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Phosphatidylinositol 4-Kinase Associated with Spinach Plasma Membranes. Isolation and Characterization of Two Distinct Forms[1]

Tomas Westergren, Lars Ekblad, Bengt Jergil, and Marianne Sommarin*

Department of Plant Biochemistry, Lund University, Box 117, SE–221 00, Lund, Sweden (T.W., M.S.); and Department of Biochemistry, Centre for Chemistry and Chemical Engineering, Lund University, Box 124, SE–221 00, Lund, Sweden (L.E., B.J.)

Highly purified plasma membranes from spinach (*Spinacia oleracea* L.) leaves contained phosphatidylinositol (PtdIns) kinase activity that was firmly associated with the membrane. The enzyme was solubilized by detergent treatment (2% [w/v] Triton X-100) and purified by heparin-Sepharose and Q-Sepharose chromatography. Two enzymically active fractions, QI and QII, both exhibiting PtdIns 4-kinase activity, were resolved and purified 100- to 300-fold over the plasma membrane. QI and QII shared similar high apparent K_m values for ATP (approximately 0.45 mM) and PtdIns (approximately 0.2 mM) and were insensitive to inhibition by adenosine. While Mg^{2+} was the preferred divalent cation, Mn^{2+} could partly substitute in the reaction catalyzed by the QII enzyme but not in that catalyzed by QI. Mn^{2+} acted synergistically with suboptimal Mg^{2+} concentrations to activate not only the QII enzyme, but also to some extent QI. Both enzymes were inhibited by millimolar concentrations of Ca^{2+} and micromolar concentrations of wortmannin. The apparent molecular mass for QI was 120 kD, which was determined by SDS-PAGE and western blotting using an antibody against a peptide unique for lipid kinases and the binding of ^{3}H-wortmannin, and for QII 65 kD as determined by immunodetection and renaturation of PtdIns kinase activity in the 65-kD region of polyacrylamide gels.

Phosphatidylinositol (PtdIns) 4-kinase catalyzes the phosphorylation of PtdIns in the D-4 position of the inositol ring. This is the committed step in the synthetic pathway leading to PtdIns 4,5-bisphosphate (PtdIns 4,5-P_2), which in animal cells is the agonist-sensitive precursor of the intracellular messengers inositol 1,4,5-trisphosphate and diacylglycerol. Apart from this function in signal transduction, both PtdIns 4-P and PtdIns 4,5-P_2 appear to have other cellular functions as well, for example, regulating cytoskeletal architecture, acting as enzyme effectors, or acting as components of vesicular fusion (for reviews, see Carpenter and Cantley, 1990; Pike, 1992; Gehrmann and Heilmeyer, 1998). This multiplicity of functions is perhaps reflected in the presence of multiple forms of PtdIns 4-kinase associated with various subcellular compartments.

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* Corresponding author; e-mail marianne.sommarin@plantbio.lu.se; fax 46–46–2224116.

Although most of the components involved in PtdIns signaling in animal cells have also been identified in plant cells (Sandelius and Sommarin, 1990; Drøbak, 1992; Coté and Crain, 1994), the functions of the plant components are less well characterized. Phosphoinositides have been implicated in a number of environmental stress signaling processes, including responses to acid and osmotic stress, light stress, and pathogen attack, as well as in regulating turgor and leaf and flower movement (Drøbak, 1992; Coté and Crain, 1994). It remains to be demonstrated conclusively, however, that environmental stress is coupled to the turnover of phosphoinositides in a manner similar to that of animal signaling systems.

Considerably more is known about animal than plant PtdIns kinases. In both types of organisms PtdIns 3-kinase and PtdIns 4-kinase activities have been identified as catalyzing the phosphorylation of the inositol ring in the 3- and 4-positions, respectively. Two major types of PtdIns 4-kinase have been isolated from animal cells, and both are preferentially membrane bound. These two types have been termed type II and type III PtdIns kinase. Type I is PtdIns 3-kinase (Carpenter and Cantley, 1990; Pike, 1992). Type II PtdIns 4-kinase is a 55-kD enzyme that is readily renatured after SDS-PAGE. It has comparatively low K_m values for PtdIns and ATP (below 100 μM) and is strongly inhibited by adenosine and Ca^{2+} (Carpenter and Cantley, 1990). The type III enzyme has an apparent molecular mass of 200 kD on gel filtration, and has been renatured from a 200-kD polypeptide after SDS-PAGE (Gehrmann et al., 1996). It has 3- to 7-fold higher K_m values for PtdIns and ATP than the type II enzyme and is insensitive to inhibition by adenosine and Ca^{2+}. In addition to these membrane-bound activities, type III PtdIns 4-kinase activity has also been found in the soluble fraction. This enzyme was isolated from adrenal tissue, and could be resolved into two forms of approximately 110 and 200 kD on gel filtration (Downing et al., 1996; Balla et al., 1997). Both of these forms were wortmannin sensitive, although 1 order of magnitude less than established earlier for PtdIns 3-kinase (Downing et al., 1996).

Several animal PtdIns 4-kinases with properties of the type III enzyme have been cloned. These represent polypeptides of either 92 kD (Balla et al., 1997; Meyers and Cantley, 1997) or 230 kD (Gehrmann et al., 1996; Nakagawa et al., 1996; Balla et al., 1997), presumably corresponding to

the isolated type III enzyme forms. This is in contrast to the only cloned kinase with type II properties (Wong and Cantley, 1994), a 97-kD polypeptide for which no corresponding enzyme has been isolated.

Apart from animal cells, multiple forms of PtdIns 4-kinases have also been isolated from yeast. Of these, there are membrane-bound forms of 45 and 55 kD (Belunis et al., 1988; Nickels et al., 1992) and a soluble form of 125 kD (Flanagan and Thorner, 1992). The 125-kD enzyme has been cloned (Flanagan et al., 1993) and exhibits characteristics closer to the type III than the type II enzyme. A 200-kD enzyme has also been cloned (Yoshida et al., 1994). The 45- and 55-kD enzymes still await cloning. They are regarded as the yeast equivalents of the mammalian type II PtdIns 4-kinase, although their characteristics are not typical of this enzyme in that they are not inhibited by adenosine and they have a high K_m for ATP (Belunis et al., 1988; Nickels et al., 1992).

In plant cells, PtdIns kinase activity was originally found to be associated with plasma membranes (Sandelius and Sommarin, 1986; Sommarin and Sandelius, 1988; Kamada and Muto, 1991), and has subsequently been found in the cytoskeleton (Tan and Boss, 1992; Xu et al., 1992), nuclei (Hendrix et al., 1989), and soluble fraction (Steinert et al., 1994; Okpodu et al., 1995). Plant PtdIns 4-kinases have been partially purified from the soluble fraction of cells of the unicellular alga *Dunaliella parva* (Steinert et al., 1994) and carrot (Okpodu et al., 1995), and from *Catharanthus roseus* plasma membranes (Hanenberg et al., 1995). The soluble carrot enzyme has an apparent molecular mass of approximately 80 kD, both on gel filtration and after renaturation following SDS-PAGE (Okpodu et al., 1995), whereas both the *D. parva* enzyme and the solubilized *C. roseus* plasma membrane enzyme eluted at 500 kD or above on gel filtration (Steinert et al., 1994; Hanenberg et al., 1995), indicating that these enzymes form large complexes.

There is a paucity of kinetic data on plant PtdIns kinases, but the *C. roseus* enzyme shows low K_m values for ATP and PtdIns similar to animal type II kinases (Hanenberg et al., 1995), whereas the carrot enzyme exhibits a K_m for ATP in the type III range (Okpodu et al., 1995). Both of the isolated soluble enzymes are inhibited by adenosine and require comparatively high concentrations of Mg^{2+} (30–40 mM) for maximum activity (Steinert et al., 1994; Okpodu et al., 1995). A particular property of the *D. parva* PtdIns kinase is that it is more active in the presence of Mn^{2+} than Mg^{2+}, and that these two ions act synergistically, enhancing enzymatic activity several-fold over that of either ion alone (Steinert et al., 1994). Thus, available evidence indicates that plant PtdIns kinases differ in significant ways from animal PtdIns kinases.

Recently, a partial and a full-length clone encoding two distinct plant PtdIns 4-kinases were isolated and characterized (Stevenson et al., 1998; Xue et al., 1999). The deduced amino acid sequence of the partial cDNA from Arabidopsis (Stevenson et al., 1998) revealed domains highly similar to the lipid kinase unique (LKU), Pleckstrin homology, and catalytic domains of known PtdIns 4-kinases. Based on sequence and structure similarities, this enzyme can be grouped together with the 230-kD type III PtdIns 4-kinases (subtype 1.2; see Gehrmann and Heilmeyer, 1998). The immunoaffinity-purified PtdIns 4-kinase (AtPI4Kα) exhibited a molecular mass of 205 kD and was identified in Arabidopsis and carrot microsomes and in an F-actin-enriched fraction from carrot cells, but was not further characterized (Stevenson et al., 1998). The deduced amino acid sequence of the Arabidopsis full-length clone encoding a 126-kD PtdIns 4-kinase (AtPI4Kβ) carried the LKU and catalytic domains, but was distinguished by a 300-amino acid domain termed NH, which has a high proportion of charged amino acids (Xue et al., 1999). The recombinant enzyme activity was stimulated by Triton X-100 and inhibited by adenosine (IC_{50} about 250 μM) and 10 μM wortmannin (Xue et al., 1999), and belongs structurally to the 92-kD type III PtdIns kinase (subfamily 1.1; see Gehrmann and Heilmeyer, 1998).

Interestingly, in higher plant cells the level of PtdIns 4-P is up to 35-fold higher than that of PtdIns 4,5-P_2 (Sandelius and Sommarin, 1990; Munnik et al., 1994), while in animal cells these phosphoinositides usually are present in approximately equal amounts. This feature may reflect a more central role of PtdIns 4-P than PtdIns 4,5-P_2 in plant phosphoinositide-dependent processes compared with animal cells, in which the classical signal transduction pathway involving hydrolysis of PtdIns 4,5-P_2 catalyzed by phospholipase C perhaps is predominant. The function of PtdIns 4-P and further phosphorylated products might be in cytoskeletal regulation and membrane trafficking or in the modulation of enzyme activities. Consistent with the assumption that PtdIns 4-P also plays multiple roles in the plant cell, the existence of multiple isoforms of PtdIns 4-kinase seems likely. Because there is so far rather scant knowledge on plant PtdIns 4-kinases and their relation to corresponding animal enzymes, we purified and characterized this enzyme from spinach leaf plasma membranes. The enzyme activity was resolved into two distinct forms with apparent molecular masses of 65 and 120 kD. The biochemical characteristics of both forms resemble those of type III PtdIns 4-kinases.

MATERIALS AND METHODS

Materials

Heparin-Sepharose CL 6B, Q-Sepharose Fast Flow, CNBr-activated Sepharose 4B, and HiTrap were from Pharmacia Biotech (Sollentuna, Sweden). Reduced Triton X-100, type I brain extract, and wortmannin were from Sigma (St. Louis). [17-^{3}H]Wortmannin-17-ol was from New England Nuclear Life Science Products (Boston). Silica gel 60 plates were from Merck (Darmstadt, Germany). Ready Safe was from Beckman Instruments (Fullerton, CA). Immobilon PVDF transfer membranes were from Millipore (Bedford, MA). ECL western blotting system, Hyperfilm β-max, and Amplify were from Amersham International (Little Chalfont, UK). PtdIns and PtdIns 4-P were purified from the type I brain extract (Schacht, 1978), and [γ-^{32}P]ATP was prepared as described previously (Chang et al., 1974).

Plant Material

Spinach (*Spinacia oleracea* L.) plants were grown in a greenhouse with supplementary light (23 W m^{-2}, 350–800 nm; G/86/2 HPLR 400 W, Philips, Eindhoven, The Netherlands). Expanding leaves of 4- to 5-week-old plants were used.

Isolation of Plasma Membranes

Plasma membranes were purified from a microsomal fraction of spinach leaves by partitioning in an aqueous polymer two-phase system, and checked for purity as described in detail previously (Olbe and Sommarin, 1998). Plasma membranes were suspended (10–15 mg of membrane protein per mL) in 330 mM Suc, 10 mM HEPES-KOH, pH 7.5, 1 mM DTT, and 0.1 mM EDTA, and stored in liquid nitrogen until use.

Purification of PtdIns Kinase

Plasma membranes were solubilized in a mixture containing 10 mM HEPES-KOH, pH 7.5, 0.15 M KCl, 10% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, and 2% (w/v) reduced Triton X-100 for 30 min under continuous agitation. Non-solubilized material was pelleted at 100,000*g* for 1 h and the supernatant was used for enzyme purification. This step and the following chromatography steps were performed at 6°C.

A heparin-Sepharose column (2.6 × 9.5 cm) was coupled to an HPLC (BioCad Sprint, PerSeptive Biosystems, Cambridge, MA) and equilibrated with 10 mM HEPES-KOH, pH 7.5, 10% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, and 0.1% (w/v) reduced Triton X-100. The solubilized material was applied to the column, which was washed with equilibration buffer before elution using a linear 0 to 1 M KCl gradient, total volume 60 mL, in the equilibration buffer mixture. The flow rate was 1 mL min^{-1} and 4-mL fractions were collected. Fractions containing PtdIns kinase activity were combined and desalted on HiTrap desalting columns equilibrated with 10 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, and 0.1% (w/v) reduced Triton X-100. The desalted material was applied to a Q-Sepharose column (1.6 × 10 cm) equilibrated with the desalting buffer. The column was developed using a linear 0 to 1 M KCl gradient in a total volume of 30 mL. The flow rate was 1 mL min^{-1} and fractions of 1.5 mL were collected. Fractions containing PtdIns kinase activity were combined and either used immediately for characterization studies or stored in liquid nitrogen until use.

PtdIns Kinase Assay

PtdIns kinase activity was assayed as described by Sommarin and Sandelius (1988) with minor modifications. The standard assay mixture contained 20 mM HEPES-KOH, pH 7.5, 20 mM $MgCl_2$, 0.28% (w/v) Triton X-100, 1 mM DTT, 20 nmol of PtdIns, 3 to 5 μg of enzyme protein, and 2 mM [γ-^{32}P]ATP (100 dpm/pmol, 167 kBq) in a total volume of 50 μL. The reaction was started with ATP and stopped after 3 min by the addition of 1 mL of cold (4°C) chloroform:methanol (1:1, v/v), followed by 20 μL of type I brain extract (containing carrier phosphoinositides) dissolved in chloroform (100 mg mL^{-1}) and 0.5 mL of 1.2 M HCl. Phase separation was facilitated by centrifugation at 2,000 rpm for 5 min in a tabletop centrifuge. The upper water phase was removed and the lower chloroform phase was washed twice, first with 0.5 mL of methanol:1 M HCl (1:1, v/v) and then with 0.5 mL of methanol:0.1 M HCl (1:1, v/v). The washed-chloroform phase was dried under a stream of nitrogen, redissolved in 30 μL of chloroform, and applied to dry Silica gel 60 TLC plates impregnated with 1% (w/v) dipotassium oxalate in 50% (v/v) ethanol. After developing the TLC plates in chloroform:methanol:ammonia:water (45:45:10:5, v/v), lipids were visualized with iodine vapor. The lipid bands of interest were scraped off and mixed with Ready Safe scintillation cocktail, and radioactivity was measured by liquid scintillation counting.

Fractions eluted from the chromatography columns were assayed with a faster method. After incubation as above, 300 μL of chloroform:methanol (1:1, v/v) was added to stop the reaction and extract the lipids. Phase separation was attained by adding 150 μL of 1.2 M HCl. Ten microliters of the chloroform phase was applied to a Silica gel 60 TLC plate with plastic backing (size 20 × 6.5 cm) impregnated with 1% (w/v) dipotassium oxalate in 50% (v/v) ethanol. The TLC was developed using chloroform:methanol:ammonia:water (45:45:10:5, v/v) as the mobile phase. In this way the separation time was shortened from 2 h to 15 min. After drying the plate, radiolabeled lipids were visualized with a phosphor imager (Molecular Dynamics, Sunnyvale, CA) after 30 min of exposure and spots were analyzed with Image Quant 1.2 software (Molecular Dynamics). This rapid analysis method was essential for efficient and high-yield purification of the labile enzyme, bringing the purification procedure from more than 2 d with the ordinary PtdIns kinase assay to 1 d.

Preparation of Rat Liver Cytosol

Liver cytosol was prepared from a male Sprague-Dawley rat. The liver was minced and homogenized in 3 volumes of 0.25 M Suc, 5 mM Tris-HCl, pH 8.0, 0.5 mM dithioerythritol, and 0.5 mM PMSF with one up-and-down stroke in a glass-teflon Potter-Elvehjem homogenizer at a pestle speed of 1,000 rpm. The homogenate was centrifuged at 1,000*g* for 10 min. The pellet was re-extracted twice by homogenization and centrifugation in the same volume of homogenization medium. The combined supernatants were centrifuged for 90 min at 100,000*g* and the supernatant was collected.

Determination of Phosphorylated Lipid Product

Rat liver cytosol (75 μg of protein), and the QI (5-μg) and QII (5-μg) fractions were each incubated in 50 μL of 50 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 0.001% (w/v) Triton X-100, 0.5 mM PtdIns, and 1 mM [γ-^{32}P]ATP (100 dpm/pmol) for 15 min. After stopping the reaction with chloro-

form and using the standard washing procedures described above, the products were separated on Silica gel 60 TLC-plates according to the method of Walsh et al. (1991) and visualized with a phosphor imager.

Antibody Production

Antibodies were raised against the peptide HPLTAQYGVKVLRSC, a LKU sequence corresponding to amino acid residues 383 to 396 in the deduced amino acid sequence of the human type II PtdIns 4-kinase PI4Kα (Wong and Cantley, 1994). The peptides were conjugated to keyhole limpet hemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester and injected into rabbits. Serum was collected and antibodies were purified on affinity matrices consisting of each peptide conjugated to BSA, as described above, and further coupled to CNBr-activated Sepharose 4B according to the manufacturer's description. The antibodies were eluted with 0.1 M Gly-HCl, pH 2.5, and stored at −20°C.

Wortmannin Sensitivity

The wortmannin sensitivity of the PtdIns kinase forms was analyzed by the standard assay method, but with the addition of a 1-μL aliquot of wortmannin in ethanol 2 min prior to the addition of ATP to give a final concentration of up to 10 μM.

In wortmannin-binding studies, 20-μL enzyme aliquots were incubated for 20 min at 20°C with 0.4 μCi of [17-^{3}H]wortmannin-17-ol (Balla et al., 1997). The samples were analyzed on mini SDS-PAGE (see below). After staining the gels with Coomassie Brilliant Blue R-250 and destaining, gels were impregnated with Amplify solution for 1 h at 20°C, dried, and exposed to Hyperfilm β-max for 2 weeks at −80°C.

SDS-PAGE and Western Blotting

Samples were solubilized at 20°C in a sample buffer mixture to the following final concentrations: 2% (w/v) SDS, 10% (v/v) glycerol, 62.5 mM Tris-HCl, pH 6.8, and 5% (v/v) 2-mercaptoethanol, before electrophoresis on a 10% polyacrylamide gel according to the method of Laemmli (1970) using a Mini-Protean II cell (Bio-Rad Laboratories, Hercules, CA). Gels were either stained with Coomassie Brilliant Blue R-250 or the polypeptides were electrophoretically transferred to an Immobilon PVDF membrane for immunostaining. The membrane was incubated overnight at 4°C with the polyclonal antibody LKU (see above). Enhanced chemiluminescent detection was used to visualize the immunodecorated bands.

Renaturation of PtdIns Kinase Activity

Enzyme samples were electrophoresed on polyacrylamide gels containing 0.05% (w/v) SDS in a Mini-Protean II cell. Each lane was cut transversely in 2-mm slices. The slices were incubated in 200 μL of standard PtdIns kinase assay mixture for 5 to 6 h. The mixture was transferred to a new tube and 1.2 mL of chloroform:methanol (1:1 by volume) was added, followed by 0.5 mL of 1.2 M HCl to extract phosphoinositides. After phase separation, the chloroform phase was washed, dried, subjected to TLC separation, and the radioactivity quantitated as described for the standard PtdIns kinase assay.

Protein Determination

Protein was measured as described by Bearden (1978) with BSA as the standard.

Reproducibility

Data presented in the figures are representative of two to five independent enzyme preparations, with the exception of the chromatograms presented in Figure 1, which are representative of 12 independent enzyme fractionations. All measurements were performed in duplicate with the exception of the chromatograms presented in Figure 1, where single measurements on each fraction were executed. The variation between duplicates did not exceed 6%.

RESULTS

Partial Purification of Two Forms of PtdIns Kinase

A major part of the total membrane-associated PtdIns kinase activity in plant cells is recovered in highly purified

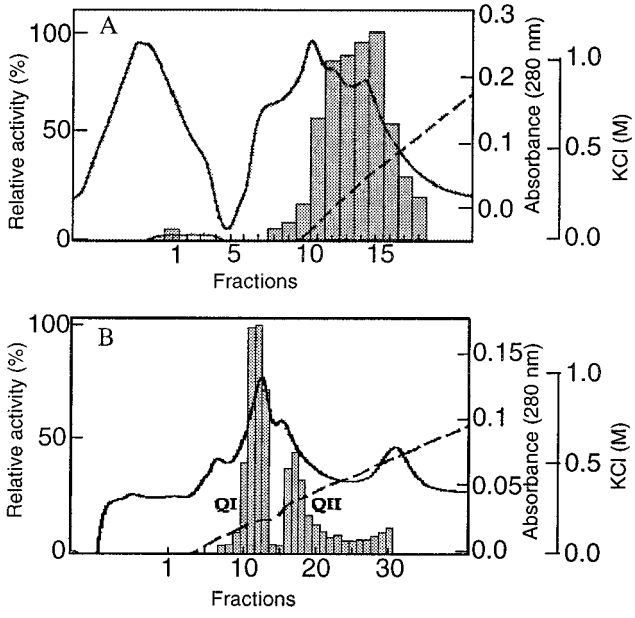

Figure 1. Heparin-Sepharose (A) and Q-Sepharose (B) chromatography of plasma membrane PtdIns 4-kinase. Spinach plasma membranes were solubilized with Triton X-100 and the extract chromatographed first on heparin-Sepharose and then on Q-Sepharose, as described in "Materials and Methods." A, Heparin-Sepharose. Four-milliliter fractions were collected. Fractions 12 to 15 were combined and desalted prior to use in the next chromatography step. B, Q-Sepharose. The fraction size was 1.5 mL. Shaded bars, PtdIns 4-kinase activity; solid line, protein (A_{280}); dashed line, KCl gradient.

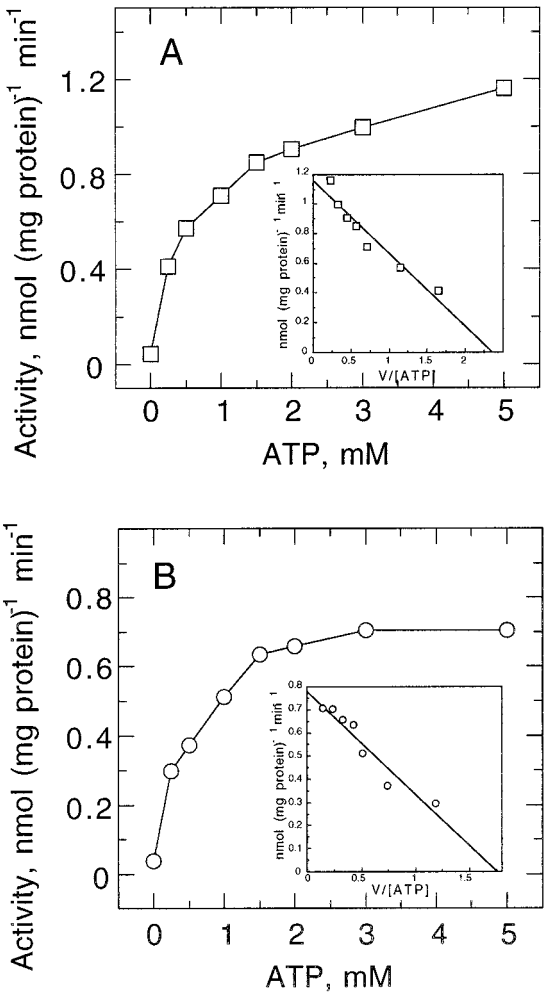

Figure 2. ATP dependency of PtdIns 4-kinases QI (A) and QII (B). PtdIns 4-kinase activity was measured in HEPES-NaOH, pH 7.5, with 0.8 mM PtdIns and 20 mM $MgCl_2$. K_m values were calculated from Eadie-Hofstee plots (insets) using nonlinear least-squares fits generated with computer software (A, $r^2 = 0.92$; B, $r^2 = 0.92$).

plasma membranes (Sandelius and Sommarin, 1990; Wissing et al., 1992). Such membranes, prepared from the microsomal fraction by aqueous polymer two-phase partitioning (Larsson et al., 1994), were therefore selected as the starting material for the purification of spinach leaf PtdIns kinase. The purified membranes were treated with reduced Triton X-100, and the solubilized fraction was chromatographed on heparin-Sepharose. PtdIns kinase activity bound to the column material under the conditions selected, and the enzyme was eluted as a broad peak in the first half of a 0 to 1 M KCl gradient (Fig. 1A). Fractions with PtdIns kinase activity were combined, desalted, and further chromatographed on Q-Sepharose (Fig. 1B). When a KCl gradient was applied, two peaks of PtdIns kinase activity, QI and QII, were resolved, which eluted rather closely at 0.2 and 0.3 M KCl, respectively. The resolution into two peaks was distinct and consistent in all purifications.

Attempts to purify either of the enzyme fractions further were unsuccessful due to the loss of most of the PtdIns kinase activity in subsequent steps. This may be due to the lability of the partly purified enzyme fractions. Storage for 12 h at 4°C or −20°C resulted in a more or less total loss of activity in both the QI and QII fractions. However, when the two fractions were snap-frozen and stored in liquid nitrogen, the activity in the QI fraction was stable for several months, while that of the QII fraction was stable for several months after an initial activity loss of 50%.

As the PtdIns kinase activity of the homogenate and the microsomes was too low to be determined accurately, the overall degree of purification could not be estimated. The specific activity of the spinach plasma membrane PtdIns kinase was approximately 1 nmol mg^{-1} min^{-1}, while the QI and QII fractions showed activities in the 100 to 300 nmol mg^{-1} min^{-1} range. Therefore, an approximately 100- to 300-fold purification over the plasma membrane was attained. The protein patterns of the plasma membrane fraction, the heparin-Sepharose fraction, and QI and QII were distinct, as shown by SDS-PAGE and silver staining (data not shown). However, no distinct enrichment of any particular protein band could be observed, indicating a low abundance of PtdIns kinase proteins.

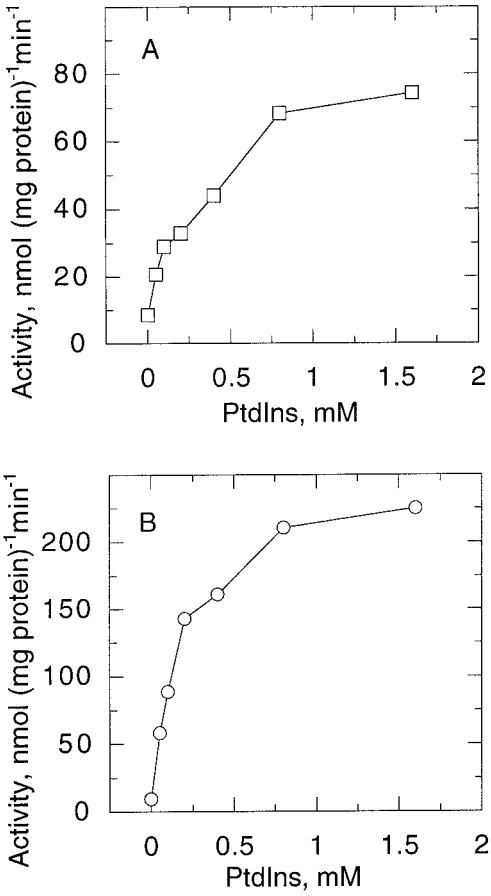

Figure 3. PtdIns dependency of PtdIns 4-kinases QI (A) and QII (B). PtdIns 4-kinase activity was measured in 50 mM HEPES-NaOH, pH 7.5, with 20 mM $MgCl_2$ and 2 mM ATP.

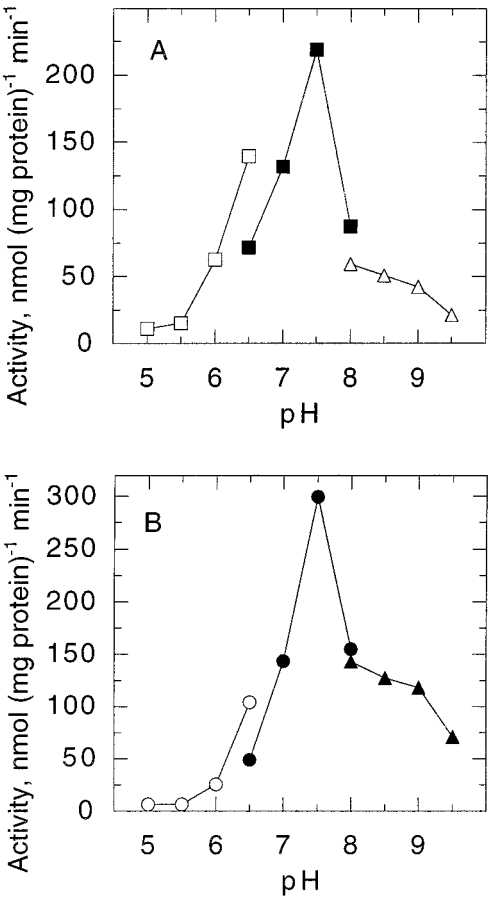

Figure 4. pH profiles of PtdIns 4-kinases QI (A) and QII (B). PtdIns 4-kinase activity was measured in the following buffers; 50 mM MES-NaOH (□, ○, pH 5–6.5), 50 mM HEPES-NaOH (■, ●, pH 6.5–8) and 50 mM 1,3-bis[Tris(hydroxymethyl)methylamino]propane (BTP)-HCl (△, ▲, pH 8–9.5).

General Properties of the PtdIns Kinase Forms

The dependency of the activities of the isolated PtdIns kinases on the concentration of ATP is shown in Figure 2. Both enzyme forms showed saturation kinetics with apparent K_m values of 0.49 and 0.44 mM for QI and QII, respectively. The saturation curves for PtdIns were also similar for the two forms with apparent K_m values of 0.23 and 0.17 mM, respectively (Fig. 3). Both QI and QII had an activity optimum at pH 7.5 displayed at a narrow pH interval (Fig. 4) and were insensitive to adenosine: a 35% inhibition at 1 mM and above and a 20% inhibition at 4 mM and above for QI and QII, respectively (data not shown).

Mg^{2+} served as the divalent cation for both the PtdIns kinase forms, and 20 mM or more was required for maximum enzyme activity (Fig. 5). Mn^{2+} could not substitute for Mg^{2+} in the QI form (Fig. 5A). In contrast, QII was partly active in the presence of $MnCl_2$, with an optimum at 4 mM (Fig. 5B). In the low-millimolar range (1–4 mM), Mn^{2+} was equally as effective as Mg^{2+}, but at higher concentrations Mg^{2+} was the preferred divalent ion; 20 mM $MnCl_2$ gave 25% of the activity seen with $MgCl_2$. Interestingly, Mn^{2+} acted in synergy with suboptimal concentrations of Mg^{2+}, not only for QII but also for QI. Thus, at 10 mM $MgCl_2$ the activity of QII was enhanced by more than 50% by the simultaneous addition of 2 mM $MnCl_2$, the activity again decreasing at higher concentrations of $MnCl_2$. For QI, an activity optimum was observed at 1 to 2 mM $MnCl_2$ in the presence of 10 mM $MgCl_2$, slightly exceeding the activity observed with $MgCl_2$ alone, whereas an increase to 10 mM Mn^{2+} completely inhibited the enzyme.

The effect of $CaCl_2$ was also tested. Both enzyme forms were inactive with Ca^{2+} only (not shown). In the presence of 20 mM $MgCl_2$, Ca^{2+} concentrations 2 mM and above approximately halved the activity of QI, while QII was less sensitive, retaining 80% of its activity at 4 mM $CaCl_2$ (Fig. 6).

PtdIns Phosphorylation Site

To examine whether the two enzyme forms catalyzed the phosphorylation of PtdIns in the 3- or 4-position, the reaction products were separated by TLC in the presence of borate (Walsh et al., 1991; Hegewald, 1996) and compared

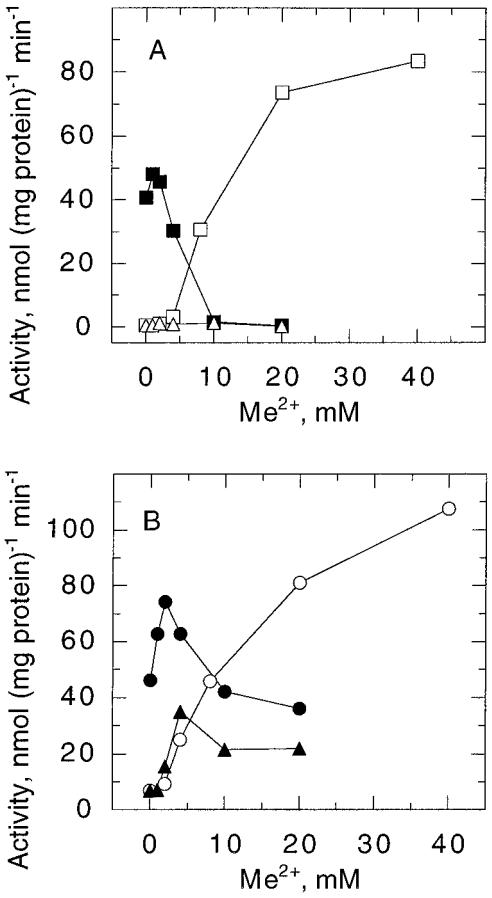

Figure 5. Effects of Mg^{2+} and Mn^{2+} on PtdIns 4-kinase activity. The activities of PtdIns 4-kinases QI (A) and QII (B) were measured in HEPES-NaOH, pH 7.5, with 0.4 mM PtdIns and 2 mM ATP. The divalent ion concentrations were varied as indicated; □, ○, Mg^{2+} concentration curve in the absence of Mn^{2+}; △, ▲, Mn^{2+} concentration curve in the absence of Mg^{2+}; ■, ●, Mn^{2+} concentration curve in the presence of 10 mM Mg^{2+}.

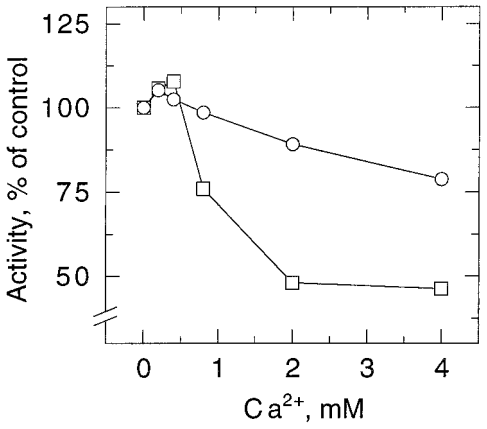

Figure 6. Effect of Ca^{2+} on PtdIns 4-kinases QI (□) and QII (○). PtdIns 4-kinase activities were measured in 50 mM HEPES-NaOH, pH 7.5, with 20 mM Mg^{2+} and 2 mM ATP. $CaCl_2$ concentrations were varied as indicated.

with the products obtained with PtdIns 3-kinase from rat liver cytosol (Fig. 7). Both forms of the plant enzyme catalyzed the formation of a phosphorylated product, which migrated more slowly than the wortmannin-sensitive product of the liver enzyme. This suggests that both QI and QII catalyze the phosphorylation of PtdIns in the 4-position rather than in the 3-position, which is further supported by the fact that both enzyme forms exhibited maximum activity in the presence of 0.2% (w/v) Triton X-100. Animal PtdIns 3-kinases are nearly inactive under such conditions (Fruman et al., 1998).

Wortmannin Inhibition

The hydrophobic, steroid-related fungal metabolite wortmannin is a potent inhibitor of most mammalian PtdIns 3-kinases (Carpenter and Cantley, 1990). Wortmannin irreversibly inhibits the PtdIns 3-kinase by reacting covalently with a Lys residue (Wymann et al., 1996) that is required for catalytic activity and is conserved in all PtdIns kinases (Fruman et al., 1998). Recent studies have shown that animal cells contain both wortmannin-sensitive and wortmannin-insensitive forms of PtdIns 4-kinases (Gehrmann and Heilmeyer, 1998). The sensitivity of PtdIns kinases to wortmannin varies considerably depending on the enzyme and enzyme source. In general, PtdIns 3-kinases are inhibited by concentrations in the nanomolar range, while wortmannin-sensitive PtdIns 4-kinases require micromolar concentrations for inhibition. An examination of the effect of wortmannin on the purified plant PtdIns 4-kinases showed that the drug inhibited QI with an IC_{50} value of approximately 7 μM (Fig. 8), 2 orders of magnitude higher than that reported for the sensitive form from animal cells (Downing et al., 1996). QII was less sensitive, being inhibited by 30% at 10 μM wortmannin.

Determination of Molecular Mass

The apparent molecular masses of QI and QII were estimated either by SDS-PAGE followed by western blotting

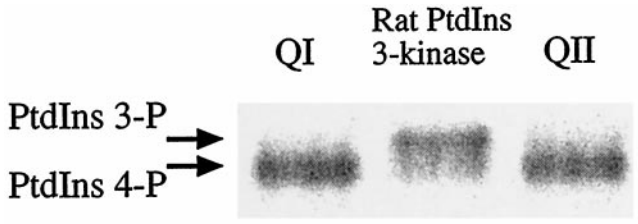

Figure 7. Identification of PtdIns phosphorylation site. Rat liver cytosol containing PtdIns 3-kinase activity and QI and QII fractions were incubated with [γ-^{32}P]ATP and the lipids extracted and phosphoinositides analyzed as described in "Materials and Methods."

using the antibody LKU raised against a peptide corresponding to a LKU sequence from human PtdIns kinase PI4Kα as the primary antibody, or by regeneration of PtdIns kinase activity from gel slices. Alternatively, enzyme fractions were incubated with [^{3}H]wortmannin-17-ol, which may irreversibly bind to wortmannin-sensitive PtdIns kinases (Balla et al., 1997), followed by SDS-PAGE and autoradiography. The results from these different approaches are shown in Figure 9.

The LKU antibody recognized two polypeptides in the heparin-Sepharose fraction (Fig. 9, lane 1) with apparent molecular masses of 65 and 120 kD, neither of which could be detected with the preimmune serum (not shown). The 120-kD polypeptide was also recognized by the antibody in the purified QI enzyme fraction (lane 2), whereas the 65-kD polypeptide was recognized in the QII fraction (lane 3). This suggests that the isolated PtdIns kinase forms are distinct proteins with these minimum molecular masses. Further support for this came from the wortmannin-binding studies showing a distinct wortmannin-labeled band at 120 kD among less strongly labeled bands after incubation with the QI enzyme fraction (lane 4), and from regeneration of PtdIns kinase activity after electrophoresis of the QII fraction showing enzyme activity around 65 kD (Fig. 9). No distinct wortmannin binding was seen in the QII fraction (lane 5), which was less sensitive to wortmannin inhibition, and no significant PtdIns kinase activity

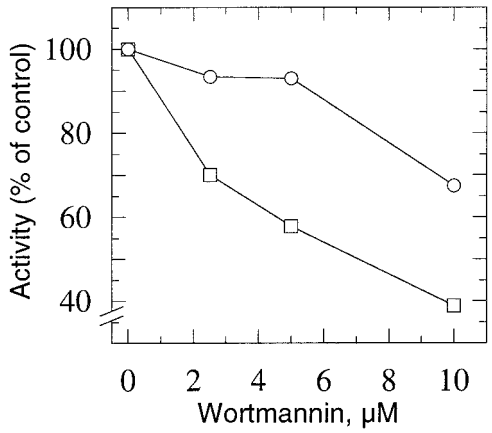

Figure 8. Effect of wortmannin on PtdIns 4-kinase activity. The QI (□) and QII (○) fractions were incubated with the indicated concentrations of wortmannin for 2 min prior to initiation of the PtdIns 4-kinase reaction by ATP.

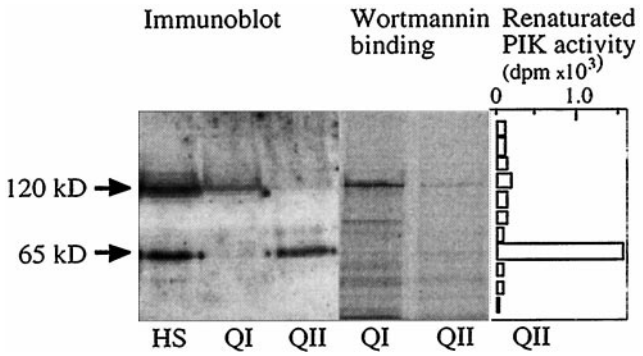

Figure 9. Estimation of PtdIns 4-kinase molecular mass. A heparin-Sepharose (HS) fraction and Q-Sepharose fractions QI and QII were analyzed by SDS-PAGE, followed by western blotting using an antibody raised against a peptide corresponding to a LKU sequence from human PtdIns kinase PI4Kα, or QI and QII lanes were sliced and analyzed with respect to their capacity to regenerate PtdIns 4-kinase activity. When QI and QII were analyzed for wortmannin binding, the fractions were incubated with ^{3}H-wortmannin-17-ol, followed by SDS-PAGE and autoradiography. Further details are described in "Materials and Methods."

could be regenerated after electrophoresis of the QI fraction (not shown).

DISCUSSION

The PtdIns kinase purification procedure, which starts with highly purified plasma membranes, resolved two PtdIns kinase fractions, QI and QII (Fig. 1), each purified 100- to 300-fold over the plasma membrane extract. As the PtdIns kinase activity of the leaf homogenate was too low to be analyzed accurately, the overall purification of the enzyme could not be calculated. We have found, however, that spinach leaf plasma membranes prepared by two-phase partitioning are enriched approximately 20-fold, suggesting that the PtdIns kinase activity in both QI and QII was enriched at least 2,000-fold over the homogenate. The specific activities of both of the purified enzyme fractions were in the range of 100 to 300 nmol mg^{-1} min^{-1} (variations between preparations mainly being due to the lability of the enzyme). This is 2- to 3-fold higher than the activity reported for PtdIns kinase purified from the soluble fraction of *D. parva* (Steinert et al., 1994), and some 20- to 30-fold higher than those purified from the soluble fraction of carrot cells (Okpodu et al., 1995) and *C. roseus* plasma membranes (Hanenberg et al., 1995). In comparison, the specific activity of PtdIns 4-kinases purified to near homogeneity from animal sources is in the range of 1 to 3 μmol $mg^{-1}min^{-1}$ (Porter et al., 1988; Walker et al., 1988), and from yeast it is 4 to 5 μmol mg^{-1} min^{-1} (Belunis et al., 1988; Flanagan and Thorner, 1992). Further purification of the QI or QII enzyme forms by any of several methods was precluded by their lability.

Although the QI and QII enzymes exhibited similar kinetic characteristics, i.e. similar K_m values for ATP (Fig. 2) and PtdIns (Fig. 3), a similar requirement for Mg^{2+} (Fig. 5), and similar pH activity profiles (Fig. 4), they differed with respect to several other important parameters, indicating that they represent distinct isoenzymes. Thus, Mn^{2+} could partly substitute for Mg^{2+} as the divalent cation in QII, while the QI kinase was inactive in the presence of Mn^{2+} (Fig. 5). QI was also more sensitive than QII to inhibition by millimolar concentrations of Ca^{2+} and micromolar concentrations of wortmannin (Figs. 7 and 8). The strongest evidence, however, that the PtdIns kinase activities of QI and QII indeed represent different molecular entities came from the analyses of their apparent molecular masses (Fig. 9), which were determined to be 120 and 65 kD, respectively, by immunoblotting after SDS-PAGE, as well as either ^{3}H-wortmannin binding (QI) or regeneration of PtdIns kinase activity following SDS-PAGE (QII). The immunoblots, which were quite specific for these two bands, also indicated little cross-contamination between the two purified fractions. As both forms catalyzed the formation of PtdIns 4-P rather than PtdIns 3-P (Fig. 7), a conclusion to be drawn is that the isolated activities represent two distinct forms of PtdIns 4-kinase and that these forms are tightly associated with spinach leaf plasma membranes.

An interesting feature of both the QI and the QII kinase was their behavior toward divalent cations. Both enzyme forms required high concentrations (above 20 mM) of Mg^{2+} for saturation (Fig. 5). While Mn^{2+} could not substitute for Mg^{2+} in the reaction catalyzed by the QI enzyme, the two divalent cations were equally efficient in supporting the activity of QII in the 1 to 4 mM concentration range. More importantly, at suboptimal Mg^{2+} concentrations, Mn^{2+} acted synergistically with Mg^{2+} to activate not only the QII enzyme, but also to some extent QI (Fig. 5). Such a synergistic activation of PtdIns kinase activity by Mg^{2+} and Mn^{2+} was observed earlier for the soluble *D. parva* enzyme (Steinert et al., 1994), in which the effect was much more pronounced than for the plasma membrane enzymes in the present study, although optimum synergistic conditions were not sought for these latter enzymes. A detailed kinetic study of the divalent ion requirement of the *D. parva* enzyme suggested that Mg^{2+} is involved in the formation of the Me^{2+}-ATP complex, whereas Mn^{2+} has a dual role: it can replace Mg^{2+} in the complex but can also bind to a separate stimulatory site of the enzyme (Steinert et al., 1994). A closer analysis of the role of Mn^{2+} might be rewarding, particularly for the QI enzyme, in which case Mn^{2+} is unable to replace Mg^{2+} in forming the catalytically active ATP complex. A similar synergistic effect has not been noted for any of the animal or yeast PtdIns 4-kinases.

Taken together, several biochemical characteristics exhibited by the QI and QII enzymes, including high K_m values for ATP and PtdIns, sensitivity to wortmannin, and comparatively low inhibition by adenosine and Ca^{2+}, indicate that these plant PtdIns 4-kinase forms share characteristics with the type III rather than the type II subclass of animal PtdIns 4-kinases. The two plant enzyme forms differed, however, in their apparent molecular masses from the two major animal types III kinases identified so far (approximately 65 and 120 kD compared with 92 and 230 kD, respectively).

In comparison with animal PtdIns 4-kinases, a strict classification of corresponding plant enzymes into distinct types is precluded in part by the incomplete data available

and in part by the disparate properties reported for the few plant enzymes isolated so far. Both the QI and the QII form apparently have properties different from those of the PtdIns kinase purified from *C. roseus* plasma membranes (Hanenberg et al., 1995), which is more similar to the animal type II enzyme with its low K_m values for ATP and PtdIns and its sensitivity to inhibition by adenosine. Instead, QI and QII share a similar high K_m for ATP with the soluble PtdIns kinases isolated from carrot (Okpodu et al., 1995) and *D. parva* (Steinert et al., 1994) cells, but seem less prone to inhibition by adenosine than these enzymes. The activity of the soluble kinases is supported equally well by Mn^{2+} and Mg^{2+} in the low millimolar concentration range, a property shared by the QII but not by the QI enzyme form.

As to the apparent molecular masses of the different PtdIns kinases, only the soluble carrot enzyme (Okpodu et al., 1995) and the QII plasma membrane enzyme have been renatured following SDS-PAGE from polypeptides of 80 and 65 kD, respectively. This should be compared with the likely molecular mass of 120 kD of the QI enzyme following western blotting and that of approximately 500 kD of both the membrane-bound *C. roseus* enzyme (Hanenberg et al., 1995) and the soluble *D. parva* enzyme (Steinert et al., 1994) following gel filtration (the latter two indicating the presence of PtdIns kinase in large complexes). Thus, from available biochemical evidence there is no unequivocal basis so far for a strict classification of plant PtdIns 4-kinases.

A further question is the relationship between the biochemically characterized soluble and membrane-bound PtdIns 4-kinases and the two plant kinases recently cloned in Arabidopsis encoding 126- and 205-kD proteins. These two cloned enzymes were not thoroughly characterized, however, precluding a closer comparison with the isolated kinases. As an increasing number of PtdIns 4-kinases are being isolated and characterized, cloned and sequenced, it is becoming evident that these enzymes exhibit divergent characteristics and are not easily assigned to any of the classical type II or III subclasses. Classification based entirely on gene sequence homologies rather than on biochemical properties may not be ideal either, as many of the recombinant enzymes have different or broader activities than previously realized (Fruman et al., 1998).

The membrane-associated PtdIns 4-kinase activity is predominantly localized to the plasma membrane fraction and seems to be tightly associated with the membrane, as it was only released upon detergent treatment. Interestingly, all PtdIns 4-kinases cloned and sequenced so far lack transmembrane domains (Martin, 1998), yet these enzymes may exist both as soluble and membrane-associated forms. The question then is whether these enzymes are integral membrane proteins or if they are associated with membranes by other mechanisms, perhaps in a reversible manner governed by physiological factors. In the latter case, potential mechanisms include post-translational lipid modification, providing tight association with the membrane through a lipid anchor, association by binding directly to specific lipids, or association via a protein anchored to the membrane. Interestingly, up to 20% of the plasma membrane-associated PtdIns 4-kinase activity in carrot could be selectively released by snake or bee venom phospholipase A_2 (Gross et al., 1992). The physiological significance of this finding is still uncertain but merits attention because phospholipase A_2 has also been implicated in several physiological processes in plants (for review, see Munnik et al., 1998). Several of the PtdIns 4-kinases cloned so far contain Pleckstrin homology domains, including the 205-kD Arabidopsis enzyme (Stevenson et al., 1998). Such domains have phosphoinositide-binding motifs that are considered essential for recruiting proteins containing these domains to membranes (for review, see Martin, 1998).

Apart from the two plasma membrane forms, distinct PtdIns 4-kinase activities are also found in spinach cytosol and in endomembranes (data not shown), indicating the existence of additional isoforms with different subcellular distributions. The physiological roles of PtdIns 4-P, and hence of PtdIns 4-kinases, in plant cells are far from clear, however. Given the high abundancy of PtdIns 4-P to PtdIns 4,5-P_2, plant PtdIns 4-P may have a more significant role in processes other than the classical signal transduction pathway involving inositol 1,4,5-trisphosphate and diacylglycerol. Thus, phosphoinositides have been shown to modulate the activities of plant enzymes such as phospholipase D, protein kinases, H^+-ATPase, and diacylglycerol kinase, and are implicated in the regulation of cytoskeletal dynamics (for review, see Munnik et al., 1998) and membrane trafficking (Matsuoka et al., 1995). Clearly, it will be of interest to further characterize the different PtdIns 4-kinases and their modes of membrane interaction to fully understand the roles of PtdIns 4-P in plant cells.

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