RESEARCH COMMUNICATION

Identification of a phosphatidylinositol 3-hydroxy kinase in plant cells: association with the cytoskeleton

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Radiolabelling experiments have revealed that plant cells contain the two 3-phosphorylated phosphoinositides: PtdIns3P and PtdIns(3,4)P₂ [Brearley and Hanke (1992) *Biochem. J.* **283**, 255–260]. However, nothing is known about the enzymes involved in the metabolism of these plant 3-phosphorylated phosphoinositides. In this study we demonstrate the presence of a PtdIns 3-hydroxy kinase(s) in plant cells. This activity was

enriched in the cytoskeletal fraction whereas only low levels of phosphoinositide 3-hydroxy kinase could be detected in plasma membranes and microsomal preparations. This cytoskeletal phosphoinositide 3-hydroxy kinase was found to be wortmannin insensitive and thus resembles PtdIns-specific 3-hydroxy kinases of which vps34p is one example.

INTRODUCTION

Increased turnover of the 4-phosphorylated phosphoinositides (4-PPIs) leading to second messenger production is now recognized as a critical step preceding many cellular responses to external stimuli (Berridge, 1993). The discovery of a novel family of 3-phosphorylated phosphoinositides (3-PPIs) has added a further degree of complexity to phosphoinositide research. These low-abundance lipids are poor substrates for phosphoinositidase C and it has therefore been hypothesized that the lipids themselves may act as signals (Serunian et al., 1989). The 3-PPIs have been implicated in regulating a variety of cellular events including: the cell cycle, protein targeting, reorganization of the cytoskeleton and cellular differentiation (Eberle et al., 1990; Dobos et al., 1993; Schu et al., 1993). However, the exact function and route of biosynthesis of the 3-PPIs in eukaryotes remains controversial.

Two main pathways for the synthesis of the 3-PPIs are currently recognized. The first involves 3-phosphorylation of PtdIns (Majerus et al., 1990; Schu et al., 1993) and the second, 3-phosphorylation of PtdIns(4,5)P₂, followed by stepwise dephosphorylation of the resulting PtdIns(3,4,5)P₃, the 3-phosphate being removed last (Stephens et al., 1991). It is becoming clear that the lipids formed by these two pathways may differ in their cellular roles and that clues to their function can be obtained from detailed studies of the enzymes involved in their biosynthesis (Stephens et al., 1994).

The first demonstration of the presence of PtdIns3P and PtdIns(3,4)P₂ in plants came from radiolabelling studies *in vivo* (Brearley and Hanke, 1992, 1993). When short labelling times were used it was found that the D4 phosphate of PtdIns(3,4)P₂ had a higher specific activity than the D3 phosphate, suggesting a biosynthetic route involving direct 3-phosphorylation of PtdIns followed by 4-phosphorylation of the resulting PtdIns3P (Brearley and Hanke, 1993). Recent findings of rapid turnover of PtdIns3P in the green alga *Chlamydomonas* (Munnik et al., 1994), and the lack of success in identifying PtdIns(3,4,5)P₃ in

this organism, supports the hypothesis that 3-PPIs in plants are likely to be synthesized by direct phosphorylation of PtdIns, rather than by dephosphorylation of PtdIns(3,4,5)P₃. Validation of this idea however depends on the demonstration of PtdIns 3-hydroxy kinase activity in plant tissues. This prompted us to search for such an enzyme in cultured carrot (*Daucus carota* L.) cells.

We present evidence for the presence in plants of a phosphatidylinositol 3-hydroxy kinase activity which is enriched in the cytoskeletal fraction; only low levels of PtdIns 3-hydroxy kinase activity could be detected in whole cell extracts and membrane fractions. The cytoskeletal phosphoinositide 3-hydroxy kinase was found to be sensitive to Triton X-100, but relatively insensitive to the fungal toxin, wortmannin (0–230 nM). These results show that the plant cytoskeletal phosphoinositide 3-hydroxy kinase has properties in common with a family of wortmannin-insensitive PtdIns-specific 3-hydroxy kinases, of which the yeast vacuolar protein-sorting protein vps34p is a member. This similarity, and the cytoskeletal location of the phosphoinositide 3-hydroxy kinase, suggests that the plant kinase may be involved in protein targeting or reorganizing the cytoskeleton.

MATERIALS AND METHODS

L- α -Phosphatidylinositol (from soybean) and wortmannin were from Sigma. [2-³H]L- α -Phosphatidylinositol 4-phosphate (185 GBq/mmol) and [γ -³³P]ATP (71.6 TBq/mmol, 370 MBq/ml) were from DupontNEN.

Cell cultures

Daucus carota L. 'Norwich' suspension cultures were maintained as described by Lloyd et al. (1980).

Cytoskeletons

Cytoskeletons were prepared from *D. carota* suspension cells essentially as described by Xu et al. (1992), with the following modifications. Cells were protoplasted in 1% (w/v) Driselase (Sigma) and either 2% (w/v) Onozuka R-10 or 1% (w/v) Onzuka RS cellulase (Yakult Pharmaceuticals and Co. Ltd, Japan). Cells were detergent extracted as described by Xu et al. (1992), but cytoskeletons were purified on a sucrose gradient after the Triton X-100 extraction. The cytoskeletons were diluted 7-fold in buffer A [100 mM Pipes, 10 mM MgCl₂, 10 mM EGTA, 0.4 M sorbitol, 10% (v/v) dimethylsulphoxide and proteinase inhibitor cocktail (10 μ M benzamidine hydrochloride, 1 mM phenylmethanesulphonyl fluoride, 1 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 μ g/ml phenanthroline, 10 μ g/ml pepstatin A]. Cytoskeletons were pelleted by centrifugation for 10 min at 320 *g* and resuspended in fresh buffer A. Cytoskeletons were immediately frozen and stored at -20°C until use.

Plasma membranes and microsomes

Plasma membranes and microsomes were purified essentially as described by Drøbak et al. (1994). Briefly, 100 g wet weight of carrot-suspension culture cells were harvested and homogenized and microsomes prepared. The microsomal uptake media contained 5 mM KCl. Microsomes were frozen at -20°C . Plasma membranes were prepared by aqueous polymer two-phase partitioning and were 6–10-fold enriched in appropriate marker enzymes e.g. 1,3 β -glucan synthetase 2 (for details, see Drøbak et al., 1994).

Whole cell extract

Cells (2 days old) were harvested by centrifugation, and homogenized in a glass homogenizer in a medium containing: 100 mM Hepes/KOH, pH 7.5, 10 mM EGTA, 10 mM MgCl₂, 10 mM dithiothreitol (DTT), and proteinase inhibitors (see buffer A). All steps were performed at 4°C . The homogenate was centrifuged for 5 min at 300 *g* and the supernatant immediately frozen at -20°C until use.

3-Hydroxy kinase assay

3-Hydroxy kinase assay was performed in a total volume of 50 μ l in medium containing 50 mM Hepes/KOH, pH 7.5, 5 mM MgCl₂, 30 mM DTT, 0.1 mM Na₂VO₄ (prepared as in Fohr et al., 1989), 50 μ M [γ -³²P]ATP (150 KBq/nmol) and proteinase inhibitors (see buffer A for list of proteinase inhibitors and concns.). The PtdIns substrate was added as a sonicated suspension of liposomes (20 μ g) in 5 mM Hepes/KOH, pH 7.5, and any detergent added after sonication as described by Carpenter et al. (1990). Cellular protein was added (15–20 μ g) and the reaction terminated after 5 min at 25°C (or as indicated).

Lipid extraction and analysis

Kinase assays were terminated by addition of 2 ml of ice-cold chloroform/methanol/conc. HCl (100:100:0.7, by vol.), and authentic [2-³H]PtdIns4P was added. After 2 min on ice, 400 μ l of 0.6 M HCl was added. The extract was agitated for 30 s and then the top phase removed. 800 μ l of chloroform/methanol/0.6 M HCl (3:48:47, by vol.) was added and the extract again agitated. This was repeated three times. Lower phases containing phosphoinositides were dried down under O₂-free N₂ then resuspended in 60 μ l of chloroform/methanol (2:1, v/v) and spotted onto t.l.c. plates and an additional 20 μ l (2:1) added to the tubes to recover

residual lipids. Two t.l.c. systems were used for lipid analysis. The first was chloroform/methanol/conc. ammonia/water (90:70:4:16, by vol.) as running solvent, using LK5 t.l.c. plates (Whatman). The second t.l.c. system was the borate system described by Walsh et al. (1991) for separating PtdIns4P and PtdIns3P. The silica gel 60 plates (Sigma) used for the borate separation were equilibrated in the tank for 1 h prior to chromatography.

Deacylation

The lipids used for deacylation had approx. 20000 d.p.m. [³H]PtdIns4P added at the stage of reaction termination. The radioactive spots containing PtdIns4P and putative PtdIns3P were recovered from t.l.c. plates after identification by autoradiography, deacylated according to Clarke and Dawson (1981) and then treated as described by Drøbak and Roberts (1992). GroPtdInsPs were separated by h.p.l.c. using a Partisil 10 SAX column (250 mm $\times$ 4.6 mm; Whatman, Maidstone, Kent, U.K.) and a gradient of (NH₄)₂HPO₄, pH 3.8, as follows: 0–10 min, 0% (NH₄)₂HPO₄; 10 min–70 min, 0%–25% (NH₄)₂HPO₄; 70 min–80 min, 25% (NH₄)₂HPO₄; 80 min–95 min, 0% (NH₄)₂HPO₄. Fractions were collected every 15 s starting at 21 min. Radioactivity was determined by liquid scintillation spectrophotometry (LKB Rack-Beta) using Pico-Fluor 15 scintillant (Canberra-Packard, Pangbourne, U.K.) and a dual ³²P/³H counting programme.

Deglyceration

³²P-labelled lipids were separated using the borate t.l.c. system and the putative PtdIns3P spot removed. Authentic [2-³H]-PtdIns4P was added and both lipids were deacylated as described above. Deglyceration was then carried out essentially as described by Brown and Steward (1966). The InsPs were separated by isocratic h.p.l.c. using a Partisil 10 SAX column (Whatman) and elution with 240 mM NaH₂PO₄ pH 3.8. (For details, see Wreggett and Irvine 1989; Drøbak et al., 1991.) Fractions were collected at 10 s intervals starting at 3 min and radioactivity was determined as above.

RESULTS AND DISCUSSION

Identification of radiolabelled PtdIns3P as a product of PtdIns phosphorylation

An initial search for 3-phosphorylated kinase products was carried out using the borate t.l.c. system. This separation system utilizes the ability of PtdIns4P, but not of PtdIns3P, to form borate complexes (Walsh et al., 1991). Figure 1 shows separation of radiolabelled lipids obtained from kinase assays employing various cellular fractions. When authentic [³H]PtdIns4P was used as an internal standard and carried through the extraction procedure, ³H could only be detected in the lower of the two spots (i.e. PtdIns4P). This confirms that the lower spot in the autoradiogram in Figure 1 contains PtdIns4P and that the other compound is unlikely to have been produced non-enzymically after termination of the reaction.

H.p.l.c. of glycerophosphoinositide and inositol phosphate derivatives

Separation by h.p.l.c. of ³²P-labelled glycerophosphoinositides derived from the two phosphorylated lipid products identified by borate t.l.c., is shown in Figure 2(a). One compound co-chromatographed exactly with an internal standard of authentic

Figure 1 T.l.c. separation of ^{33}P -labelled kinase products

Lanes A–D: whole cell extract, purified plasma membranes, microsomes and cytoskeletons (all carrot) respectively. The whole cell extract, microsomes and cytoskeletons contained 18 μg protein and the plasma membrane only 10 μg . The amounts of PtdIns3P formed were 5.0 pmoles/mg, 14.2 pmoles/mg, 17.5 pmoles/mg and 37.4 pmoles/mg for whole cell extract, plasma membranes, microsomes, and cytoskeletons respectively. All assays were terminated after 5 min.

$[\text{H}]\text{GroPtdIns4P}$, whereas the other eluted 1.25 min earlier. This elution profile is identical to that obtained by Auger et al., (1990) when separating authentic GroPtdIns3P and GroPtdIns4P. Isocratic h.p.l.c. separation of authentic $[\text{H}]\text{Ins}(1,4)\text{P}_2$ (derived from an internal $[\text{H}]\text{PtdIns4P}$ standard) and the ^{33}P -labelled inositol bisphosphate (derived by deacylation and deglyceration of putative PtdIns3P recovered from borate t.l.c. plates) is illustrated in Figure 2(b). The absence of any $[\text{H}]\text{Ins}(1,4)\text{P}_2$ indicates that borate t.l.c. effectively separates PtdIns4P from putative PtdIns3P. Similar conclusions were reached by Munnik et al. (1994).

Given the D-1 linkage of the diester phosphate of PtdIns, only five possible inositol bisphosphates can be derived by deglyceration of its monophosphorylated derivatives [i.e. $\text{Ins}(1,X)\text{P}_2$, $X = 2-6$]. The chromatographic properties of the InsP_2 eluting before $\text{Ins}(1,4)\text{P}_2$ are entirely consistent with this isomer being $\text{Ins}(1,3)\text{P}_2$ (Stephens et al., 1989; Wreggett and Irvine 1989; Munnik et al., 1994). The co-chromatography of PtdIns4P and the other labelled PtdInsP in conventional t.l.c.-systems and their separation in borate systems, combined with the chromatographic behaviour of the obtained GroPtdInsP and InsP derivatives, all indicate that the unknown kinase product is PtdIns3P.

Properties of cytoskeletal phosphoinositide 3-hydroxy kinase

The properties of the plant cytoskeletal phosphoinositide 3-hydroxy kinase were examined under linear reaction conditions with respect to time and protein.

The effect of Triton X-100 on the cytoskeletal phosphoinositide 3-hydroxy kinase was tested at a range of Triton concentrations from 0.02 % to 0.2 % (v/v) (results not shown). Triton was found to inhibit the phosphoinositide 3-hydroxy kinase at least 70 % at

Figure 2 H.p.l.c. separation of GroPtdInsPs and inositol phosphates

(a) Separation of GroPtdIns derivatives obtained after deacylation of the compounds contained in the two spots marked PtdIns4P and PtdIns3P in Figure 1. (b) Separation of InsPs obtained after deglyceration of the PtdInsP spot spiked with authentic $[\text{H}]\text{PtdIns4P}$. ^3H label is indicated by ∇ ; ^{33}P label is indicated by $\bullet$. For further details see Materials and methods/Discussion.

all the concentrations used (i.e. both above and below the critical micellar concentration). The mammalian phosphoinositide 3-hydroxy kinases are also inhibited by Triton X-100, although in the case of the PtdIns 3-hydroxy kinase the inhibition displays quantitative differences (Whitman et al., 1987; Stephens et al., 1994).

The marked Triton X-100 sensitivity of the cytoskeletal 3-hydroxy kinase, and the specific assay conditions used in this study (very low levels of high-specific radioactivity ATP), may help to explain why Xu et al. (1992) did not find any evidence for any significant PtdIns 3-hydroxy kinase activity in their study of lipid kinases in carrot cytoskeletons.

Sensitivity of the cytoskeletal phosphoinositide 3-hydroxy kinase to the fungal toxin, wortmannin is shown in Figure 3. Kinase activity is inhibited only approximately 20 % by 230 nM wortmannin. Wortmannin is a very potent inhibitor of the mammalian PtdIns(4,5) P_2 3-hydroxy kinases with an IC_{50} of 1 nM (Arcaro and Wymann, 1993). The mammalian PtdIns 3-hydroxy specific kinase however, is only 20 % inhibited by approximately 200 nM wortmannin and the yeast PtdIns-specific 3-hydroxy kinase, vps34p is also resistant to inhibition by this toxin (Stephens et al., 1994; Woscholski et al., 1994). With regard to its sensitivity to wortmannin, the cytoskeletal phospho-

Figure 3 Effect of wortmannin on the cytoskeletal PtdIns 3-hydroxy kinase

The cytoskeletal 3-hydroxy kinase was assayed at various concentrations of wortmannin.

inositide 3-hydroxy kinase most closely resembles the PtdIns-specific 3-hydroxy kinases of mammals and yeast.

Possible physiological roles for the plant PtdIns 3-hydroxy kinase

The points of similarity between the plant cytoskeletal phosphoinositide 3-hydroxy kinase and the other known eukaryotic PtdIns 3-hydroxy kinases may help to give clues to its function.

The mammalian PtdIns-specific 3-hydroxy kinase is only thought to be active at specific times during the cell cycle or induced by agonists, as little activity was found in unsynchronized cell populations (Stephens et al., 1994). The idea that such a kinase could be involved in the cell-cycle progression is given credence by the study of Dobos et al. (1993) who found that PtdIns3P was elevated in G2/M populations of synchronized HT-29 cells compared with a G1 population. Such a function for the cytoskeletal phosphoinositide kinase cannot be ruled out.

Evidence suggests that the yeast PtdIns 3-hydroxy kinase, vps34p, is involved in protein sorting to the yeast vacuole equivalent (Schu et al., 1993). The large vacuole, which is a conspicuous feature of plant cells, may also have a similar protein targeting system. The idea that the cytoskeletal phosphoinositide 3-hydroxy kinase may be involved in this targeting process must also be considered.

The cytoskeletal location of this plant phosphoinositide 3-hydroxy kinase is somewhat surprising. A PtdIns(4,5)P₂ 3-hydroxy kinase has been reported to associate with the membrane skeleton in activated platelets (Zhang et al., 1992), but so far there is no evidence that either vps34p or the mammalian PtdIns-specific 3-hydroxy kinases are cytoskeletally associated. One of the suggested functions for 3-PPIs is in modulation of cytoskeletal dynamics in response to extracellular stimuli (Eberle et al., 1990). Cytoskeletal dynamics are especially important in plant cells given the suggested role of the cyto-

skeleton in determining the plane of cell division. It is therefore possible that the cytoskeletal phosphoinositide 3-hydroxy kinase is a component of a signal transduction cascade allowing the plant cytoskeleton to respond to the environment.

The data presented above support evidence from labelling studies *in vivo* using intact plant cells that the probable pathway of synthesis of PtdIns3P and PtdIns(3,4)P₂ in plants follows the following scheme: PtdIns → PtdIns3P → PtdIns(3,4)P₂ (Brearley and Hanke 1993; Munnik et al., 1994). The demonstration that PtdIns 3-hydroxy kinase activity can be assayed *in vitro* in a number of higher plant extracts should facilitate further progress in the study of the plant phosphoinositide 3-hydroxy kinase(s) and their role(s) in a variety of cellular events.

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