Phosphorylation Down-regulates the RNA Binding Function of the Coat Protein of Potato Virus A*

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Plant viruses encode movement proteins (MPs) to facilitate transport of their genomes from infected into neighboring healthy cells through plasmodesmata. Growing evidence suggests that specific phosphorylation events can regulate MP functions. The coat protein (CP) of potato virus A (PVA; genus Potyvirus) is a multifunctional protein involved both in virion assembly and virus movement. Labeling of PVA-infected tobacco leaves with $^{32}$Porthophosphate demonstrated that PVA CP is phosphorylated in vitro. Competition assays established that PVA CP and the well characterized 30-kDa MP of tobacco mosaic virus (genus Tobamovirus) are phosphorylated in vitro by the same Ser/Thr kinase activity from tobacco leaves. This activity exhibits a strong preference for Mn$^{2+}$ over Mg$^{2+}$, can be inhibited by micromolar concentrations of Zn$^{2+}$ and Cd$^{2+}$, and is not Ca$^{2+}$-dependent. Tryptic phosphopeptide mapping revealed that PVA CP was phosphorylated by this protein kinase activity on multiple sites. In contrast, PVA CP was not phosphorylated when packaged into viroids, suggesting that the phosphorylation sites are located within the RNA binding domain and not exposed on the surface of the virion. Furthermore, two independent experimental approaches demonstrated that the RNA binding function of PVA CP is strongly inhibited by phosphorylation. From these findings, we suggest that protein phosphorylation represents a possible mechanism regulating formation and/or stability of viral ribonucleoproteins in planta.

A delicate balance between protein phosphorylation and dephosphorylation regulates the function of a vast variety of proteins in the cell. Recently, several lines of evidence have suggested that phosphorylation of plant virus-encoded movement proteins (MPs) by host plant protein kinases may be involved in the process of virus movement (1–3). The functional role of MPs is to assist the spread of viral progeny from cell to cell and over long distances (reviewed in Refs. 4–7). There is evidence that the 30-kDa MP of tobacco mosaic virus (TMV; genus Tobamovirus) is phosphorylated when expressed in insect cells from a baculovirus vector (8), in TMV-infected protoplasts (9, 10), and in the cell wall-enriched fractions of transgenic plants expressing the wild-type MP and its mutants (3, 11). The 17-kDa MP of potato leafroll virus (genus Luteovirus) was shown to be phosphorylated in a reconstituted system containing bacterially expressed protein and membrane preparations from potato leaves (12). In another report, phosphorylation of the 69-kDa MP of turnip yellow mosaic virus (genus Tymovirus) was demonstrated when the MP gene was expressed in insect cells using a baculovirus vector (13).

It is not yet clear whether MP phosphorylation is essential for the general process of virus movement; however, there is growing evidence suggesting that phosphorylation can affect several MP functions. Originally, it was proposed that phosphorylation represents a mechanism for MP inactivation and sequestration in the cell walls of mature plants (11). Polyolysis processing was found to be an alternative mechanism to phosphorylation for inactivation of TMV MP in Arabidopsis thaliana (14). Recently, new evidence has accumulated that suggests that phosphorylation of TMV MP may directly affect its function. Either phosphorylation or the presence of serine 37 in MP of tomato mosaic virus (genus Tobamovirus) was shown to be essential for the protein intracellular localization and stability and, therefore, required for the efficient spread of the virus (1). These results indicated that phosphorylation of MPs by cellular protein kinase(s) may represent an active process required by the plant viruses to execute their movement function. Second line of evidence in support of the possible involvement of MP phosphorylation in the cell-to-cell movement came from in vitro studies showing that the phosphorylation of TMV MP abolishes its ability to repress RNA translation (2). This finding suggested a possible mechanism for how MP phosphorylation may regulate the function of movement ribonucleoprotein intermediates in the course of their cell-to-cell translocation. According to this hypothesis, MP phosphorylation converts the translation-incompetent movement intermediates into the translation-ready state, thus allowing the virus to replicate in the newly infected cell. In another recent study (3), TMV MP mutant mimicking phosphorylation was reported to be deficient in plasmodesmal transport, suggesting that phosphorylation is involved in down-regulation of the MP activity.

Although much is already known about the phosphorylation of MPs of tobamoviruses, data on the phosphorylation of potyvirus proteins involved in movement are completely missing. In contrast to tobamoviruses, potyviruses do not encode a particular dedicated movement protein, using instead several polyfunctional proteins to execute their movement function. These proteins, designated movement-related proteins (MRPs), include the coat protein (CP), the helper component protease (HC-Pro), the cylindrical inclusion protein (CIP), and the ge-

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The abbreviations used are: MP, movement protein; TMV, tobacco mosaic virus; MRP, movement-related protein; CP, coat protein; HC-Pro, helper component protease; CIP, cylindrical inclusion protein; PVA, potato virus A; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; SAP, shrimp alkaline phosphatase; PPTase-2A, protein phosphatase-2A; DMC, divalent metal cation.
Phosphorylation of the Coat Protein of Potato Virus A

Alkaline phosphatase-coupled anti-rabbit IgG (diluted 1:5000) was used to reveal the presence of the primary antibodies. Prior to RNA binding or immunoblotting, amounts of protein were normalized by comparing band intensity on Amido Black- or Ponceau S-stained membranes. Radioactively labeled proteins or RNA-protein complexes were visualized by autoradiography on Kodak Co. BMR film or quantified by using a phosphor imager (Fuji) and Tina 2.0k software (Raytest).

In Vivo Phosphorylation—Source leaves of PVA-infected or mock-infected tobacco plants were cut in disks (1 cm in diameter) and incubated in 25 mM HEPES, pH 7.4, containing 1 mCi (0.5 mCi/mmol) of [γ-32P]ATP (3000 Ci/mmol) in the presence or absence of 1 μM staurosporine (Sigma). Vacuum was applied until the leaf discs darkened and the mixture was incubated overnight at 22 °C. Following removal of the incubation solution, the leaf discs were thoroughly rinsed, dried on filter paper, and cut into smaller pieces. The resulting leaf fragments were homogenized in NET buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Nonidet P-40 [ Sigma], 0.02% NaN3, 100 units mL-1 of Trasylol [Bayer]) containing 1% SDS and immediately boiled for 15 min. The lysates were cleared by centrifugation and diluted (1:10) with NET buffer for immunoprecipitation. Presoaked protein A-Sepharose (Amersham Pharmacia Biotech) was added at 1% (w/v) to the diluted lysates and incubated for 1.5 h at 4 °C to remove Sepharose-binding proteins. After centrifugation at 3200 × g for 10 min in a cold centrifuge, the supernatant was applied to the immobilized protein A-Sepharose, washed five times with NET buffer, and the Sepharose-bound proteins were resolved by SDS-PAGE and electrotransferred to Immobilon-P membranes. The membranes were probed with mouse anti-PVA IgG (Bioreba) as described above and autoradiographed.

In Vitro Kinase Assays—Phosphorylation was measured as the incorporation of radioactivity from [γ-32P]ATP into the purified substrate proteins. Redivue [γ-32P]ATP ([γ-32P]ATP; ≥2500 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Assays were performed at room temperature for 30 min with occasional swirling in a final volume of 15 μL containing 0.5 μL [γ-32P]ATP (~10 μCi), 1 μg of substrate protein, 25 mM HEPES, pH 7.4, in the presence or absence of divalent cations (Mg2+, Mn2+, Ca2+, Zn2+, and Cd2+ at the indicated concentrations). Unless stated, freshly prepared total plant protein extract (≥3 μg) was used as a kinase source. For Western analysis with anti-phosphotyrosine antibodies, the kinase assays were performed with 20 μL of [γ-32P]ATP together with 50 μL unlabelled ATP. To study the effect of staurosporine on protein phosphorylation, the compound was added into the kinase assays at a final concentration of 1 μM. Reactions were terminated by adding 5 μL of 5× SDS-PAGE sample buffer, followed immediately by boiling for 5 min. The phosphorylated proteins were analyzed by SDS-PAGE as described above.

Enzymatic Dephosphorylation Assay—Protein dephosphorylation was analyzed as the loss of [32P]Phosphate from labeled proteins following their separation by SDS-PAGE. Two types of enzymes were used in dephosphorylation assays: general shrimp alkaline phosphatase (SAP; 1 unit/μL; Amersham Pharmacia Biotech) and Ser/Thr-specific protein phosphatase-2A (PPTase-2A, 0.5 units/μL, Promega). Digestion of phosphoproteins with SAP was performed at 37 °C for 15 min in a final volume of 20 μL containing 1 μL of phosphorylated substrate protein, 3 units of SAP, 0.5 mM MgCl2, and 20 mM Tris-HCl, pH 8.0. Protein dephosphorylation with PPTase-2A was carried out by incubation of the 20-μL sample containing 1 μg of phosphorylated substrate protein, 0.5 units of PPTase-2A, 1 mM MnCl2, 1 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.5, for 20 min at 37 °C. Control reactions in the same buffer with 1 μM okadaic acid (Calbiochem) were performed. The dephosphorylation reactions were terminated by adding 5 μL of 5× SDS-PAGE sample buffer followed immediately by boiling for 5 min.

Tryptic Phosphopeptide Mapping—Tryptic phosphopeptide mapping was carried out as described in Ref. 21.

RNA-Protein Blotting—The RNA-protein binding assays were performed according to the method described in Ref. 22 with some modifications. Total amounts (100 μg) of RNA target proteins purified in vitro in the presence of 500 μL unlabeled ATP and 10 mM Mn2+ as described above. The phosphoproteins were separated from unincorporated ATP by SDS-PAGE and transferred to Immobilon-P membranes. The membranes were blocked for 1 h in RNA binding buffer (20 mM HEPES, 6 mM Tris-HCl, pH 7.0, 25 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol) containing 5% (w/v) nonfat milk powder. After
three washes with RNA binding buffer, SDS was removed from the blotted proteins by guanidine HCl extraction. For this purpose, membranes were incubated for 10 min in RNA binding buffer with 6 M guanidine HCl. The proteins were further renatured by successive washes of 100, 300, or 500 mM NaCl (100, 300, or 500 mM). The sufficient number of washes were removed from the membranes by several 10-min washes with RNA binding buffer containing 0.1% Nonidet P-40 and different concentrations of NaCl (100, 300, or 500 mM). The efficient number of washes was determined by measuring the radioactivity of the discarded buffer. Finally, the membranes were dried, and the remaining radioactively labeled RNA-protein complexes were analyzed as described above.

RNA-Protein Binding Assays Using Metal Chelate Magnetic Beads—Prior to RNA-protein binding assays, *in vitro* phosphorylation of His$_6$-tagged recombinant protein was carried out in the presence of 500 μM unlabeled ATP and 10 mM Mn$^{2+}$ as described above. Phosphorylated protein was checked for degradation by SDS-PAGE, and control reactions were performed without the addition of plant protein extract. The 20-μl reaction mixtures containing 1 μg of phosphorylated or nonphosphorylated protein were incubated with Ni$^{2+}$-NTA magnetic agarose beads (Qiagen) for 30 min at room temperature with occasional swirling. The particle-protein complexes were separated from unincorporated ATP and plant extract contaminants using a PickPen magnetic particle transfer device (Bio-Nobile, Turku, Finland). The beads were washed up from the solution, transferred to fresh tubes, and washed with 150 μl of RNA binding buffer (50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 50 mM NaCl, 1 mg ml$^{-1}$ BSA, 10% glycerol (v/v)). The procedure was repeated again, and the pellet was resuspended in 50 μl of RNA binding buffer. The beads were then incubated with 5 × 10$^6$ cpm of 32P-labeled PVA 5‘-untranslated region (+) transcript for 20 min on ice with occasional gentle shaking. Two more washes with RNA binding buffer followed each time by particle transfer to fresh tubes were performed. This procedure removed the unbound RNA from the beads. Finally, the beads were resuspended in 50 μl of RNA binding buffer and the residual radiolabel was checked using a liquid scintillation counter.

Calculations—The total concentration of manganese ions required for 95% saturation of ATP in the assay (0.5 μM) was calculated using the $K_a$ value taken from Ref. 23.

RESULTS

**CP and VPg of PVA Are Phosphorylated in a Reconstituted System Containing Protein Kinases from N. tabacum**—The four MRPs of PVA (CIP, CP, HC-Pro, and VPg) and the MP of TMV were expressed in *Escherichia coli* as fusion proteins with N-terminal hexahistidine affinity tags and purified by immobilized metal affinity chromatography. The Coomassie-stained gels presented in Fig. 1 show that the purified protein preparations were free from any major contaminants. The purified PVA MRPs were further assayed for phosphorylation in a reconstituted system containing total plant protein extract and [$\gamma$-32P]ATP. The phosphorylation reaction mixtures were subjected to SDS-PAGE followed by staining with Coomassie Brilliant Blue and autoradiography. The 30-kDa MP of TMV was used as a positive control in all assays, since its phosphorylation by plant protein kinases is well characterized. Two negative controls were performed in each assay to verify that recombinant proteins do not themselves bind radiolabeled ATP and that bands on the autoradiogram do not correspond to cellular phosphoproteins. In these control experiments, either purified plant protein kinases or plant protein extracts were alone incubated in the presence of [$\gamma$-32P]ATP. As shown in Fig. 1, two MRPs of PVA, CP and VPg were found to be phosphorylated by plant protein kinases in the reconstituted system, but the phosphorylation of two other proteins, CIP and HC-Pro, was not detected. Autoradiography did not reveal any labeled protein in the negative controls, confirming that bands identified in other lanes correspond to phosphorylated recombinant proteins. As expected, TMV MP was phosphorylated in all control reactions, showing that plant extracts used in the assays contained active protein kinases (Fig. 1, lanes 1).

The VPg of PVA is covalently linked to the 5‘-end of the viral genome, whereas the CP of PVA is involved in the noncovalent
interactions with viral RNA in virions and putative movement intermediates. This apparent difference suggests distinct functions for these two proteins in the genome transport process, those of PVA CP more closely resembling the functions attributed to TMV MP. Therefore, the current study was aimed at comparison of the plant protein kinases involved in phosphorylation of PVA CP and TMV MP and evaluation of a possible effect exerted by phosphorylation on the RNA binding properties of PVA CP.

**Effect of Staurosporine on PVA CP and TMV MP Phosphorylation**—A powerful approach to dissect the functional role of protein phosphorylation is to follow the change in the activity of the target protein after specific inhibition of its phosphorylation. Therefore, our next goal was to determine an effective strategy for interfering with the phosphorylation of viral MRPs. Staurosporine, which is a potent and broad spectrum inhibitor of protein kinases, was introduced at 1 μM concentration into kinase assays containing recombinant PVA CP and TMV MP, plant protein extracts, and [γ-32P]ATP. Fig. 2A shows that staurosporine had an inhibitory effect on the phosphorylation of both studied proteins. This finding allowed us to use staurosporine in the subsequent experiments as an inhibitor of PVA CP phosphorylation.

**PVA CP Is Phosphorylated in Infected Plants**—Our next goal was to verify that PVA CP is phosphorylated in PVA-infected tobacco leaves. For this purpose, a series of experiments in which pieces of PVA-infected leaves were incubated in [32P]orthophosphate were performed. After overnight incubation, virus-infected cells were lysed and immunoprecipitated with goat anti-PVA antibodies. The resulting immunoprecipitates were analyzed on protein gel blots with mouse anti-PVA IgG, followed by autoradiography. To determine the phosphorylation status of in vivo synthesized PVA CP, the radioactively labeled spots were superimposed on the specific spots observed on protein gel blots. The 32P-labeled band with an electrophoretic mobility similar to that of PVA CP was observed in immunoprecipitates of infected plants, demonstrating that it corresponds to a phosphorylated, virus-encoded protein (Fig. 2B, lane 2). From these results, we concluded that PVA CP is phosphorylated in vivo. To further support this conclusion, we tested the effect of staurosporine on PVA CP phosphorylation in infected plants. In agreement with the results obtained in vitro, phosphorylation of PVA CP in vivo was also inhibited by 1 μM staurosporine (Fig. 2B, lane 3).

**The Plant Protein Kinase Activity That Phosphorylates PVA CP Is Ser/Thr-specific**—The substrate specificity of the plant protein kinases phosphorylating the viral MRPs was previously determined only for MPs of tocmoviruses. The phosphorylation sites within TMV MP and tomato mosaic virus MP were mapped to serine or threonine residues (1, 10, 11). To identify the specificity of the enzyme(s) involved in phosphorylation of PVA CP, we employed a two-step approach. As a first step, we analyzed phosphorylated PVA CP (designated PVA pCP) by probing protein gel blots with polyclonal phosphotyrosine-specific antibodies. Phosphorylated TMV MP (TMV pMP), known to be modified at Ser/Thr, was used in these experiments as a negative control. To verify that the blotted proteins were indeed phosphorylated, we performed protein kinase assays with radiolabeled ATP. Thus, the bands identified by immunoblotting could be compared with the phosphorylation pattern. As shown in Fig. 3A (lanes 2 and 4), the phosphotyrosine-specific antibodies did not recognize any of the bands corresponding to phosphorylated PVA CP or TMV MP detected by autoradiography. At the same time, the antibodies specifically interacted with tyrosine-phosphorylated proteins from the epidermal growth factor-stimulated A431 cell line lysate, which was used as a positive control (Fig. 3A, lane 5). Therefore, these data ruled out the possibility that the tyrosine residues of PVA CP are phosphorylated. To obtain further evidence that PVA CP is phosphorylated on Ser/Thr, we employed a second strategy based on enzymatic dephosphorylation of recombinant proteins. PVA CP was phosphorylated in vitro using radiolabeled ATP and then treated with nonspecific SAP or PPTase-2A. Nonspecific alkaline phosphatases are known to strip the bound phosphate from any phosphoester-containing compound including phosphoserine, phosphothreonine, or phosphotyrosine. On the other hand, PPTase-2A selectively hydrolyzes phosphoserine and phosphothreonine but does not remove phosphate from phosphotyrosine. By comparing the extent of radioactivity incorporated into PVA pCP before and after treatment with these two phosphatases, it was possible to estimate the phosphorylation state of target proteins. For this purpose, enzymatically dephosphorylated PVA CP was analyzed by gel electrophoresis, and incorporated label...
was visualized by autoradiography. One lane in each dephosphorylation assay contained phosphoprotein incubated with PPTase-2A in the presence of its potent inhibitor (1 μM okadaic acid). A separate assay was performed with TMV pMP, which was used as a Ser/Thr-phosphorylated positive control. Fig. 3B shows that nearly all phosphorylation within PVA CP was reversed both by SAP and by PPTase-2A, confirming that the protein is a substrate for Ser/Thr-specific protein kinases. In the control lanes, the activity of PPTase-2A was significantly inhibited by okadaic acid. Enzymatic assays with TMV pMP produced similar results, with PPTase-2A having even higher capacity to dephosphorylate this substrate than SAP. Thus, the results obtained with phosphatase assays together with those obtained by immunoblotting show that PVA CP is phosphorylated by plant protein kinase activity with Ser/Thr-substrate specificity.

The Plant Protein Kinase Activity That Phosphorylates PVA CP and TMV MP Exhibits a Preference for Mn2+ over Mg2+ and Is Not Ca2+-dependent—It is well established that protein kinases do not employ free ATP as a substrate for protein phosphorylation, using instead the noncovalent complex between ATP and divalent metal cation (phosphate-donating complex). Therefore, the presence of divalent metal cation (DMC) is an essential requirement for protein kinase activity. However, metal cations can also play another important role in the catalysis through direct or indirect (via water molecules) interaction with the enzyme and/or coordination of the reaction intermediates. Because the intracellular concentration of free DMCs is tightly regulated (24), the enzymatic activity of certain protein kinases in the cell may be controlled through dynamic changes in the local concentration of metal ions (25). One of the purposes of the present study was to examine the divalent cation requirement for the plant protein kinase activity phosphorylating viral MRPs. It was found that phosphorylation of PVA CP by the cellular protein kinase(s) from total protein extract of tobacco was stimulated by Mn2+. The dependence of kinase activity on the concentration of Mn2+ is shown in Fig. 4A. Maximal activity was achieved at about 1 mM of MnCl2, and a minor inhibition of protein phosphorylation was observed at concentrations of MnCl2 higher than 1 mM. Such a decrease in activity may be due to the nonspecific interaction between metal ion and protein that is often observed at concentrations higher than 5 mM (26), reducing the amount of free DMC in the assay. When free Mn2+ was re-
moved from the phosphorylation reaction mixture in a complex with 10 mM EDTA, the kinase activity dropped down to the control level (Fig. 4A).

To determine the cation specificity of the plant protein kinase activity, we compared levels of PVA CP and TMV MP phosphorylation at the same 10 mM concentrations of Mn$^{2+}$, Mg$^{2+}$, and Ca$^{2+}$. The results presented in Fig. 4B demonstrate that the plant protein kinase activity exhibited a clear preference for Mn$^{2+}$ over Mg$^{2+}$ with each of the substrate proteins. However, in contrast to Mn$^{2+}$ and Mg$^{2+}$, the addition of 10 mM Ca$^{2+}$ into the assays did not stimulate substrate protein phosphorylation (Fig. 4B). This is in agreement with the recent report (3) that protein kinase activity phosphorylating TMV MP requires the presence of Mg$^{2+}$ but not Ca$^{2+}$ cations.

**Micromolar Concentrations of Zn$^{2+}$ and Cd$^{2+}$ Inhibit the Mg$^{2+}$-dependent Phosphorylation of PVA CP and TMV MP**—It is well recognized that, compared with Mn$^{2+}$ and Mg$^{2+}$, Zn$^{2+}$ and Cd$^{2+}$ have remarkably different effects on the activity of several protein kinases including Csk, a soluble protein-tyrosine kinase. While Mn$^{2+}$ and Mg$^{2+}$ function as essential activators of these enzymes, Zn$^{2+}$ and Cd$^{2+}$ strongly inhibit them. In the case of Csk, Zn$^{2+}$, and Cd$^{2+}$, concentrations as low as 10 μM were found to inhibit enzymatic activity by more than 95% in solutions that already contain high concentrations of Mg$^{2+}$ (23). In the current study, we monitored phosphorylation of two substrate proteins (PVA CP and TMV MP) at increasing concentrations of zinc or cadmium (0 mM, 10 μM, 100 μM, and 1 mM) in the presence of 10 mM Mg$^{2+}$. It is important to note that since micromolar concentrations of Zn$^{2+}$ and Cd$^{2+}$ were used in these experiments, precautions were made to avoid nonspecific binding of these cations to ATP, substrate proteins, and buffer constituents. Zn$^{2+}$ and Cd$^{2+}$ were always the last components added to the assay together with the plant protein extract (kinase source), while ATP was already saturated with Mg$^{2+}$, and buffers were made with HEPES rather than Tris. Fig. 5 shows that both Zn$^{2+}$ and Cd$^{2+}$ acted as inhibitors of protein kinase activity. The addition of 10 μM Zn$^{2+}$ or Cd$^{2+}$ in the presence of 10 mM Mg$^{2+}$ already
markedly reduced phosphorylation levels of both substrate proteins: PVA CP and TMV MP. The 1000-fold difference in concentrations of zinc and cadmium (10 μM) and magnesium (10 mM) ensured that the actual concentrations of free Zn²⁺ and Cd²⁺ were not affected by complexing with ATP.

Thus, we concluded that PVA CP and TMV MP are both phosphorylated by the same type of plant protein kinase activity having a distinct mode of kinetic response to metal cations. This enzymatic activity is not Ca²⁺-dependent but shows a clear preference for Mn²⁺ over Mg²⁺ and may be inhibited by very low concentrations of Zn²⁺ and Cd²⁺. To further support this conclusion, competition assays were performed in which PVA CP and TMV MP were together incubated in a reconstituted system containing plant protein kinases and [γ-³²P]ATP.

PVA CP and TMV MP Are Competing Substrates for the Same Mn²⁺-activated Plant Protein Kinase Activity—To determine whether PVA CP and TMV MP are phosphorylated by the same enzyme, constant amounts of each protein were assayed for phosphorylation in the presence of increasing amounts of the other substrate protein. Two types of experiments were carried out. In one set of experiments, kinase assays contained a constant amount of TMV MP and increasing concentrations of PVA CP. Alternatively, phosphorylation reactions were performed with a constant amount of PVA CP and increasing concentrations of TMV MP. Following incubations with plant protein extracts in the presence of 10 μM Mn²⁺, the radiolabeled phosphoproteins were separated by gel electrophoresis, transferred to membranes, and autoradiographed. Fig. 6A shows that at higher competitor concentrations, less label was incorporated into the substrate proteins. These results strongly suggest that PVA CP and TMV MP are able to compete with each other as substrates for the same Mn²⁺-activated protein kinase. To eliminate the possibility that PVA CP and TMV MP are phosphorylated by a plant protein kinase with low specificity, we compared the ability of another PVA-encoded protein, VPg, to compete for phosphorylation with PVA CP and TMV MP. The putative movement complex of PVA is composed of CP and viral RNA, which, in turn, has VPg covalently attached to its 5'-end. Therefore, VPg is likely to colocalize with CP in the subcellular compartments during virus assembly and movement. Should there be a protein kinase having low specificity and using several proteins as substrates, it would phosphorylate not only CP but also VPg. On the contrary, the results presented in Fig. 6B show that PVA VPg could not compete as a kinase substrate with either PVA CP or TMV MP. This finding allowed us to conclude that the phosphorylation of PVA CP and TMV MP by the Mn²⁺-activated plant protein kinase activity is substrate-specific and that PVA VPg is a substrate for another kinase.

Comparison of the Phosphorylation Patterns of the PVA CP Purified from Bacteria with That of the Virion-extracted Protein—One of the key questions raised in studies employing bacterially expressed proteins is whether the polypeptide obtained from bacteria is folded similarly to the native protein synthesized in an eukaryotic system. This point is of extreme importance in the current study, since the location of specific phosphorylation sites strongly depends on the protein conformation. To minimize its effects on protein folding, we introduced the shortest possible histidine tag into the recombinant proteins. In addition, purified proteins were tested for their ability to bind RNA to verify that they preserve active conformations. However, additional evidence was required to prove that the bacterially expressed protein is correctly folded, and, therefore, has a similar phosphorylation pattern with the protein synthesized in vivo. For this purpose, PVA CP was extracted by LiCl from virus particles and phosphorylated in a
reconstituted system in parallel with the bacterially expressed protein. Tryptic peptide analysis of the resulting phosphoproteins revealed that the phosphorylation patterns of the bacterially expressed and the virion-extracted PVA CP were nearly identical. As shown in Fig. 7, five spots with similar mobilities were detected on both peptide maps, suggesting that PVA CP is phosphorylated at multiple sites. However, one extra spot (marked VI) was reproducibly observed on the peptide map of the bacterially expressed coat protein. This spot had a very low chromographic mobility, suggesting that it may correspond to a hydrophilic nucleic acid derivative. Nevertheless, we cannot completely discount it as a nucleotide contaminant and rule out the possibility that it represents a phosphopeptide. Despite this one possible contradiction, we conclude from the comparison of the peptide maps that the bacterially expressed and the in vivo synthesized proteins share the same conformation and are similarly phosphorylated by the plant protein kinase activity.

PVA CP Is Not Phosphorylated When Packaged into Virions—To determine whether virion formation has any effect on PVA CP phosphorylation, in vitro kinase assays with purified virus particles were performed. As shown in Fig. 8 (lane 2), autoradiography revealed no bands corresponding to 32P-labeled phosphorylated PVA CP. Two possible explanations for this finding are that PVA CP is already extensively phosphorylated in virions or its phosphorylation is blocked upon virus packaging. To investigate the first possibility, we extracted the coat protein from the same virion preparation and again examined its phosphorylation. For this purpose, the virus was disassembled in guanidine HCl or LiCl, and the released CP was tested in kinase assays. Fig. 5A (lanes 4 and 6) shows that both guanidine HCl-extracted and LiCl-extracted CP were effectively phosphorylated in vitro by the plant protein kinase activity. Thus, we ruled out the possibility that the absence of detectable phosphorylation of intact virions was simply the result of preliminary phosphorylation of the coat protein in infected plants. Taken together, the results presented above strongly suggest that PVA CP is not phosphorylated when packaged into virions. This fact may be explained by the existence of conformational restrictions preventing the exposure of the PVA CP phosphorylation sites upon virion assembly. One possibility is that the CP phosphorylation sites are not exposed on the surface of the virus particle because they are located within the core domain involved in interaction with the viral RNA. Multiple alignments of the amino acid sequences of the potyviral coat proteins revealed at least 10 consensus Ser/Thr residues within the highly conserved core region (27, 28). Therefore, we suggested that phosphorylation of some Ser/Thr residues may potentially affect the RNA binding properties of the coat protein. To address this possibility, we next studied the effect of phosphorylation on the RNA binding properties of PVA CP using two different experimental approaches.

Phosphorylation Down-regulates Binding of PVA CP to RNA—To begin our analyses, we implemented a multistep technique based on blotting of phosphorylated PVA CP and probing it with radioactively labeled RNA transcript (RNA-protein blotting). The procedure comprised five stages: 1) target protein was phosphorylated in the presence of Mn2+ in a reconstituted system containing plant protein kinases from total cellular extract; 2) the phosphoprotein was separated from unincorporated ATP and protein extract contaminants by SDS-PAGE and transferred to membranes; 3) SDS was removed from the blotted protein by guanidine HCl extraction; 4) immobilized protein was renatured and incubated with radio-labeled RNA transcript; 5) unbound RNA was washed from the membranes with a NaCl solution, and the remaining RNA-protein complex was visualized by autoradiography.

An important aspect of the above assay system is the complete removal of unincorporated ATP from the phosphorylation reaction mixture by gel electrophoresis prior to analysis of the target protein. Furthermore, successful detection of the effect of phosphorylation on RNA binding largely depends on the final amount of the phosphoprotein in the assay. As long as purified preparations of the plant enzymes involved in MRP phosphorylation are not available, the only sources of protein kinases for the assays are cellular extracts that contain natural protein kinase inhibitors and protein phosphatases. Therefore, the yield of phosphorylated protein in such assays may be insufficient and should be artificially increased. This can be achieved by inhibition of the protein phosphatase activity using polypeptide inhibitors such as fluoride ion or by direct stimulation of the plant protein kinase activity. We determined that a stimulation of the plant protein kinase activity by Mn2+ is the most straightforward and efficient strategy to obtain a large pool of phosphorylated MP molecules.
Phosphorylation of the Coat Protein of Potato Virus A

Fig. 8. A, PVA CP is not phosphorylated when packaged into virions. PVA CP in intact virions (lane 2) was assayed for phosphorylation in parallel with equal amounts of the coat protein extracted from the same virion preparation using guanidine HCl (Gu HCl; lane 4) or LiCl (lane 6) methods. Bacterially expressed PVA CP was used as a positive control for phosphorylation (lane 8). Control reactions without the addition of plant protein extracts (lanes 1, 3, 5, and 7) were performed. The lower panel represents a Coomassie-stained gel of CP amounts used in the experiment. B, binding of PVA CP to RNA is regulated by phosphorylation. PVA CP was phosphorylated in vitro in the presence of unlabeled ATP and 10 mM MnCl₂. The phosphoprotein (lane 2) was separated from unincorporated ATP by SDS-PAGE, transferred to membrane, and probed with ³²P-labeled RNA transcript. Control lanes 1 and 3 contained the nonphosphorylated PVA CP and BSA. After protein transfer, the membranes were either stained with Amido Black or incubated with radioactively labeled RNA. The positions of molecular mass markers (lane M) are indicated in kilodaltons. C, the addition of 1 μM staurosporine into the protein kinase assay preserves the RNA binding activity of PVA CP. The protein incubated with plant extract in the presence of staurosporine (+/+) possesses the same high affinity to RNA as the unphosphorylated protein (−/−). In the control lane, protein was phosphorylated in the absence of staurosporine (+/−), and its binding to RNA was inhibited. D, analysis of the effect of protein phosphorylation on the RNA binding properties of PVA CP using magnetic beads (see “Experimental Procedures”). Graphic representation of the results obtained from three independent experiments. Each bar represents mean ± S.E. values. Phosphorylated protein (+) was checked for degradation by SDS-PAGE and compared with nonphosphorylated protein (−) (amounts of protein are shown in the inset). All proteins shown are histidine fusions except in A, lanes 1–6.

The results obtained with the technique described above demonstrated that the RNA binding activity of PVA CP was strongly affected by phosphorylation. Fig. 8B shows that the affinity of phosphorylated PVA CP for RNA was remarkably lower compared with that of nonphosphorylated protein. Densitometry revealed that the RNA binding affinity of phosphorylated protein dropped about 100-fold compared with that of nonphosphorylated protein. To ensure that no other protein modification except phosphorylation was responsible for the observed effect, we performed a control assay in which staurosporine was introduced into the protein kinase assay. Because staurosporine was previously shown to inhibit phosphorylation of PVA CP, its presence in the kinase assay was thought to preserve protein functions affected by phosphorylation. Indeed, the RNA binding activity of PVA CP remained at the control level after the protein was subjected to mock treatment with the plant protein extract in the presence of 1 μM staurosporine (Fig. 8C).

To further support the conclusion that phosphorylation down-regulates the RNA binding function of PVA CP, we implemented another experimental approach. It has been demonstrated that His₆(TMV MP) can effectively bind RNA while immobilized on the column with Ni²⁺-NTA adsorbent (19). In this study, we developed a convenient modification of this method based on the magnetic bead technology. This approach combined the advantages of the noncovalent protein immobilization with the ease and speed of magnetocapture assays. First, the target His₆(PVA CP) was extensively phosphorylated in a reconstituted system containing plant protein extract, unlabeled ATP, and Mn²⁺ as an essential protein kinase activator. The phosphorylated protein was next immobilized on the Ni²⁺-NTA magnetic agarose beads in parallel with the negative control, the nonphosphorylated protein. The beads were magnetically separated from unincorporated ATP and contaminating plant proteins, washed with RNA binding buffer, and incubated with radiolabeled RNA transcript. Further washings removed free RNA from the beads, and the remaining radioactivity was measured by liquid scintillation counting. Control experiments with no immobilized protein indicated that RNA itself does not bind to the beads. It is important to note that the RNA binding buffer contained BSA to prevent nonspecific interactions, and PVA CP was checked for degradation by gel electrophoresis (Fig. 8D, inset). In agreement with the results obtained by RNA-protein blotting, analysis using magnetic
beads also revealed that the RNA binding of PVA CP was strongly down-regulated by phosphorylation (Fig. 8D).

**DISCUSSION**

In recent years, the role of MP phosphorylation in virus movement has begun to be revealed. There is a growing body of evidence that MP phosphorylation represents a cellular function that controls spread of the virus (1–3). In the current study, we demonstrate that phosphorylation by the plant protein kinase activity with a distinct mode of activation and inhibition by divalent metal cations strongly down-regulates interactions of PVA CP with RNA. Furthermore, we provide evidence that the same protein kinase activity phosphorylates the well characterized TMV MP. Our results indicate that this activity can be highly stimulated by the manganese cations. The possible mechanism behind such stimulation, at first sight, seems obvious; increasing concentrations of Mn$^{2+}$ can activate the enzyme catalytic activity through a noncovalent interaction with ATP and the formation of the ATP-DMC complex, which is the true donor of phosphate for protein kinases. However, the concentrations of Mn$^{2+}$ used in our assays were well above the limit necessary to completely saturate 0.5 μM ATP in the reaction mixture. We calculated that 95% saturation of 0.5 μM ATP is achieved at 0.19 mM Mn$^{2+}$. If metal cations can affect catalytic activity only through an interaction with ATP, there will be no stimulation of phosphorylation after all ATP molecules become saturated with Mn$^{2+}$. On the contrary, our results show that the plant kinase activity phosphorylating PVA CP rises further when Mn$^{2+}$ concentrations increase beyond those needed to saturate ATP. This suggests that in addition to interaction with ATP, divalent metal cations play another important role in the activation of this plant enzyme. Previously, the problem of kinase activation by DMCPs has been extensively studied for several animal tyrosine kinases including Csk, Src, and the fibroblast growth factor receptor kinase (23, 25). The catalytic activity of these enzymes has been also shown to depend on the concentration of DMC in the assay at levels higher than those required to completely saturate ATP. This led to the conclusion that protein-tyrosine kinases possess a second metal binding site that is essential for enzyme activation (25). It has been suggested that an additional metal cation can activate the kinase by binding directly to the enzyme or via a metal-substrate complex. In the course of the current study, we observed that the plant protein kinase that phosphorylates PVA CP and TMV MP is activated in a fashion similar to Csk and Src at concentrations of Mn$^{2+}$ higher than those required for ATP saturation. This suggests that the plant enzyme also requires additional divalent metal cations as essential activators. Similarly to tyrosine kinase Csk, the plant Ser/Thr kinase activity involved in MRP phosphorylation was also inhibited by micromolar concentrations of Zn$^{2+}$ and Cd$^{2+}$ in the presence of a 1000-fold excess of Mg$^{2+}$. Taken together, these results suggest that the plant enzyme involved in PVA CP and TMV MP phosphorylation as well as certain animal protein kinases including Csk may share the common functional domains responsible for the regulation of catalytic activity through interactions with metal ions. Interestingly, inhibition of protein phosphorylation by cadmium suggests a possible mechanism behind the recently reported cadmium-induced resistance of tobacco plants to turnip vein clearing virus (genus *Tobamovirus*) (29, 30).

The noncovalent binding to RNA represents one of the essential functions attributed to the movement-related proteins of plant viruses. An elegant hypothesis that the translational of viral RNA through plasmodesmata occurs in the form of vRNA complexes was put forward after it was shown that TMV MP strongly interacts with single-stranded nucleic acids in vitro (31). Recently, major support for the assumption that vRNP is indeed a movement intermediate was gained when it was shown that viral RNA colocalizes with TMV MP in infected tobacco proteoplasts (32). It is assumed that MP has the capacity to unfold viral RNA, creating vRNP complexes that are structurally compatible with modified plasmodesmata (33, 34). The finding that TMV MP serves as an efficient repressor of TMV RNA translation in vitro (35) suggested an interesting possibility that a strong interaction between MP and viral RNA switches the RNA function from replication to movement. In other words, viral RNA-MP complexes may represent movement intermediates excluded from replication and destined solely for cell-to-cell translocation. This hypothesis, in turn, raised another question: what is the mechanism converting the translation-incompetent movement intermediates back into the infectious form when they reach the neighboring cell? The possible answer to this question emerged when phosphorylation of TMV MP was shown to abolish its ability to repress RNA translation in vitro (2). It has been suggested that MP phosphorylation represents a molecular strategy to restore translation of viral RNA in the newly infected cell.

Although the cell-to-cell movement of tobamoviruses is assumed to occur in a form of vRNP complexes, the nature of the transport complex of potyviruses is still largely unknown. One possibility is that potyviruses move from cell to cell as ribonucleoprotein complexes, analogous to that proposed for TMV but containing the coat protein (36). If the latter is true, a similar phosphorylation-mediated mechanism may exist to control the stability of potyvirus movement intermediates. Our finding that phosphorylation of PVA CP strongly inhibits its RNA binding function suggests to us a hypothetical model describing how the function of the PVA movement intermediates is regulated by phosphorylation. According to this model, at middle and late stages of infection, a certain pool of viral RNA becomes excluded from replication through a strong cooperative interaction with CP and then is targeted to structurally modified plasmodesmata. The unfolded vRNP movement intermediates are then actively transported through plasmodesmata into the neighboring cells. During or after translocation through plasmodesmata, cellular protein kinases phosphorylate CP molecules, decreasing their affinity toward viral RNA. Consequently, the translation-incompetent vRNP movement intermediates dissociate, allowing the viral RNA to start replication in the newly infected cell.

Alternatively, potyviruses may require virion assembly for cell-to-cell translocation. In this case, phosphorylation may control movement of potyviruses by regulating the amount of CP available for interaction with viral RNA. Our results demonstrate that the protein extracted from virions but not the virions themselves could be phosphorylated. This finding suggests that phosphorylation may sequester a certain amount of nonvirion CP from interactions with viral RNA, thus regulating the process of virus packaging.

What is the molecular mechanism behind the phosphorylation-mediated regulation of the RNA binding properties of PVA CP? The most straightforward explanation requires that multiple phosphorylations add extra negative charges to the RNA binding domains of the protein and inhibit its electrostatic interactions with RNA. However, regulation of PVA CP function by phosphorylation may be a more complex process. It may not be ruled out that phosphorylation causes a conformational change within the target protein. The fact that phosphorylation of PVA CP produced a very strong effect suggests that its function is probably affected by phosphorylation on a structural level. Ultimately, three-dimensional crystal structures of movement-related proteins will be required to determine the
Phosphorylation of the Coat Protein of Potato Virus A

effect of phosphorylation on their domain conformations.

Finally, still another role played by phosphorylation in viral movement may be proposed. Specific interactions of MPs with cellular proteins having the characteristics of plasmodesmal receptors have been recently reported (37–39). It needs to be established whether specific phosphorylation events control functions of these proteins that potentially regulate the cell-to-cell molecular pathway. There is a possible correlation between the developmental regulation of cell wall-associated plant protein kinase activity (11) and the transition from simple to branched forms of plasmodesmata (40). Assuming that protein phosphorylation is involved in specific macromolecular trafficking through plasmodesmata, changing patterns of protein kinase expression in the leaves undergoing the sink-source transition may reflect a part of a complex developmental strategy used by plants to regulate plasmodesmal permeability.

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