The potato leafroll virus 17K movement protein is phosphorylated by a membrane-associated protein kinase from potato with biochemical features of protein kinase C

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Abstract The 17 kDa protein (pr17), the phloem-limited movement protein (MP) of potato leafroll luteovirus (PLRV), is associated with membranous structures and localized to plasmodesmata [Tacke et al. (1993) Virology 197, 274–282; Schmitz, J. (1995) Ph.D. Thesis, University of Cologne]. In planta the protein is predominantly present in its phosphorylated form, but it is rapidly dephosphorylated during isolation under native conditions. In an effort to examine the nature of the protein kinase(s) involved in the phosphorylation reaction, pr17 deletion mutants were expressed as fusion proteins in a bacterial expression vector system and tested for their ability to be phosphorylated by potato membrane preparations as well as by commercially available kinases. A fusion protein containing the nucleic acid-binding, basic, C-proximal domain (pr17C1) was identified to be phosphorylated by a Ca^{2+}- and phospholipid-dependent, membrane-associated protein kinase. This protein kinase activity was inhibited by the addition of (19–36) protein kinase C (PKC) inhibitory peptide, known to be a highly specific inhibitor of mammalian PKC. Moreover, also the mammalian PKC from rat was able to phosphorylate pr17 in vitro. The results suggest that phosphorylation of pr17 takes place at membranous structures, possibly at the deltoid plasmodesmata connecting the sieve cell-companion cell complex of the phloem, by the activity of PKC-related, membrane-associated protein kinase activity.

Key words: Cell-to-cell transport; Phloem; Phosphorylation; Post-translational modification; Protein kinase

1. Introduction

Potato leafroll virus (PLRV), a phloem-specific luteovirus causing severe yield losses in potato, is an aphid-transmitted virus with a single-stranded, non-polyadenylated, (+)-sense RNA genome of 5.9 kb [3]. Six large open reading frames (ORFs) are organized into two gene clusters separated by a small intergenic region (Fig. 1a). The 17 kDa protein (pr17) of PLRV is encoded by ORF4 in the 3′ half of the viral genome. It is translated from subgenomic PLRV RNA1 (sgRNA1) by internal translation initiation at an in vitro translation efficiency which is sevenfold higher as compared to that of the coat protein, the main structural protein of the PLRV virion, which is translated from the identical sgRNA1 [4,5].

Biochemical characterization of pr17 has identified two functional domains: the basic C-terminal half of the protein binds to single-stranded nucleic acids in a sequence-unspecific manner [6]. Secondly, pr17 dimerizes in vivo and in vitro, and this functional property resides in an amphipathic α-helix within the acidic pr17 amino-terminus [1]. In PLRV-infected and pr17-transgenic potato plants, pr17 protein is predominantly detected in the typical, deltoid-shaped plasmodesmata which in the phloem system connect the sieve cells to the companion cells [2]. On the basis of these observations, pr17 was proposed to be the phloem-specific PLRV movement protein mediating cell-to-cell transport by shaping PLRV RNA into complexes that are capable of transfer through the plasmodesmata of the phloem [7].

Such interaction of pr17 with PLRV RNA to form pr17/PLRV RNA complexes has to be subject to regulatory mechanisms, such that after cell-to-cell transport a (partial) dissociation of the complex precedes PLRV RNA replication. The molecular mechanisms which are involved in the regulation of pr17 activity are unknown. However, we have shown previously that in planta pr17 exists predominantly as a phosphoprotein, and that this post-translational modification does not inhibit the nucleic acid-binding activity of the protein [1]. Therefore, phosphorylation/dephosphorylation reactions could represent one possible mechanism in the regulation of pr17 activity. This view is supported by studies on the 30 kDa movement protein (pr30) of tobacco mosaic virus (TMV) which is post-translationally modified by a cell wall-associated serine/threonine protein kinase [8]. This phosphorylation may possibly represent a mechanism for cell wall localization of pr30, although from pr30 phosphorylation studies in tobacco protoplasts it has been suggested that this modification could prevent non-specific binding of nucleic acids to the basic part of the protein [9].

In the present work we demonstrate that the in vitro phosphorylation of the PLRV 17 kDa protein is catalyzed by a membrane-associated potato protein kinase with biochemical features similar to that of animal PKC.

2. Materials and methods

2.1. Materials

Calf thymus histone H1 (type III-S), compounds 48/80 and R24571 (calmidazolium) were purchased from Sigma. The specific inhibitor of protein kinase A (PKA) catalytic subunit and PKC inhibitory peptide (19–36) were from Calbiochem Biochemicals. [γ-^{32}P]ATP was supplied by Amersham International.

2.2. Preparations of pr17 mutant proteins in E. coli

Pr17 fusion proteins were expressed and purified from *E. coli* by a modification of a previously described method [10]. For the expression of pr17N (residues 1–54), pr17C1 (residues 55–95) and pr17C2 (residues 93–156) fusion proteins, the respective pr17 gene sequences were amplified by the polymerase chain reaction (PCR) using pCPL1 plasmid DNA as a template [11] and oligodeoxynucleotide primers containing the *Bam*HI and *Eco*RI restriction sites, respectively. Appropriate PCR fragments were isolated as *Bam*HI/*Eco*RI fragments, subcloned into the vector pGEX-3X (Pharmacia Biotech), and their

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sequences verified by sequence analysis. Plasmid DNAs were transformed into *E. coli* strain BL21 [12], colonies were grown at 37°C, and expression of the glutathione *S*-transferase (GST)/pr17 fusion proteins was induced by the addition of 1 mM IPTG for 2 h. Cells were harvested, resuspended in 1×PBS buffer containing 1% Triton X-100, lysed by sonification, and subjected to centrifugation at 10000×*g* for 5 min at 4°C. The supernatant was mixed with a 50% slurry of glutathione sepharose 4B (Pharmacia Biotech) and the mixture was incubated at room temperature for 30 min to ensure fusion protein binding. The fusion proteins were eluted with buffer containing 10 mM reduced glutathione (Sigma) in 50 mM Tris-HCl pH 8.0 and stored at −20°C until used. Protein concentrations were determined by the Bradford assay [13].

2.3. Preparation of membrane fractions from potato leaves

Wild-type potato plants (*Solanum tuberosum* var. Sieglinde) were used as sources of plant tissue. Leaves (10 g) were frozen in liquid nitrogen, ground to a fine powder and homogenized in 20 ml of H buffer (50 mM MES-NaOH pH 7.0, 250 mM sucrose, 5 mM $MgCl_2$, 0.5 mM DTT, 5 mM EDTA, 1 mM PMSF) as described previously [14]. After the first centrifugation (3000×*g* for 10 min at 4°C), the supernatant was divided into aliquots and centrifuged at 16000×*g* for 1 h at 4°C in a microfuge. The resulting membrane pellets were frozen at −70°C before use.

2.4. In vitro kinase assay

Membrane pellets were resuspended in a small volume of 2×assay buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 200 μM sodium pyrophosphate, 2 mM NaF, 200 μM PMSF). For in vitro phosphorylation, membrane proteins (15 μg) were mixed with 5 μg of pr17 mutant proteins (plus further ingredients as indicated). The reaction was started by the addition of 0.5 μl of [γ-^{32}P]ATP (5 μCi, specific radioactivity 5000 Ci/mmol), and stopped after 25 min at 30°C by adding Laemmli sample buffer. The samples were boiled for 5 min and separated by polyacrylamide gel electrophoresis (PAGE) on 15% SDS-containing polyacrylamide gels [15]. Proteins were stained with Coomassie brilliant blue R-25 before autoradiography. Radiolabeled pr17 bands were excised from the gel and their radioactivity was determined in a liquid scintillation counter.

2.5. Extraction of lipids from the membrane preparations

Crude membrane preparations were treated with 90% (v/v) acetone at 0°C for 10 min as described [16]. Following centrifugation the membrane pellet was washed twice with H buffer and finally homogenized in 2×kinase assay buffer. The lipid fraction was concentrated in a rotary evaporator, resuspended in 10 mM Tris-HCl pH 8.0, and added to the reaction mixture as required.

Removal of lipids from membrane preparations with phospholipase C (200 units/ml) from *Bacillus cereus* (Sigma) was carried out in 25 mM Tris-HCl pH 7.5 at 30°C for 20 min. Following this treatment the membrane preparations were added to the phosphorylation reaction as required.

2.6. In vitro PKC assay

Protein kinase C activity was measured in a buffer containing 25 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM DTT, 0.1 mg/ml L-α-phosphatidyl-L-serine (Sigma) and 0.1 mg/ml 1,2-diolein (Sigma). The reaction was started by adding 0.5 μl of [γ-^{32}P]ATP (5 μCi, specific radioactivity 5000 Ci/mmol) and 20 ng of purified PKC from rat brain (mixture of α, β and γ isoforms, Biomol), continued for 25 min at 30°C, and stopped by adding Laemmli sample buffer. After PAGE separation, radiolabeled proteins were visualized by autoradiography as described in Section 2.4.

3. Results

The results of subcellular localization studies provided evidence that pr17 protein is predominantly associated with fractions enriched with nuclei, chloroplasts, mitochondria, and membrane structures with only minimal amounts of pr17 detected in the soluble fractions [1]. Therefore, the possibility that pr17 is phosphorylated by a membrane-associated protein kinase(s) was investigated in preliminary experiments by identifying such activities in membrane fractions. In fact, crude membrane preparations from potato leaves could phosphorylate in a time-dependent manner histone H1 – a poorly selective substrate commonly used for the measuring of kinase activity in vitro (data not shown).

Further we tested the ability of potato membrane preparations to phosphorylate pr17 protein. For this, three deletion mutants of pr17 were produced in *E. coli* by the pGEX expression vector system yielding fusion proteins with the N-terminus of the glutathione *S*-transferase protein [10]. The GST fusion proteins were purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilized glutathione. Pr17N fusion protein contained the acidic N-terminal half of protein (amino acid residues 1–54), pr17C1 and pr17C2 proteins represented the carboxy-terminus of pr17 (residues 55–95 and 93–156, respectively; Fig. 1b,c). The full-length GST/pr17 fusion protein was not included in the experiment, because it was produced in bacteria in an insoluble form.

For in vitro phosphorylation experiments, pr17 fusion proteins were incubated with crude membrane preparations from potato leaves in the presence of [γ-^{32}P]ATP and subjected to PAGE followed by staining with Coomassie blue and autoradiography. As shown in Fig. 2, under these experimental conditions, membrane preparations were able to phosphorylate only pr17C1 protein (lane 6). No phosphorylation was detected when GST protein alone was used as a substrate (Fig. 2, lane 3). Also, the membrane preparations themselves did not show endogenous substrate activity for protein kinase (Fig. 2, lane 1). These results suggest that a membrane-associated protein kinase activity of potato is involved in the phosphorylation of pr17, and that the phosphorylation sites are located within the N-proximal half of the pr17 carboxy-terminus.

This in vitro phosphorylation of the pr17C1 protein by membrane-associated protein kinase(s) was not dependent on exogenously added stimulators of the reaction. The time course of pr17C1 phosphorylation increased linearly and reached a plateau at about 25 min after the start of the reaction (data not shown). As several Ca^{2+}- and Ca^{2+}/calmodulin-dependent membrane-associated protein kinases are known in plants [14,16–20], we investigated the possibility that the membrane-associated protein kinase activity from potato leaves is a calcium- and calmodulin-dependent or a calcium- and phospholipid-dependent enzyme activity. In order to examine the Ca^{2+} dependence of pr17C1 phosphorylation, the enzyme activity was measured by adding increasing amounts of calcium. Efficient phosphorylation was detected in the presence of 1 μM Ca^{2+} (Fig. 2, lane 6), a concentration which is close to the physiological range [21]. As shown in Fig. 3a, the addition of Ca^{2+} to the reaction mixture could weakly stimulate the phosphorylation of pr17C1 protein. In the presence of higher concentrations of Ca^{2+}, the enzyme activity was slightly inhibited. The addition of EGTA (Ca^{2+}-chelating agent) inhibited the phosphorylation catalyzed by the enzyme from membrane preparations (Fig. 3b). In the presence of 2 mM EGTA the phosphorylation of pr17C1 was inhibited by 74%. The same effect of Ca^{2+} and EGTA was observed when histone H1 was used as a substrate for phosphorylation (data not shown).

A possible phospholipid dependence of the membrane-associated protein kinase activity was tested by extracting crude

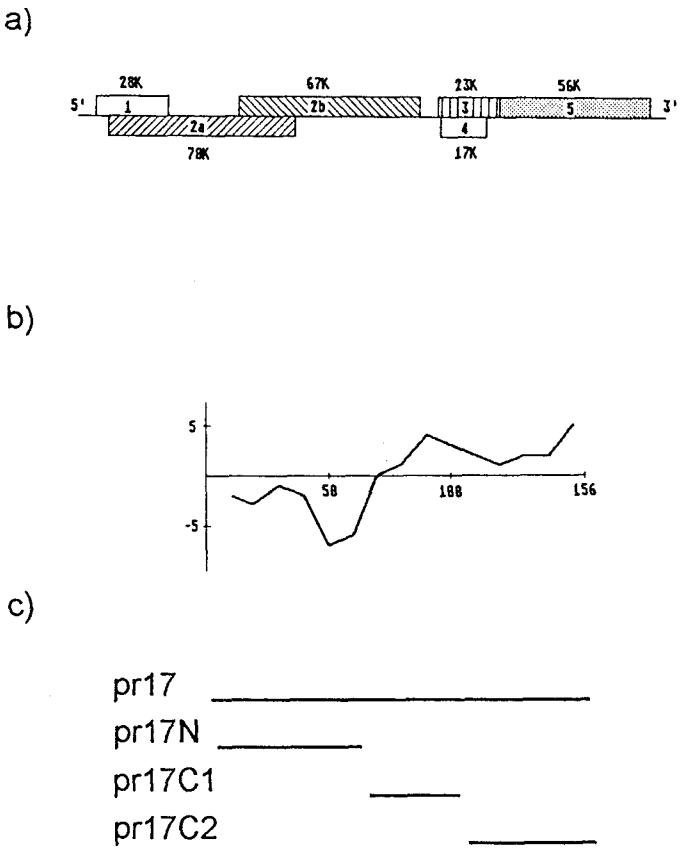

Fig. 1. Analysis of the pr17 protein. a: Structure of the PLRV genome. Positioning and numbering of ORFs are indicated. b: Charge distribution in the PLRV 17kDa protein. c: Schematic representation of pr17 deletion mutants expressed by the pGEX expression vector system and yielding fusion proteins with N- (pr17N) or C- (pr17C1 and pr17C2) terminal domains of the PLRV 17kDa protein.

membrane preparations with acetone. The enzyme activity of acetone-treated membranes was completely lost (Fig. 3e, lane 2). On the other hand, the addition of the lipid fraction extracted from the membranes restored the phosphorylation activity (Fig. 3e, lanes 3–6). In a different set of experiments, the removal of lipids from the membrane preparations by treatment with phospholipase C decreased the protein kinase activity as well (Fig. 3d, lane 5). These results suggested that the membrane-associated kinase from potato leaves is dependent on phospholipids for enzyme activity.

Pr17C1 protein contains several motifs corresponding to known consensus phosphorylation sites for serine/threonine protein kinases including protein kinase C, protein kinase A and calmodulin-dependent protein kinase II (7, 3 and 1 putative phosphorylation sites, respectively). In order to identify the class of protein kinase(s) involved in the phosphorylation of pr17C1, highly selective protein kinase inhibitors were included in the phosphorylation assay. Calmidazolium, a specific inhibitor of calmodulin-regulated enzymes (Fig. 3d, lane 3) and the calmodulin antagonist compound 48/80 (Fig. 3d, lane 4) did not inhibit the phosphorylation of pr17C1 protein. Also, the addition of a highly specific inhibitor of PKA catalytic subunit did not affect the kinase activity from potato membrane fractions (Fig. 3d, lane 2). In contrast, the inhibitory PKC peptide (19–36), derived from the pseudosubstrate autoinhibitory sequence of animal protein kinase C and known to selectively inhibit the PKC activity [22], showed a dramatic effect on the phosphorylation of pr17C1 protein when its phosphorylation by the membrane-associated protein kinase activity was measured in the presence of increasing amounts of inhibitor (Fig. 3c). In the presence of 200 μM PKC inhibitor, the phosphorylation activity of pr17 C1 protein was inhibited by 82%.

When pr17C1 protein was tested as a substrate for an ani-

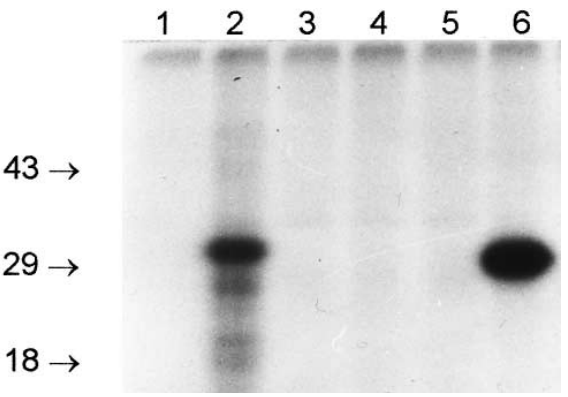

Fig. 2. Phosphorylation of bacterially produced pr17 deletion mutant proteins by crude membrane preparations from potato leaves. Histone H1 type III-S (lane 2), GST (lane 3), pr17N (lane 4), pr17C1 (lane 6), and pr17C2 (lane 5) were incubated with crude membrane preparations from potato leaves in the presence of [γ-^{32}P]ATP and subjected to SDS-PAGE followed by staining with Coomassie blue and autoradiography. Lane 1, membrane preparations were incubated with [γ-^{32}P]ATP without exogenous substrate. Molecular mass values are indicated on the left side of the gel.

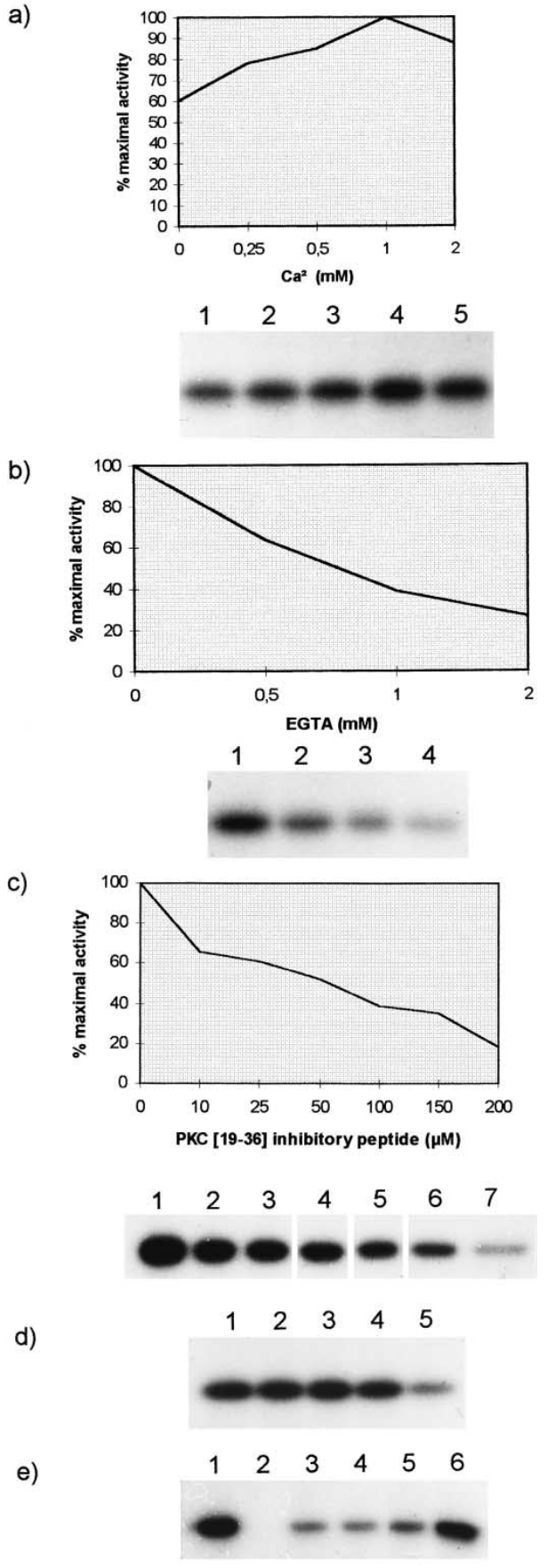

Fig. 3. Characterization of pr17C1 phosphorylation by a membrane-associated protein kinase activity. a: The Ca^{2+} dependence of pr17 phosphorylation by membrane preparation was measured by adding increasing amounts of calcium ions: 0.25 mM (lane 2), 0.5 mM (lane 3), 1 mM (lane 4), 2 mM (lane 5) or no added Ca^{2+} (lane 1). b: pr17C1 phosphorylation in the presence of 0.5 mM EGTA (lane 2), 1 mM EGTA (lane 3), 2 mM EGTA (lane 4) or no added EGTA (lane 1). c: The inhibitory effect of the PKC pseudosubstrate peptide (19–36) on the phosphorylation of pr17C1 protein. Phosphorylation of pr17C1 protein in the presence of increasing amounts of PKC inhibitor: 10 μM (lane 2), 25 μM (lane 3), 50 μM (lane 4), 100 μM (lane 5), 150 μM (lane 6), 200 μM (lane 7). Lane 1: phosphorylation of pr17C1 without PKC (19–36) inhibitor peptide. d: Phosphorylation of pr17C1 by membrane preparations in the presence of protein kinase inhibitors: 50 U of specific inhibitor of PKA catalytic subunit (lane 2), 50 μM calmidazolium (lane 3), 50 μg/ml calmodulin antagonist compound 48/80 (lane 4). Lane 1: phosphorylation of pr17C1 without additions; lane 5: phosphorylation of pr17C1 by membrane preparations treated with phospholipase C. e: Effect of acetone treatment on the activity of membrane-associated protein kinase activity. Lane 1: phosphorylation of pr17C1 by membrane preparations not treated with acetone. Lanes 2–5: phosphorylation of pr17C1 by acetone-treated membrane preparations plus 5 μl lipid fraction (lane 3), 10 μl lipid fraction (lane 4), 15 μl lipid fraction (lane 5), and 30 μl lipid fraction (lane 6). ←

mal protein kinase C, the Ca^{2+}- and phospholipid-dependent protein kinase C from rat brain was able to phosphorylate both pr17C1 and pr17C2 proteins (Fig. 4, lanes 6 and 5). Phosphorylation was not observed when GST and pr17N proteins were used as the substrates (Fig. 4, lanes 3 and 4), nor did PKC display autophosphorylation activity (Fig. 4, lane 2). The amounts of incorporated radioactive label for ^{32}P-labeled histone H1 and pr17C1 were in the same range as those phosphorylated by membrane-associated protein kinase from potato (not shown).

4. Discussion

Post-translational modification of proteins by protein kinases is an important regulatory mechanism of the various activities exhibited by proteins. These regulatory activities of PKs are also part of signal transduction pathways, where extracellular signals are amplified and propagated by a cascade of protein phosphorylation and/or dephosphorylation events. In plants, protein phosphorylation by PKs may regulate unique processes, such as photosynthesis, photomorphogenesis and gravitropism, cytoplasmic streaming, stress adaptation, and pathogen response [23,24]. Protein kinases can be classified into various groups according to their substrate or cofactor specificities The Ca^{2+}-/phospholipid-dependent serine/threonine kinases, represented by protein kinase C

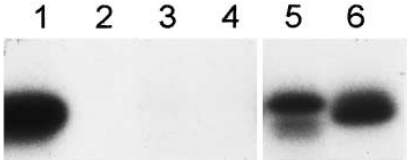

Fig. 4. Phosphorylation of bacterially produced pr17 fusion proteins by PKC from rat brain. The autoradiogram shows incubations with histone 1 type III-S (lane 1), PKC without exogenously added substrate (lane 2), GST (lane 3), pr17N (lane 4), pr17C2 (lane 5), and pr17C1 (lane 6). Incubations were carried out with 20 ng of PKC from rat brain in the presence of [γ-^{32}P]ATP and analyzed by PAGE as described in the legend to Fig. 2.

(PKC), play an important role within the intracellular activation cascade. They are typically activated by the second messenger diacylglycerol and involved in cellular responses to various agonists [25,26]. Several protein kinase activities with the properties of animal PKC have been characterized and partially purified from different organs of many plant species [14,16,27–30]. Like in the animal system, protein kinase C is apparently involved in the regulation of many cellular functions in plants.

Here we provide evidence for the presence in potato membrane fractions of a protein kinase(s) with biochemical features similar to animal protein kinase C. This potato protein kinase activity phosphorylates the 17kDa movement protein of the luteovirus PLRV. Further observations suggest that (i) this protein kinase is stimulated by Ca^{2+} and inhibited by EGTA, (ii) the protein kinase activity is phospholipid-dependent, as phospholipase C treatment of membrane fractions decreased the phosphorylation activity and acetone treatment resulted in the complete loss of enzymatic activity which could be restored by the addition of membrane lipids, (iii) the highly selective PKC inhibitory peptide (19–36) inhibited the phosphorylation of pr17C1 protein, and (iv) the protein kinase C from rat brain was able to phosphorylate pr17C1 in in vitro kinase assay.

In the present phosphorylation studies we used a reconstruction system in which bacterially produced purified pr17 fusion proteins were incubated with potato membrane preparations in the presence of [γ-^{32}P]ATP. Under these experimental conditions only the pr17C1 mutant protein was phosphorylated by protein kinase from crude membrane preparations. This result correlates with the amino acid sequence of pr17C1 which contains 7 putative phosphorylation sites for PKC. The fact that PKC from rat brain phosphorylated also pr17C2 protein could best be explained by the fact that PKC is a multigene family with at least 10 identified members [31]. Our results, however, do not exclude the possibility that in vivo protein kinase(s) other than one of the PKC family is involved in the phosphorylation of pr17. Phosphorylation within this region of the pr17 protein is nevertheless an interesting aspect of the putative regulatory function exerted by phosphorylation. This pr17 portion is part of the basic nucleic acid-binding domain, and the introduction of one or more phosphate residues may lower its basic charge to such an extent that the specific interaction with PLRV RNA is weakened and leads to the dissociation of the ribonucleoprotein complex.

Phosphorylation studies on another virus-specific movement protein – the 30 kDa MP protein of TMV known to be phosphorylated by a cell wall-associated protein kinase – suggested that the phosphorylation may represent a mechanism for the host plant to sequester pr30 following its localization to cell walls [8]. On the other hand, the phosphorylation of pr30 in TMV-infected protoplasts revealed that pr30 is multiply phosphorylated at serine residues in at least five internal tryptic peptides [9] in addition to the C-terminal in vitro phosphorylated phosphopeptide reported earlier [8]. These conflicting results on in vitro and in vivo phosphorylation patterns emphasize the necessity to isolate PLRV pr17 protein from PLRV-infected and pr17-transgenic potato plants in order to study the in vivo phosphorylation pattern.

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