a, b), and the reaction with mAb 8D12 was very poor (the relative efficiency of these mAb–MP reactions, calculated using the Scion Image program, was no more than 7 % of
the reaction of the region of residues 223–257 with mAb 1A10 or the region of residues 1–30 with mAb 4A3 as a control). It could be suggested that the C-terminal epitope of TMV MP in the ER and CW fractions was considerably protected from mAb binding. It is possible that this effect was due to phosphorylation of the C-terminal epitope. Likewise, epitopes including residues 76–89 (the phosphorylatable MP region; Haley et al., 1995) were unable to react with the corresponding mAbs (Fig. 4a, b).

Since the negative effect of mimicking phosphorylation on MP function was host-dependent, occurring in N. tabacum but not in N. benthamiana (Waigmann et al., 2000), we examined the accessibility to mAbs of MP from ER and CW fractions of TMV-infected N. tabacum ‘Samsun’. Fig. 5 shows that, similarly to N. benthamiana, neither epitope (residues 258–268 and 76–89) of MP isolated from ER-MP and CW-MP fractions of N. tabacum plants reacted with mAbs 1D8 and 7D9.

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**Fig. 3.** Accessibility of TMV MP epitopes to different mAbs in the ER-MP and CW-MP fractions isolated from TMV-infected N. benthamiana at 2 days p.i. mAbs bound to the MP in either of the two fractions dissociated into H- and L-chains upon SDS-PAGE. mAbs specific to the corresponding region of MP are shown above the gel. The positions of the IgG chains and MP are indicated by arrows. (a) CW-MP fraction from TMV-infected leaves; (b) ER-MP fraction from TMV-infected leaves. (c) MP used as a loading control.

**Fig. 4.** Immunoblot analyses of TMV MP extracted under denaturing PAGE from the ER-MP and CW-MP fractions of TMV-infected N. benthamiana plants at 2 days p.i. mAbs specific to the corresponding region of TMV MP are shown above the gel. The position of MP is indicated by arrows. (a) TMV MP isolated from the ER-MP fraction of TMV-infected leaves; (b) TMV MP isolated from CW-MP fraction of TMV-infected leaves. Lanes: 1, TMV MPREC; 2, MP isolated from ER fraction; 3, MP isolated from CW fraction of infected leaves.
**Factors affecting binding of mAbs to TMV MP**

**Influence of C-terminal substitutions and phosphorylation/dephosphorylation on TMV MP interactions with antibodies**

**Experiments with isolated MP preparations.** We analysed the effect of C-terminal point mutations and phosphorylation/dephosphorylation on the efficiency of MPREC interaction with antibodies. Fig. 6(a) shows that substitution of three C-proximal Ser/Thr residues (Ser258, Thr261 and Ser265; sb3A) led to very strong, but not complete, inhibition of the interaction of mAb 7D9 with mutant MP. Substitution of all four of the C-proximal Ser/Thr residues (Ser258, Thr261 and Ser265; sb3A) led to very strong, but not complete, inhibition of the interaction of both proteins with mAbs (Fig. 6a). Phosphorylation of MPREC and sb3A sharply reduced, but did not abolish, their reaction with polyclonal Abs (Fig. 6b), indicating that some epitopes outside the C-terminal region were reacting with polyclonal Abs. However, subsequent MPREC(P) dephosphorylation by bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIAP) restored the efficiency of antibody interaction with MPREC (Fig. 6c).

**Dephosphorylation of TMV MP in ER-MP and CW-MP fractions.** To demonstrate that dephosphorylation of TMV MP in ER-MP and CW-MP fractions restored the binding capacity of C-terminal epitopes with mAbs, we incubated these fractions with BAP and then analysed them by immunoblotting. The results, presented in Fig. 6(d, e), indicate that, in both fractions (ER and CW), dephosphorylation dramatically stimulated the reaction of mAb 8D12 (specific to the MP C terminus) with TMV MP. mAb 1A10, specific for amino acids 223–257 of MP, was used as a control (Fig. 6f, g), showing that the binding efficiency of mAb 1A10 at this site did not depend on ER or CW MP dephosphorylation.

These observations provided strong evidence that, in both cell fractions, phosphorylation of C-terminal epitopes of TMV MP alone converted them into a state that was poorly recognizable by antibodies, whereas dephosphorylation of MP restored its ability to interact with mAbs.

Taken together, our data suggest that the majority of MP molecules undergo C-terminal phosphorylation at an early stage of virus infection, on ER membranes, i.e. not after, but before reaching the PD (CW).

Structural and bioinformatic studies have considered the C-terminal region of MP to be a flexible tail, well suited to a regulatory function (Brill et al., 2000; Melcher, 2000), as has been suggested for other proteins (Cornell et al., 1995). It is noteworthy that the negative regulatory effect of TMV MP mimicking phosphorylation on MP function was not observed in N. benthamiana, N. clevelandii or N. glutinosa plants, even though the protein kinase activity specific for the C-terminal sites of TMV MP was found in the CW fraction of these plants (Waigmann et al., 2000; Trutnyeva et al., 2005).

Apparently, the C-terminal region of TMV MP is not essential for virus spread in plants, but may be necessary for regulation of other processes, e.g. sequestration of excess MP from ER membranes by C-terminal phosphorylation. This assumption is supported by our data presented above and is consistent with the reports of Arce-Johnson et al. (1995) and Szécsi et al. (1999), who concluded that only a negligible amount of MP was required for efficient intracellular spread of the virus genome. In accordance with this idea, Tyulkina et al. (2006) reported that complementation of crTMVMP::GFP by a non-phosphorylatable MP mutant (sb3A) led to the production of four times more infection foci than were produced by wild-type MP.

**METHODS**

**Plasmid construction.** The binary vector expressing the TMV MP gene was described previously (Tyulkina et al., 2006). *Agrobacterium tumefaciens* strain GV3101 was transformed by this plasmid and used in agroinjection experiments.

**Antibodies.** Hexahistidine-tagged TMV MP (His-MP), its mutant forms and BiP were expressed in *Escherichia coli* XL-1 Blue using an expression vector pQE30 and purified according to the Qiagen expressionist manual (Qiagen). Polyclonal antibodies against
Fig. 6. Influence of C-terminal substitutions and phosphorylation/dephosphorylation on the ability of TMV MP to bind mAbs and polyclonal antibodies. sb3A represents Ser258→Ala, Thr261→Ala, Ser265→Ala; sb4A represents Ser258→Ala, Thr261→Ala, Ser265→Ala, Ser267→Ala. The position of MP is indicated by arrows. (a, b) Immunoblot analyses of recombinant MPREC substitution mutants sb3A and sb4A and their phosphorylated forms using mAb 7D9 (a) and polyclonal antibodies to TMV MP (b). (c) Dephosphorylation of recombinant MPREC, previously phosphorylated by PKC [MP(P)], with BAP or CIAP. (d–g) Dephosphorylation of TMV MP in CW-MP (d, f) and ER-MP (e, g) fractions from TMV-infected leaves of N. benthamiana. mAb 1A10, specific for MP residues 223-257, was used as a control (f, g).

TMV MP (MPREC) and BiBREC (BiP, a resident ER protein that we used as an ER marker) were raised in mice using His-MP as antigen and in rabbits using His-BiP as antigen. Production of mAbs to MPREC and mapping of specific MP domains using MP deletion mutants were described previously (Sukhacheva et al., 2005).

Isolation of plant tissue fractions. Young leaves of N. benthamiana or N. tabacum were infected mechanically with TMV (10 µg ml⁻¹) or agroinjected with MP expression vector (Tyulkina et al., 2006). At different times post-inoculation (from 1 to 5 days), the leaves were treated as described by Citovsky et al. (1993) with slight modifications. All operations were carried out at 4 °C. Inoculated leaves were homogenized in 3 vols (w/v) H buffer (100 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM EDTA, 5 mM DTT, 25 mM sucrose). In some experiments, a mixture of phosphatase inhibitors was used (5 mM NaF, 100 mM Na3VO4 and 100 nM okadaic acid). The homogenate was centrifuged at 5000 × g for 5 min to generate two fractions: the cell wall (CW) and ER-containing (P-30) fractions. The pellet was suspended in an initial volume of H buffer and washed (with subsequent centrifugations) three times for 60, 40 and 20 min with 1% Triton X-100 and twice without 1% Triton X-100. The final, grey pellet was used as the CW fraction. The initial supernatant was centrifuged at 10,000 × g for 10 min and then at 30,000 × g for 30 min to generate the supernatant fraction (S-30) and the membrane-containing pellet (P-30). The P-30 pellet was used as the ER fraction. The pellets of the CW and ER fractions were heated in SDS-PAGE sample buffer at 95 °C for extraction. After centrifugation at 10,000 × g for 5 min, the supernatants were separated by SDS-PAGE and subjected to immunoblotting.

Immunoblot analysis. Cell fractions were resolved on 12.5% SDS-PAGE gels (Laemmli, 1970) and proteins were then transferred to PVDF membranes (Hybond-P; Amersham). After transfer, membranes were probed with a 10,000-fold dilution of primary antibodies (polyclonal mouse anti-MP antibodies or polyclonal rabbit anti-BiP antibodies or mAbs) and a horseradish peroxidase conjugate as the secondary antibody (Sigma). Bound antibodies were detected using ECL Western blotting detection reagents (Amersham). After immunoblotting, membranes were stained with Amido black solution for evaluation of the amount of total protein loaded in each lane (loading control).

Accessibility of TMV MP epitopes to mAbs in fractions from infected leaves. Pellets of the CW and ER fractions were resuspended in TBS-T buffer, divided into aliquots and probed with different mAbs under standard conditions for Western immunoblotting (Sambrook et al., 1989). After repeated washings, the complexes were heated in SDS-PAGE sample buffer at 95 °C for extraction. The samples were then centrifuged at 10,000 × g for 5 min and soluble proteins were subjected to SDS-PAGE and blotted onto PVDF membranes. The blots were developed using a horseradish peroxidase-conjugated secondary antibody (Sigma).

Agrobacterium-mediated transient expression of MP gene. Agrobacterium-mediated transient expression assays were performed as described by Scofield et al. (1996). A. tumefaciens strain GV3101 bearing the expression plasmids was grown to stationary phase at 28 °C in LB medium supplemented with 50 µg rifampicin ml⁻¹, 25 µg gentamicin ml⁻¹ and plasmid-specific antibiotic (Sambrook et al., 1989). After centrifugation, the cells were resuspended in infiltration buffer (MES, pH 5.6, 10 mM MgSO4) to an OD600 of 0.4. The suspension was pressure-infiltrated into leaves of 4-week-old N. benthamiana plants using a syringe, soaking several square centimetres of leaf mesophyll tissue.

Phosphorylation and dephosphorylation of MP in vitro. Phosphorylation of bacterially expressed MPREC and its substitution mutants was done in a 10 µl reaction mixture containing 0.4 µg purified protein, 300 µg phosphatidylserine ml⁻¹, 30 µg diacylglycerol ml⁻¹, 20 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 250 µM EGTA, 400 µM CaCl₂, 100 µg BSA ml⁻¹, 3 mM ATP and 1 µl PKC (Promega). After incubation at room temperature for 30 min,
reactions were terminated by adding SDS-PAGE sample buffer with urea and heated at 95 °C. Phosphoproteins were resolved by SDS-PAGE and subjected to immunoblotting.

For MP dephosphorylation, pellets of the CW or ER fractions from N. benthamiana leaves infected with TMV were resuspended in 10 mM Tris/HCl, pH 8.0, 10 mM MgCl₂. Aliquots from both fractions were treated with BAP (Promega) or CIAP (Promega) at final concentrations of 0.1 U µl⁻¹. After 60 min at 37 °C, reactions were terminated by heating at 95 °C and the products were subjected to immunoblotting with appropriate mAbs or polyclonal Abs. Dephosphorylation of bacterially expressed MP, previously phosphorylated by PKC, was done in the same way.

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