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Communication

Association of Phosphatidylinositol Kinase, Phosphatidylinositol Monophosphate Kinase, and Diacylglycerol Kinase with the Cytoskeleton and F-Actin Fractions of Carrot (*Daucus carota* L.) Cells Grown in Suspension Culture[1]

Response to Cell Wall-Degrading Enzymes

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ABSTRACT

Phosphatidylinositol kinase (PI), phosphatidylinositol monophosphate (PIP) kinase, and diacylglycerol (DAG) kinase activities were detected in the cytoskeletal fraction isolated from microsomes and plasma membranes of carrot (*Daucus carota* L.) cells grown in suspension culture. The lipid kinase activities were associated with the actin filament fraction (F-actin fraction) isolated from the cytoskeleton. The PI and PIP kinase activity in the F-actin fraction significantly increased after cells were treated with Driselase, a mixture of cell wall-degrading enzymes; however, the DAG kinase activity in the F-actin fraction was unaffected by the Driselase treatment. These data indicate that at least one form of PI, PIP, and DAG kinase preferentially associates with actin filaments and/or actin binding proteins and that cytoskeletal-associated PI and PIP kinase activities can change in response to external stimulation.

The inositol phospholipids PIP[2] and PIP_2 play important roles in signal transduction (2, 6, 9, 17). The enzymes involved in the biosynthesis of these lipids, i.e. PI 4-kinase and PI 4-monophosphate 5-kinase, have been found to exist in the plasma membrane (5, 22), cytosol (19), and nucleus (13) of plant cells. In addition to being the source of second messengers, PIP and PIP_2 may be involved in regulating the organization of the cytoskeleton (12, 14, 16, 23, 25, 26).

It has been demonstrated in animal cells that the enzymes involved in phosphoinositide metabolism, i.e. PI, PIP, and DAG kinase activities, also are associated with the actin filament fraction of the cytoskeleton (20). Recently, we have shown that PI kinase can be released from the plasma membrane of carrot (*Daucus carota* L.) cells by PLA_2 and activated by a soluble, heat-stable activator (11). One question posed by these findings was, where would the released PI kinase go within the cell? In this article, we show that PI, PIP, and DAG kinase can associate with the cytoskeleton and that PI and PIP kinase respond to short-term treatment with cell wall-degrading enzymes.

MATERIALS AND METHODS

Materials

Carrot (*Daucus carota* L.) cells grown in suspension culture were transferred weekly as previously described (3). The cells were used for experiments on the fourth day.

PI, PIP, and DAG (dioctanoyl-glycerol) were purchased from Sigma, Triton X-100 was obtained from Pierce, and Driselase came from Plenum Scientific Research, Inc. [γ-^{32}P]ATP and monoclonal anti-chicken muscle actin antibody (clone C4) were purchased from ICN; anti-mouse antibody conjugated with alkaline phosphatase and substrates were from Promega, and hydrophobic polyvinylidenedifluoride film was from Gelman Sciences.

Preparation of Cytoskeleton from Microsomes

The cells were collected on filter paper (Whatman No. 1) and homogenized at 4°C with low ionic strength CSB as described by Abe and Davies (1) at a ratio of 1 g fresh weight of cells to 1 mL of buffer with minor modifications. The CSB was modified to contain 5 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 2 mM EGTA, 1 mM PMSF, 1 mg leupeptin/100 mL, and 0.1 mM sodium vanadate. A 5 mM stock solution of sodium vanadate was prepared according to Gallagher and Leonard (10). The homogenate was centrifuged at 1,000*g* for 4 min, and the resulting supernatant was centrifuged at 40,000*g* for

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[2] Abbreviations: PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate; CSB, cytoskeleton isolation buffer; DAG, diacylglycerol; PI, phosphatidylinositol; PLA_2, phospholipase A_2.

45 min. The microsomal pellet was suspended in CSB containing 1% (v/v) Triton X-100 and shaken on ice at 110 rpm for 20 min. The cytoskeleton (the Triton-insoluble fraction) was collected by centrifugation at 50,000*g* for 20 min, and was subsequently washed twice with 1% Triton X-100 and once without Triton X-100 in CSB. The washed cytoskeletal fraction was used to assay for lipid kinase activity except when determining the relative lipid kinase activities in the Triton-soluble and -insoluble fractions. For these experiments, the Triton-insoluble pellet was not washed, but was resuspended in 1% Triton X-100 to the same volume as the supernatant and used for the lipid kinase assays.

Driselase Treatment and Preparation of Cytoskeleton from Plasma Membrane

Cells were treated with 2% (w/v) Driselase at 25°C for 10 min, and plasma membranes were prepared using aqueous two-phase partitioning as previously described (5). The cytoskeleton was isolated in the same manner described for microsomes.

Actin Depolymerization and Repolymerization

The F-actin fraction, which contained actin filaments and actin-binding proteins, was prepared as described by Payrastre et al. (20) with some modifications to the buffers. Briefly, the cytoskeletal fraction (approximately 0.5 mL obtained by Triton X-100 extraction as described above) was treated in a centrifuge tube with 2.0 mL of depolymerizing buffer containing 5 mM Hepes (pH 7.0), 0.6 M KI, 100 mM KCl, 2 mM EGTA, 1 mM PMSF, 1 mg leupeptin/100 mL, and 0.1 mM sodium vanadate. After 20 min on ice with gentle shaking, the solution was centrifuged at 40,000*g* for 20 min. The supernatant was dialyzed two times against 500 mL of CSB containing 5 mM benzamidine instead of leupeptin and centrifuged at 12,000*g* for 5 min. The resultant pellet (denoted the F-actin fraction) was suspended in 0.3 mL of polymerization buffer (CSB with 5 mM benzamidine). Protein was determined according to Smith et al. (21) with BSA as a standard. The volume was adjusted to give the desired concentration of protein as indicated, and aliquots were used for phosphorylation or gel electrophoresis. For some experiments, the F-actin fraction was subject to a second cycle of depolymerization and repolymerization.

Lipid Kinase Assay

The lipid phosphorylation assays were performed using [γ-^{32}P]ATP (0.2 μCi/nmole) in the presence of 25 μg of exogenous substrate (DAG, PI, or PIP) and 0.25% Triton X-100 in a final volume of 50 μL as previously described (5). The reaction was stopped after 15 min unless otherwise noted. The lipids were extracted and chromatographed on thin-layer plates (7). Radioactivity was quantitated by a Bioscan System 500 Image scanner.

SDS-PAGE and Immunoblotting

Proteins were solubilized in SDS-PAGE sample buffer, boiled for 4 min and separated on a 12% SDS-PAGE (15), and transferred onto polyvinylidenedifluoride film. Immunoblotting was performed using a kit from Promega according to the accompanying instruction manual, except the incubation time with the primary antibody was overnight, and with the secondary antibody was 3 h. A monoclonal anti-actin antibody (clone C4) from ICN was used as the primary antibody, and anti-mouse IgG conjugated with alkaline phosphatase was the secondary antibody.

RESULTS

PI, PIP, and DAG Kinase Are Associated with the Cytoskeleton

Extraction with nonionic detergent such as Triton X-100 or Nonidet P-40 typically has been used to obtain cytoskeleton from membranes and cells (18). As shown in Figure 1A, PI, PIP, and DAG kinase activities were found in the Triton-insoluble fraction, indicating the presence of these enzymes in the cytoskeletal fraction. Of the total PI kinase activity in the microsomal fraction, greater than 40% was recovered with the cytoskeleton, and when plasma membranes were used, greater than 70% of the total PI kinase activity was recovered with the cytoskeleton. The PIP and DAG kinase activities associated with the cytoskeletal fraction were greater than 90% of the total activity when either plasma membrane or microsomes were used. The lipid kinases in the Triton-soluble and -insoluble (cytoskeletal) fractions were assayed with similar amounts of protein and under identical assay conditions, and there was no loss of total activity.

To determine whether or not lipid kinase activity was associated with the actin filaments, we depolymerized the actin filaments in the cytoskeletal fraction to obtain soluble actin and actin-binding proteins. The solubilized actin was repolymerized to form actin filaments (20) and was centrifuged at 12,000*g* for 5 min. The 12,000*g* pellet is denoted the F-actin fraction. Under these conditions, large actin filaments and actin bundles and the associated actin-binding proteins pellet (8, 20); however, proteins such as BSA remain in the supernatant (W. Yang and W.F. Boss, unpublished results).

Lipid kinase activity was concentrated in the F-actin fraction (pellet), whereas the supernatant contained little activity (Fig. 1, B-D). The strong preference of the lipid kinases to pellet with F-actin indicated an association of the lipid kinases with actin and/or actin-binding proteins. To more accurately compare the enzyme activities in the supernatant and the pellet, the supernatant was concentrated to give a protein concentration equal to that of the resuspended F-actin pellet. Under these conditions, PIP- and PI kinase-specific activities were approximately 2 and 3 times higher, respectively, in the F-actin fraction than in the supernatant (Table I).

After two cycles of depolymerization-repolymerization, lipid kinase activity still was recovered preferentially in the F-actin fraction (Table I); however, there was a loss of PI and DAG kinase activity with the second polymerization cycle, and not all of the lipid kinase activity was recovered in the F-actin pellet. This would be expected if on the second cycle, not all of the actin had repolymerized into large filaments, and, thus, the amount of actin and actin-binding proteins in the supernatant had increased. Western blot analysis of the

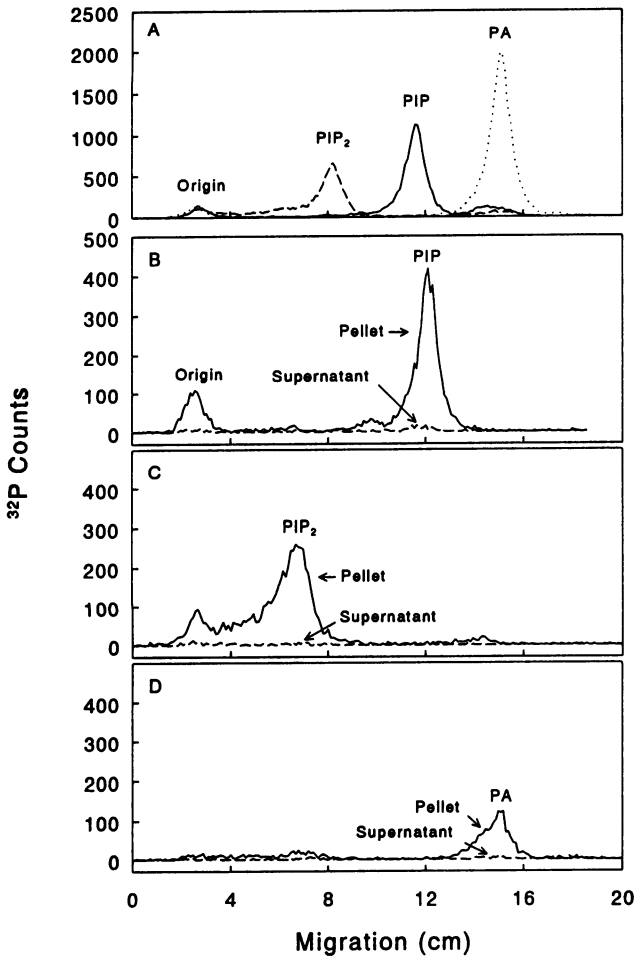

Figure 1. A, Phosphorylation of exogenous PI (—), PIP (- -), and DAG (···) by carrot cell cytoskeleton. Cytoskeleton was obtained by extracting microsomes with 1% (v/v) Triton X-100 as described in "Materials and Methods." The phosphorylation was performed using [γ^{32}P]ATP with 25 μg of exogenous lipid and 16 μg of cytoskeletal protein. The reaction time was 15 min for PI kinase and 30 min for PIP and DAG kinase. The products were separated on LK-5D thin-layer plates and analyzed with a Bioscan System 500 Image scanner. B–D, Phosphorylation of exogenous PI (B), PIP (C), and DAG (D) by the F-actin fraction. The F-actin fraction was isolated from microsomal cytoskeleton. Equal aliquots (20 μL) of both the F-actin fraction (pellet, 20 μg of protein) and supernatant (1.9 μg of protein) were used for phosphorylation as described in "Materials and Methods." The reaction time was 15 min for PI and 30 min for PIP and DAG kinase. The products were separated on LK-5D thin-layer plates and analyzed with a Bioscan System 500 Image scanner.

proteins of the supernatant and F-actin pellet using anti-actin antibodies confirmed that the actin in the supernatant increased with the second repolymerization cycle (Fig. 2, compare lanes 4 and 6). In addition, even though protease inhibitors were present, there was evidence for increased proteolysis of actin during the second cycle. Actin has a molecular mass of 43 kD. As a result of the second polymerization cycle, there was an increase in a 40-kD peptide that cross-

Table I. *A Comparison of Lipid Kinase Activity in the F-Actin Fraction from Microsomes and the Remaining Supernatant after One and Two Depolymerization-Repolymerization Cycles*

Cytoskeleton obtained from microsomal membranes by Triton X-100 extraction was subject to two cycles of depolymerization-repolymerization as described in "Materials and Methods." The supernatants were concentrated to give 8 μg protein/20 μL and were used for the lipid kinase assay. An equal concentration of resuspended F-actin pellet was used. The products were separated on thin-layer plates and analyzed with a Bioscan System 500 Image scanner.

	[^{32}P] Phospholipids Formed		
	PIP	PIP_2	PA
	pmol min^{-1} mg^{-1} protein		
First cycle[a]			
F-actin pellet	212 ± 34	25 ± 6	14 ± 3
Supernatant	74 ± 11	14 ± 3	8 ± 2
Second cycle[b]			
F-actin pellet	124.5	38.4	5.9
Supernatant	108.6	26.0	4.5

[a] Numbers are the means ± SD of four values from two experiments. [b] Numbers are the average of two values from one experiment.

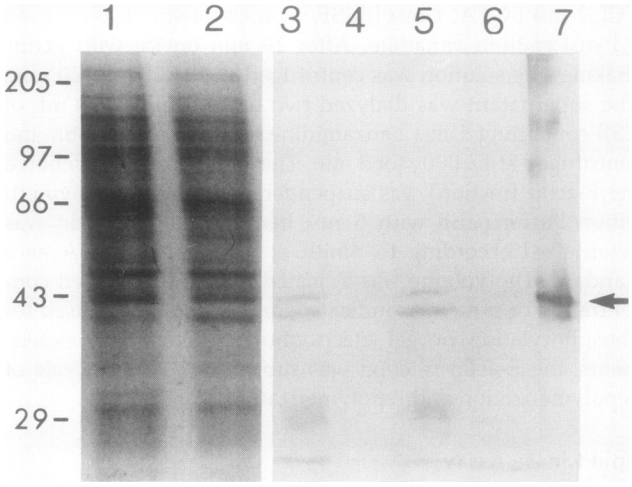

Figure 2. A comparison of the proteins present in the F-actin fraction (pellet) (lanes 1–3 and 5) and supernatant (lanes 4 and 6). Lanes 1 and 2 are a Coomassie-stained SDS gel and lanes 3 to 7 are an immunoblot with actin antibody. Lanes 1–3 and 5 contained 15 μg of protein and lanes 4 and 6 contained 6 μg of protein. Lanes 1 and 3, F-actin fraction obtained after a single cycle of depolymerization-repolymerization; lanes 2 and 5, F-actin fraction obtained after two cycles of depolymerization-repolymerization; lane 4, the supernatant obtained after a single cycle of depolymerization-repolymerization; lane 6, the supernatant fraction obtained after two cycles of depolymerization-polymerization; lane 7, chicken muscle actin (10 μg). Arrow denotes the migration of chicken muscle actin (43 kD).

reacted with actin antibodies (Fig. 2, compare lanes 1 and 2 and lanes 3 and 5).

A 40-kD peptide that cross-reacted with actin antibodies also was present in the supernatant after the second polymerization cycle (Fig. 2, lane 6). A similar 40-kD peptide was observed in commercial chicken actin (Fig. 2, lane 7), and it was assumed to result from proteolytic breakdown of actin. The lower molecular mass peptides that cross-reacted with the anti-actin antibody in the carrot preparations also were assumed to result from proteolysis. Increased proteolysis during the second polymerization-repolymerization cycle would have contributed to both the loss of F-actin and lipid kinase activity. For these reasons, the second cycle was not studied further.

Response of Cytoskeleton-Associated Kinases to Driselase Treatment

Because it was shown that a short-term treatment with Driselase led to an increase in PI and PIP kinase activity in the isolated plasma membranes of carrot cells (5), we studied the effect of Driselase treatment on cytoskeletal-associated lipid kinases. For these experiments, cytoskeleton was isolated from plasma membrane vesicles obtained from treated and control cells. As shown in Table II, the PI and PIP kinase activities increased after Driselase treatment, whereas there was no significant change in DAG kinase activity. This result is consistent with the data previously obtained by Chen and Boss using isolated plasma membranes (5).

The increase in the PI and PIP kinase activity was even greater when the cytoskeleton was subjected to a cycle of depolymerization-repolymerization, and the repolymerized F-actin fraction was assayed (Table II). The specific activity increased to 5 and 10 times the control values for PI and PIP kinase, respectively.

Table II. *The Effect of Driselase Treatment on the Lipid Kinase Activity in the Cytoskeleton and F-Actin Fractions Obtained from Plasma Membranes*

Cells were treated with 2% (w/v) Driselase at 25°C for 10 min and, plasma membranes were prepared using aqueous two-phase partitioning. Cytoskeleton was isolated by extracting with 1% Triton X-100, and F-actin was isolated by one cycle of depolymerization-repolymerization. For the lipid kinase assays, 16 μg of cytoskeletal protein or 20 μg of F-actin protein were used. Results are the means ± SD of four values from two different experiments.

Lipids Formed	Cytoskeleton[a]		F-Actin[b]	
	Control	Driselase	Control	Driselase
	% of control			
PIP	100	169 ± 25	100	495 ± 178
PIP_2	100	350 ± 124	100	1122 ± 194
PA	100	70 ± 21	100	135 ± 21

[a] Representative values of products formed for the controls are: PIP, 176 ± 34; PIP_2, 10 ± 0.6; and PA, 105 ± 6 pmol min^{-1} mg^{-1} protein. [b] Representative values of products formed for the controls are: PIP, 34 ± 4; PIP_2, 15 ± 2.5; and PA, 5.8 ± 0.2 pmol min^{-1} mg^{-1} protein.

DISCUSSION

In this article, we have shown the association of PI, PIP, and DAG kinase activity with cytoskeleton isolated from carrot membranes. Furthermore, when the F-actin fraction was separated from the cytoskeleton by depolymerization and repolymerization, the lipid kinases were found to be associated with the F-actin fraction. This is in agreement with the data from animal cells (20). While this paper was being revised, Xu et al. (24) reported that PI kinase activity was associated with cytoskeleton isolated from carrot protoplasts; however, they could not detect DAG or PIP kinase activity. The fact that these authors could not detect DAG and PIP kinase activity and that we could may be due to differences in the starting material, the methods for preparing cytoskeleton, the assay conditions, or, in the case of DAG kinase, the substrate used.

A major portion of the membrane-associated lipid kinase activities was recovered in the cytoskeletal fraction. The preferential loss of PI and DAG kinase activity during the isolation of the F-actin fraction from plasma membranes (Table II) may have resulted from differences in enzyme stability or binding affinity; however, the fact that the specific activity of the PIP kinase increased in the plasma membrane F-actin fraction while PI and DAG kinase activities decreased suggested that binding affinity was an important factor.

Using A431 cell cultures, Payrastre et al. (20) reported that when the cells were treated with epidermal growth factor, the cytoskeletal-associated PI, PIP, and DAG kinase activities increased. Chen and Boss (5) have shown that treating carrot cells with cell wall-degrading enzymes resulted in an increase in the [^{3}H]inositol-labeled PIP_2 recovered from whole cell extracts and in both PI and PIP kinase activities of isolated plasma membranes. Using the same protocol, we have shown that Driselase treatment increased the lipid kinase activity associated with the cytoskeleton and, more specifically, with the F-actin fraction isolated from plasma membranes. The specific activity of PI and PIP kinase associated with the F-actin increased 5-and 10-fold, respectively, compared to the control values.

The increase in PI and PIP kinase specific activity in the F-actin fraction as a result of the short-term Driselase treatment could result from (a) an increase in the relative amount of enzyme or of an activator, (b) a decrease in inhibitors of the lipid kinases, or (c) covalent modification of the lipid kinases or regulatory proteins. Movement of PI and PIP kinase or an activator protein from the membrane to the cytoskeleton is possible. We have shown that PI kinase can be released from isolated plasma membranes by PLA_2 and activated by a soluble protein (11). In addition, PI kinase activity is present in the soluble fraction (19) and, thus, might move from the cytosol to the membrane upon stimulation. Changes in the distribution of the enzymes will be studied when antibodies to the lipid kinases become available. Although there is no evidence for covalent modification of PI-4 kinase or PIP-5 kinase (4), we cannot rule out the possibility that either the lipid kinases or an activator was modified in response to external stimuli, and that this covalent modification either increased the specific activity of the enzyme already associ-

ated with the cytoskeletal fraction or enhanced the movement of the enzyme to the cytoskeleton.

Several laboratories have demonstrated that the inositol phospholipids PIP and PIP_2 promote actin polymerization through interaction with a number of actin-binding proteins that depolymerize actin filaments (12, 14, 16, 25, 26). Thus, changes in PIP or PIP_2 levels in discrete regions of the plasma membrane could affect cytoskeletal structure and, thereby, affect cell growth and differentiation. The fact that the enzymes involved in inositol phospholipid biosynthesis are associated with plant cytoskeleton and that lipid kinase activities change in response to external stimuli indicates that the inositol phospholipids may play a critical role in regulating cytoskeletal structure in stimulus-response mechanisms in higher plants.

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