Plant Physiol. (1995) 107: 491–500

Purification and Characterization of a Soluble Phosphatidylinositol 4-Kinase from Carrot Suspension Culture Cells[1]

Camellia Moses Okpodu[2], Wolfgang Gross, William Burkhart[3], and Wendy F. Boss*

Department of Botany, North Carolina State University, Raleigh, North Carolina 27695–7612 (C.M.O., W.B., W.F.B.); and Institut für Pflanzenphysiologie und Mikrobiologie, Freie Universität Berlin, 1000 Berlin 33, Germany (W.G.)

Previously we reported the presence of a soluble phosphatidylinositol 4-kinase (PI 4-kinase) in carrot (*Daucus carota* L.) suspension culture cells (C.M. Okpodu, W. Gross, W.F. Boss [1990] Plant Physiol 93: S-63). We have purified the enzyme over 1000-fold using Q-Sepharose ion exchange, hydroxylapatite, and G-100 gel filtration column chromatography. The M_r of the enzyme was estimated to be 83,000 by gel filtration. PI 4-kinase activity was recovered after renaturation of the 80-kD region of polyacrylamide gels, and an 80-kD peptide cross-reacted with antibodies to the yeast 55-kD membrane-associated PI 4-kinase on western blots. The isolated lipid kinase phosphorylated PI but not lysophosphatidylinositol or phosphatidylinositol monophosphate. Maximal PI kinase activity occurred when the substrate was added as Triton X-100/PI mixed micelles at pH 8. The enzyme required divalent cations. At low concentrations (1–5 mM), Mn^{2+} was more effective than Mg^{2+} in increasing enzyme activity; however, maximal activity occurred at 25 to 40 mM Mg^{2+}. Calcium from 0.01 μM to 1 mM had no effect on the enzyme activity. The K_m of the enzyme for ATP was estimated to be between 400 and 463 μM. The enzyme was inhibited by adenosine (100 μM); however, ADP (up to 100 μM) had no effect on the activity. The biochemical characteristics of the carrot soluble PI 4-kinase are compared with the previously reported PI 4-kinases from animals and yeast.

The enzyme phosphatidylinositol 4-kinase (ATP:phosphatidylinositol 4-phosphotransferase, EC 2.7.1.67) catalyzes the formation of PI-4-P from PI. PI-4-P can be further phosphorylated to PI-4,5-P_2, which is the source of the second messengers IP_3 and diacylglycerol (Majerus et al., 1986; Berridge, 1987; Coté and Crain, 1993; Drøbak, 1993). In addition, PI-4-P and PI-4,5-P_2 directly affect the activity of membrane enzymes (Varsayni et al., 1983; Schäfer et al., 1987; Memon et al., 1989). PI 4-kinase has been studied extensively because it catalyzes the first committed step in the synthesis of PI-4,5-P_2 (for review, see Carpenter and Cantley, 1990; Carman et al., 1992, 1994; Pike, 1992).

In mammalian systems, PI kinase has been classified into three subclasses (types I, II, and III). The type I enzyme, PI 3-kinase, phosphorylates the D-3 position of the inositol ring. The enzyme consists of a 110-kD catalytic subunit and an 85-kD regulatory subunit and has an apparent K_m for ATP in the range of 30 to 60 μM (Whitman et al., 1987; Carpenter and Cantley, 1990; Morgan et al., 1990). Divalent cations (Mg^{2+} or Ca^{2+}) are required for maximal activity and Mn^{2+} cannot be substituted for Mg^{2+} (Whitman et al., 1987). The type I PI 3-kinase is sensitive to nonionic detergents and is not involved in the classical signal transduction pathway that produces inositol-1,4,5-trisphosphate. In mammalian cells, the 110-kD PI 3-kinase appears to be essential for receptor Tyr kinase-mediated cell transformation (Carpenter and Cantley, 1990; Cantley et al., 1991; Fantl et al., 1992).

The type II and type III enzymes phosphorylate inositol on the D-4 position (i.e. both are PI 4-kinases). They are classified according to their biochemical properties (for review, see Carpenter and Cantley, 1990; Pike, 1992). Briefly, the type II enzymes range in molecular mass from 45 to 55 kD. They have been purified from a number of sources, including human erythrocytes (Graziani et al., 1992), bovine uterus (Porter et al., 1988), A431 tissue culture cells (Walker et al., 1988), and porcine liver (Hou et al., 1988). The type II kinases are stimulated by nonionic detergents and are inhibited by low levels of adenosine (K_i 20–100 μM) and ADP. They have a K_m for ATP in the range of 30 to 100 μM, and divalent cations are required for maximal activity; however, calcium at 0.3 to 0.4 mM inhibits the activity.

Type III PI kinases are stimulated by Triton X-100, have a higher K_m for ATP (250–742 μM) than the type II PI kinase, and are relatively insensitive to adenosine (K_i 1.5 mM; Pike, 1992). The molecular mass of the type III PI kinase in a crude preparation of bovine brain was first

[1] This work was supported in part by grant No. DCB-8812580 from the National Science Foundation and in part by the North Carolina Agricultural Research Service.

[2] Present address: Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnical Institute, Blacksburg, VA 24061–0331.

[3] Present address: Glaxo Research Institute, Glaxo Inc., Research Triangle Park, NC 27709.

* Corresponding author; e-mail wendy_boss@ncsu.edu; fax 1–919–515–3436.

Abbreviations: BiP, binding protein; HSP, heat-shock proteins; HSC, heat-shock cognate; IP_3, inositol trisphosphate; LPI, lysophosphatidylinositol; MeOH, methanol; PI, phosphatidylinositol; PI-4-P phosphatidylinositol 4 monophosphate; PI-4,5-P_2, phosphatidylinositol 4,5 bisphosphate.

estimated to be 230 kD using Suc density gradients in the presence of Triton X-100 (Endemann et al., 1987); however, a type III PI 4-kinase, with similar biochemical properties, was purified from rat brains and the molecular mass was estimated to be between 76 and 80 kD by SDS-PAGE (Yamakawa and Takenawa, 1988), suggesting that the higher estimate might have resulted from the formation of detergent micelles.

The plant plasma membrane PI 4-kinase has been extensively characterized (Sommarin and Sandelius, 1988) but never purified. Using gel filtration as well as antibodies to a yeast PI 4-kinase, W. Gross and W.F. Boss (unpublished data) estimated the M_r of the plant membrane PI kinase to be 55,000. Attempts to isolate the membrane enzyme from carrots (*Daucus carota* L.) led to the characterization of two protein activators. Gross et al. (1992) reported that phospholipase A_2 released a plasma membrane PI 4-kinase that could be activated by a 70-kD soluble, heat-stable, and trypsin-sensitive component. Yang et al. (1993) have purified a 49-kD PI 4-kinase activator protein, PIK-A49, that binds actin and has translational elongation factor-1α activity. Studies of cytoskeleton-associated proteins showed that the PI 4-kinase activity was associated with the F-actin-rich fraction isolated from membranes (Payrastre et al., 1991; Tan and Boss, 1992).

In addition to the membrane-associated PI 4-kinases, soluble forms have been reported in mammalian cells but never purified or thoroughly characterized (Michell et al., 1967; Imai et al., 1986). Recently, Flanagan and Thorner (1992) characterized and purified a soluble PI 4-kinase from yeast. The yeast enzyme required Triton X-100 for maximal activity and had an apparent K_m for ATP of 100 μM, similar to the type II enzymes; however, unlike the type II enzymes, it was insensitive to micromolar concentrations of adenosine. Furthermore, the purified enzyme had a subunit apparent mol wt of 125,000, which was larger than the 45- and 55-kD yeast membrane-associated PI 4-kinases previously reported (Belunis et al., 1988; Nickels et al., 1992) or other type II enzymes. Garcia-Bustos et al. (1994), using antibodies raised against the nuclear envelope, cloned the gene encoding a yeast nuclear PI 4-kinase that had the same predicted amino acid sequence and calculated molecular mass reported by Flanagan et al. (1993).

The yeast soluble PI 4-kinase co-purified with HSP82 and HSC82, which are both members of the HSP90 family of heat-shock proteins. Extensive work has been done on the interaction of HSP90 with the steroid receptor complexes (Sanchez et al., 1987; Smith and Toft, 1990) and other proteins, including protein kinases (Miyata and Yahara, 1992; Smith et al., 1993). The problem of co-purification of the yeast soluble PI 4-kinase with HSP90s was circumvented by a combination of genetic construction of null mutants in HSC82 as well as high ionic-strength fractionation to remove the HSP82 (Flanagan and Thorner, 1992).

Until recently, the soluble PI 4-kinase was thought not to exist in plants (Wissing et al., 1992). However, we found evidence for a soluble form of the PI 4-kinase in carrot suspension culture cells (Okpodu et al., 1990) and report here the characterization and purification of the enzyme. The soluble plant PI 4-kinase differs from the soluble yeast enzyme with regard to its sensitivity to micromolar concentrations of adenosine, relative molecular mass, and kinetic properties.

MATERIALS AND METHODS

Materials

Two lines of carrot (*Daucus carota* L.) cell cultures were grown and maintained as previously described (Boss and Ruesink, 1979; Chen and Boss, 1990). Cells were transferred every 7 d and used for experiments 3 d after transfer. In both cell lines, the soluble PI 4-kinase had the same molecular mass and enzyme kinetics. The initial characterizations were done with the cells derived from carrot roots (Boss and Ruesink, 1979). Even though there was no difference in specific activity in the crude homogenates from the two cell lines, the specific activity recovered after the first purification step (Q-Sepharose) was higher with the culture derived from wild carrot cells, indicating that the enzyme was either more stable or that there were fewer contaminating proteins or both. Therefore, the purification was done with the cell line derived from hypocotyls of wild carrot (Chen and Boss, 1990).

Chemicals

All chemicals were reagent grade. [γ-^{32}P]ATP (7000 Ci mmol^{-1}) was purchased from ICN (Costa Mesa, CA). Soybean phosphatidylinositol, Sepharose G-100, electrophoresis reagents, and two-dimensional pI molecular mass markers were purchased from Sigma. Q-Sepharose and Sephacryl S-300 was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). Prestained SDS-PAGE molecular mass markers, hydroxylapatite (Bio-Gel HT), and ampholytes were purchased from Bio-Rad. Monoclonal anti-mouse (IgG) antibodies conjugated to alkaline phosphatase and substrates were from Promega (Madison, WI). Triton X-100 and the bicinchoninic acid protein assay were purchased from Pierce (Rockford, IL). Pro-green staining kit was purchased from Integrated Separation Systems (Natick, MA).

Enzyme Assay

PI 4-kinase activity was assayed using the following procedure: 20 μL of enzyme preparation was added to 30 μL of reaction mixture to give a final concentration of 55 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 0.69 mM PI, 0.2 mM sodium molybdate, 0.25% Triton X-100, and 1.3 mM ATP containing [γ-^{32}P]ATP (0.18 μCi/nmol). PI was added to the reaction mixture as Triton X-100 mixed micelles. The stock solution of PI (10 mg/mL) was made in 3.0% Triton X-100 unless otherwise stated. When LPI was used as a substrate, it was reconstituted in CH_3Cl:MeOH (1:2). Aliquots of 20 μL (from a 1 mg/mL stock of LPI) were placed in test tubes and dried under nitrogen as described by Wheeler and Boss (1989). When the effects of cations were tested, $MgCl_2$ or $MnCl_2$ were added at the concentrations

indicated; calcium was added as $CaCl_2$/EGTA to give the free calcium concentrations indicated as described by Buckhout (1984). After 10 min at 25°C, the reaction was stopped by the addition of 1.5 mL of ice-cold $CHCl_3$:MeOH (1:2). Lipids were extracted according to Cho et al. (1992) and chromatographed on LK5D thin-layer plates that had been presoaked for 85 s in 1% (w/v) potassium oxalate and dried overnight at 120°C. $CHCl_3$:MeOH:NH_4OH:H_2O (86:76:6:18) was used as a solvent. Radioactivity was quantitated with a Bioscan System (Washington, DC) 500 Imaging Scanner.

Purification of Soluble PI 4-Kinase

All steps were carried out at 4°C. To obtain the soluble enzyme, 50 g fresh weight of carrot suspension culture cells were homogenized in 50 mL of buffer A (50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 g of polyvinylpolypyrrolidine, and 15% glycerol) and in the presence of 50-mL glass beads (0.2 mm diameter) in a Waring blender for 2 min at low setting. The resulting homogenate was filtered through Miracloth and centrifuged for 15 min at 5,000*g*. The supernatant was then centrifuged for 1 h at 45,000*g*. The supernatant was fractionated on a Q-Sepharose column (2.5 × 25 cm) preequilibrated with buffer B (25 mM Tris-HCl, pH 7.5, 1 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol, and 15% glycerol). The column was eluted with 0.2 M NaCl in buffer B (150 mL) followed by a linear gradient from 0.2 to 0.9 M NaCl in buffer B (200 mL). The flow rate was 75 mL/h and 7.5-mL fractions were collected. Fractions containing PI 4-kinase activity were pooled and diluted 4-fold with buffer C (0.01 M sodium phosphate, pH 8.0, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 10% glycerol). The diluted fractions were loaded directly onto a hydroxylapatite column (2.5 × 5 cm) and eluted with a 150-mL linear gradient of sodium phosphate (0.01–0.3 M) made in buffer C. The flow rate was 15 mL/h and 5-mL fractions were collected. Two peaks of activity eluted. The second peak of activity, which eluted at 0.22 M sodium phosphate, was pooled and ammonium sulfate was slowly added to the eluate to a final concentration of 65% (w/v) saturation. The solution was centrifuged at 45,000*g* for 15 min and the supernatant was removed. The precipitate from this step was resuspended in 6 mL of buffer D (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 20% [v/v] glycerol, 10 mM 2-mercaptoethanol, and 0.1 M NaCl) and loaded onto a Sepharose G-100 or Sephacryl S-300 column. The column was eluted with 100 mL of buffer D (at a flow rate of 5 mL/h) and 2-mL fractions were collected. Attempts to purify the enzyme by PI-Sepharose, Heparin, Cibacron Blue 3GA Agarose, and reactive Blue Agarose chromatography were unsuccessful.

Native Mol Wt Determination

The relative molecular mass of soluble PI 4-kinase was estimated by gel filtration on a Sephacryl S-300 column (1.5 × 30 cm) or G-100 column and eluted with buffer D. Lactate dehydrogenase (140,000), glutamyltransferase (80,000), BSA (66,000), horseradish peroxidase (40,000), and Cyt *c* (12,500) were used as markers.

SDS-PAGE Electrophoresis

Electrophoresis was performed using 10% (w/v) polyacrylamide gels containing SDS and 2-mercaptoethanol as described by Laemmli (1970). The relative molecular mass of PI 4-kinase was estimated using M_r markers. The gels were stained with 0.1% (w/v) Coomassie blue in 50% (v/v) methanol and 10% (v/v) acetic acid and destained with 50% (v/v) methanol and 10% (v/v) acetic acid.

Renaturation Assay from Gel Slices

Gel slices were assayed for activity using a modified method of Satiel et al. (1987). Briefly, enzyme preparations were solubilized in 62 mM Tris-HCl, pH 8.0, 0.5% (v/v) Triton X-100, 2 mM DTT, 10% (v/v) glycerol and applied to a 7% (w/v) polyacrylamide gel. After electrophoresis at 4°C, two lanes (plus and minus protein) were cut into 1-cm slices, minced in assay buffer, incubated for 12 h at 4°C, and assayed for PI 4-kinase activity. A third lane was silver stained.

Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). Samples were electrophoresed in the first dimension using an IEF gel with a pH gradient of 5 to 7. Electrophoresis for the second dimension was performed using a 10% polyacrylamide slab gel with SDS. The pI values of the polypeptides were calculated by comparison with the migration of proteins with known pI values: trypsin inhibitor from soybean (pI 4.6), ovalbumin (pI 5.1), carbonic anhydrase I (pI 5.9), and myoglobin from horse heart (pI 7.6). For sequencing, after electrophoresis in the second dimension, the gels were stained with progreen. The band with pI 5.6 was excised from the gel, electroeluted, and sequenced.

Peptide Sequencing

The protein sample was applied to a Hewlett-Packard hydrophobic sequencing column according to the manufacturer's protocol. In situ pyridylethylation and digestion with endoproteinase Lys-C (Wako Chemicals, Richmond, VA) was performed according to Burkhart (1993). After digestion, peptides were separated by reverse-phase HPLC using a Hypersil ODS column (2.1 × 100 mm, Hewlett-Packard) employing a linear gradient of 8 to 48% acetonitrile in 0.1% TFA over 80 min with a flow rate of 100 μL/min.

Protein Assays

Protein was measured by the Bradford (1976) and the modified bicinchoninic acid methods (Wiechelman et al., 1988). With the bicinchoninic acid method, DTT was reduced with iodoacetamide.

Immunoblotting

Protein samples were separated by SDS-PAGE (10% [w/v] polyacrylamide) and transferred onto nitrocellulose according to the method of Bjerrum and Schafer-Nielsen (1986). The primary antibodies were raised in mice, and incubation with the primary antibody was done overnight at 25°C. Anti-mouse IgG conjugated to alkaline phosphatase was used to visualize the cross-reactivity. Antibodies to HSP90 (AC19) were provided by Dr. David O. Toft (Mayo Clinic, Rochester, MN). AC19 is a mouse monoclonal IgG that was prepared against purified HSP90 from water mold, *Achlya ambisexualis*, essentially as described for a similar antibody (Riehl et al., 1985). This antibody does not recognize native HSP90, but it provides specific detection of HSP90 from several plant and animal species by western blotting. Dr. George Carman (Rutgers University) provided antibodies to the yeast PI 4-kinase (Buxeda et al., 1991). The antibodies to the yeast PI 4-kinase had been purified by DEAE-Affi-Gel blue column chromatography. Western blots were done with and without primary antibody to check for nonspecific binding.

RESULTS

Enzyme Purification

The soluble PI 4-kinase activity represented only about 5 to 10% of the total PI 4-kinase activity recovered from the carrot homogenate (data not shown); however, it proved to be easier to purify than the membrane-associated form of the enzyme. Preliminary experiments indicated that the specific activity of the soluble PI kinase of the carrot cells grown in suspension culture was similar to that of grocery store carrot roots, celery, cabbage, spinach, and cauliflower. Because the carrot cells could be grown easily in large quantities under controlled conditions, they were used to purify the soluble form of the PI 4-kinase as outlined in Table I. In contrast to the purification of a soluble PI 4-kinase from yeast (Flanagan and Thorner, 1992), where ammonium sulfate could be used as the first step in the purification, with the soluble carrot enzyme all activity was lost if ammonium sulfate was used as the initial fractionation step. Therefore, the 40,000*g* supernatant was loaded directly onto a Q-Sepharose ion-exchange column. Although the sample was applied immediately to the Q-Sepharose column, the low yield obtained may result in part from the presence of proteolytic enzymes in the crude supernatant. Once fractionated on Q-Sepharose, the enzyme retained activity in 20% glycerol (v/v) at −20°C for more than 4 weeks.

Previously we described the use of heparin affinity chromatography as the final step in the purification (Okpodu et al., 1990). However, less than 0.01% of the kinase activity was recovered from the heparin column; therefore, we switched to hydroxylapatite and routinely used the three columns indicated in the purification scheme (Table I). The hydroxylapatite column resulted in a 24-fold increase in specific activity. Using this procedure the recovery was 11%, and we obtained an active fraction containing one major Coomassie-staining band at approximately 80-kD on SDS-PAGE (Fig. 1). Importantly, PI 4-kinase activity could be recovered from the 80-kD region of a 7% (w/v) polyacrylamide gel (Fig. 2). The M_r of the PI 4-kinase was estimated to be 83,000 by gel filtration (Fig. 3).

Characterization of Soluble PI 4-Kinase

Because the enzyme activity was more stable in the Q-Sepharose fractions than in the crude supernatant and because relatively large quantities of active enzyme could be recovered after Q-Sepharose, this fraction was used to optimize assay conditions and to characterize the enzyme. The product formed, [^{32}P]PIP, was analyzed by A. Graziani in the laboratory of L. Cantley (Department of Physiology, Tufts University Medical School, Boston, MA). HPLC analysis of the deacylated reaction product verified that more than 99% was PI-4-P and that less than 1% was PI-3-P (C.M. Okpodu, W.F. Boss, unpublished data). The specificity of PI 4-kinase was tested using LPI and PI-4-P as substrates. Neither LPI nor PI-4-P was phosphorylated under the assay conditions used (Fig. 4).

The influence of divalent cations on the soluble PI 4-kinase activity was studied as demonstrated in Figure 5. For these experiments, the enzyme was dialyzed against buffer containing 1 mM DTT and 10% (v/v) glycerol in 25 mM Tris-HCl (pH 8). Cations were added to the reaction mixture at the concentrations noted. Mn^{2+} was more effective than Mg^{2+} at low concentrations (<5 mM); however, at high concentrations Mg^{+2} was the most effective cation. The enzyme activity in the presence of 5 to 40 mM Mn^{2+} was only 40% of that obtained with 40 mM Mg^{2+} (Fig. 5A). Increasing the free calcium from 0.01 μM to 1 mM using a calcium/EGTA buffer (Fig. 5B) had no significant effect on the soluble PI 4-kinase activity compared with the control (EGTA alone) according to Student's *t* test (confidence interval of 95%).

Table I. *Purification of the soluble PI 4-kinase from carrot suspension culture cells*

Sample	Total Protein	Total Activity	Specific Activity	Fold	Percent Recovery
	mg	*pmol/min*	*pmol min^{-1} mg^{-1}*		
Crude	44	3025	6.8	1	100
Q-Sepharose	8	788	98.5	14.5	26
Hydroxylapatite	0.2	480	2400	343	16
$(NH_4)_2SO_4$ precipitant	0.09	420	4666	686	14
Sepharose G-100	0.04	320	7500	1070	11

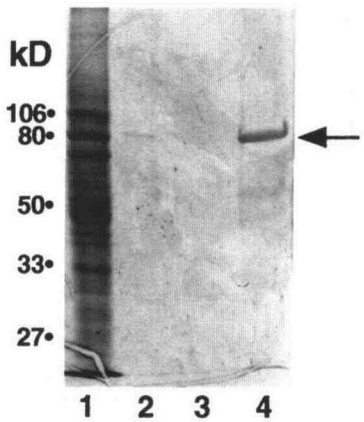

Figure 1. SDS-PAGE of purified PI 4-kinase. Each step of the purification scheme was analyzed by SDS-PAGE. An 80-kD polypeptide correlated with the PI 4-kinase activity on a 10% SDS-PAGE stained with Coomassie blue. Lane 1, Crude supernatant (20 μg); lane 2, purified soluble PI 4-kinase from the G-100 fraction (5 μg); lane 3, 0 to 65% (w/v, saturation) ammonium sulfate supernatant (20 μg); lane 4, the PI 4-kinase-enriched hydroxylapatite fraction (20 μg).

The activity of the soluble PI 4-kinase was almost 6-fold higher in the presence of Triton X-100 (Fig. 6A). Activity was maximal at 0.05 to 0.1% (v/v) Triton and decreased slightly at higher Triton concentrations; however, Triton at up to 0.5% (v/v) did not inhibit enzyme activity. Enzyme activity increased with increasing pH up to pH 8.0 (Fig. 6B). Lineweaver-Burk plots estimated the K_m for ATP to be 400 μM (Fig. 7A). Using Eadie-Hostee plots the apparent K_m for ATP was 463 μM (Fig. 7B). Adenosine (100 μM), which inhibits type II PI 4-kinases (Carpenter and Cantley, 1990; Pike, 1992), decreased the soluble PI 4-kinase (Fig. 8A). ADP (100 μM) had no effect on the soluble PI 4-kinase (Fig. 8B).

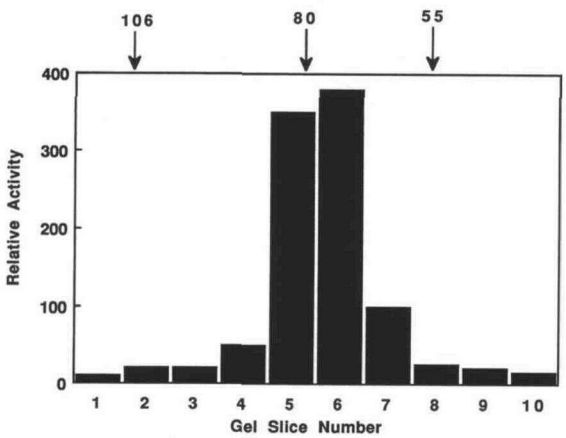

Figure 2. Renaturation of PI 4-kinase activity after polyacrylamide electrophoresis. Duplicate samples (20 μg) of partially purified PI 4-kinase from the hydroxylapatite column were subjected to 7% (w/v) polyacrylamide electrophoresis in a min-slab gel apparatus. One lane was stained with Coomassie blue. Two other lanes (plus and minus protein) were cut into 0.5-cm slices (1–10) and assayed for PI 4-kinase activity. Note that the gel was overloaded in order to recover active enzyme, and therefore, enzyme activity was detected in a broad band with maximum activity corresponding to the 80-kD region. Migration of the molecular mass markers (kD) are indicated at the top of the figure.

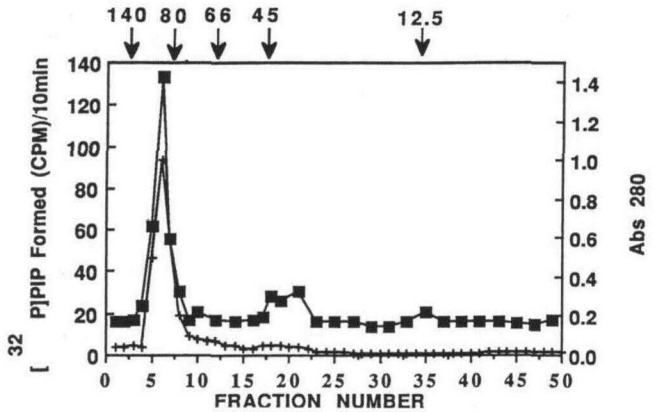

Figure 3. Elution profile of PI 4-kinase activity on a G-100 column. PI 4-kinase activity is reported as [^{32}P]phosphatidylinositol monophosphate (cpm) formed in 10 min (■). Protein was monitored as A_{280} (+). The arrows indicate the elution profiles of protein standards.

The carrot PI 4-kinase also was characterized with regard to cross-reactivity with antibodies raised against the 55-kD membrane-associated PI 4-kinase from yeast (Buxeda et al., 1991). When the most purified fraction from the G-100 column was analyzed by one-dimensional gel electrophoresis, we were able to detect only one polypeptide with Coomassie staining (Fig. 1). This 80-kD polypeptide band cross-reacted with antibodies raised against the yeast membrane PI 4-kinase (Fig. 9a). When the 80-kD band was analyzed by two-dimensional electrophoresis, antibodies

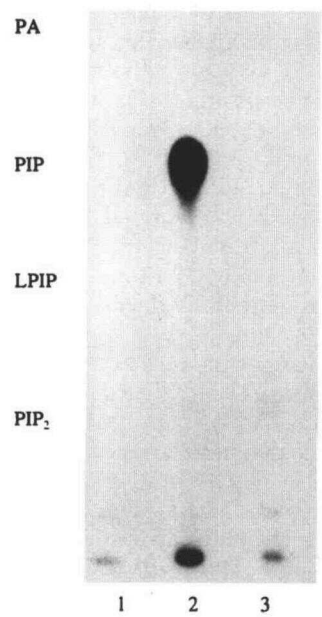

Figure 4. Substrate specificity. An autoradiograph showing the ^{32}P-lipids formed using different substrates (lane 1, LPI; lane 2, PI; lane 3, phosphatidylinositol monophosphate). The assay conditions were as described in "Materials and Methods." The same amount of enzyme was used for each assay.

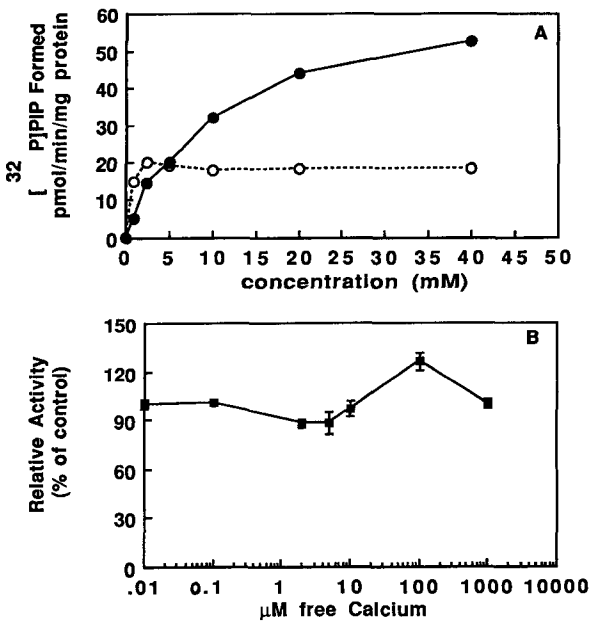

Figure 5. Effect of divalent cations on the soluble PI 4-kinase activity. Soluble PI 4-kinase activity was measured at the indicated concentrations of $MnCl_2$ (○), $MgCl_2$(●) (A) and $CaCl_2$ (B). In A, representative results are presented as pmol [^{32}P]phosphatidylinositol monophosphate min^{-1} mg^{-1} protein. In B, results are presented as percent control. The control contained 2.5 mM EGTA and the specific activity was 78 pmol [^{32}P]phosphatidylinositol monophosphate min^{-1} mg^{-1} protein. The numbers are the averages of duplicate values. In both A and B, the experiments were repeated three times and the trends were consistent. The differences in the control values in A and B reflect the differences in the enzyme preparations.

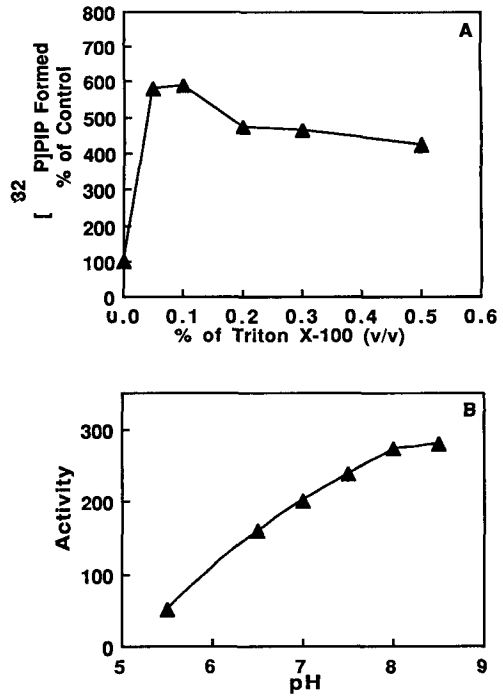

Figure 6. A, Effect of Triton X-100 and pH on the soluble PI kinase activity. The soluble enzyme was assayed for PI kinase activity with increasing concentrations of Triton X-100. B, PI kinase activity (cpm) was measured at the indicated pH values with 30 mM Mes-Tris or Tris-Mes buffer in the presence of exogenous substrate. The experiments were repeated three times and the trends were consistent (representative data are shown).

to yeast membrane PI 4-kinase recognized one polypeptide with a pI of 5.6 (Fig. 9b). However, when this fraction that was enriched in PI 4-kinase activity was used for amino acid sequence analysis, the sequence obtained from 15 fragments (142 amino acids) was 86% identical to HSC82 from tomato (Koning et al., 1992) and *Arabidopsis* (Takahashi et al., 1992), suggesting that a HSC had co-purified with the PI 4-kinase as was reported in yeast (Flanagan and Thorner, 1993). Unfortunately, the HSC was much more abundant than the soluble PI 4-kinase, and even when the peptides were proteolytically degraded, only sequence for the HSC was detectable.

HSC82 is a member of the HSP90 family of stress-induced polypeptides. The cognates are constitutively expressed. In mammalian systems, the HSP90 family of stress proteins has been shown to co-purify with known steroid receptors (Catelli et al., 1985) and to enhance protein kinase activity (Miyata and Yahara, 1992). Dissociation of the HSP90s from the receptors can be achieved in vitro with the addition of ammonium sulfate (Sanchez et al., 1987). Similarly, Flanagan and Thorner (1992) precipitated the soluble PI 4-kinase with ammonium sulfate (0–55% [w/v] saturation) and recovered HSC82 in the supernatant. When we treated the PI 4-kinase fraction from the G-100 column with ammonium sulfate (0–65% [w/v] saturation), all of the PI 4-kinase activity (100%) was in the precipitate. However, the HSC also precipitated with ammonium sulfate based on cross-reactivity with antibodies raised against *Achlya* HSP90 (Fig. 10), and on the fact that no 80-kD polypeptides were present when the supernatant was analyzed on silver-stained SDS-polyacrylamide gels (data not shown).

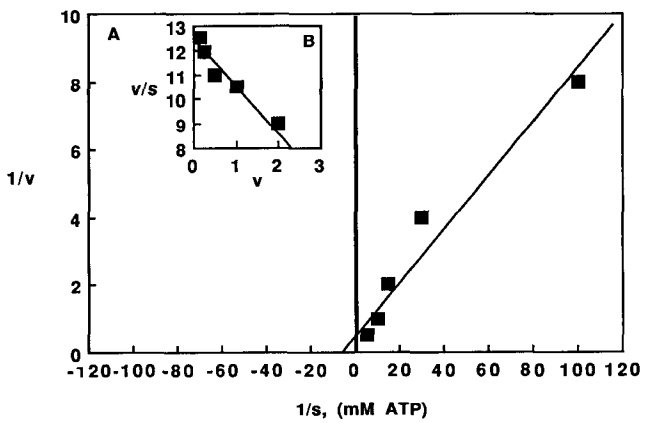

Figure 7. Kinetics for the utilization of ATP by the soluble PI kinase. A, Lineweaver-Burk plot. B, Eadie-Hofstee plot. PI kinase activity (10 µg of protein) was assayed in duplicate as described in "Materials and Methods." Activity in the presence of varying concentrations of ATP is shown (v is measured in nmol min^{-1} mg^{-1}).

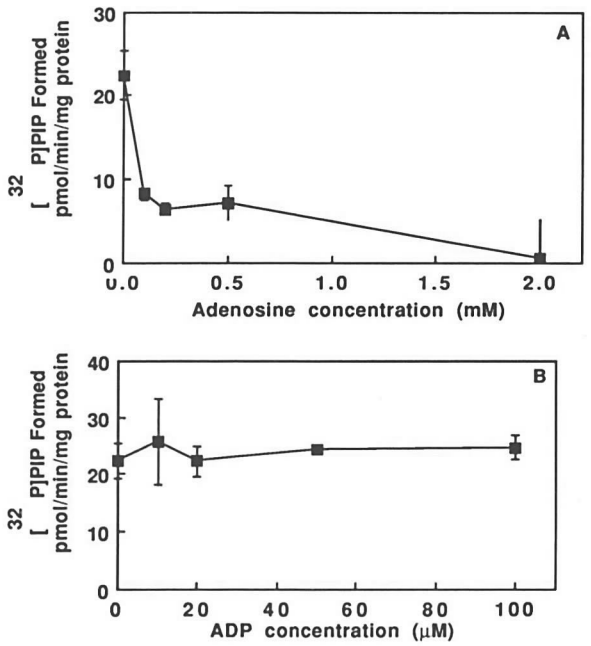

Figure 8. The effects of adenosine and ADP on the soluble PI kinase activity. A, Enzyme activity was assayed in the presence of increasing concentrations of adenosine. The numbers are the averages of duplicate values. The experiment was repeated two times and the trends were similar. B, Enzyme activity was assayed in the presence of increasing concentrations of ADP. The numbers are the mean of triplicate values. The deviation from the mean is indicated when it is larger than the symbol. The experiment was repeated three times and the trends were consistent.

In separate experiments, using a step gradient of 0 to 35% and 35 to 55% (w/v) ammonium sulfate, the PI 4-kinase activity did not separate from the HSP90 antibody-cross-reacting polypeptide (i.e. both precipitated, based on enzyme activity and western blot analysis; data not shown). Thus, we were not able to separate the HSC82 from the carrot soluble PI 4-kinase under the conditions

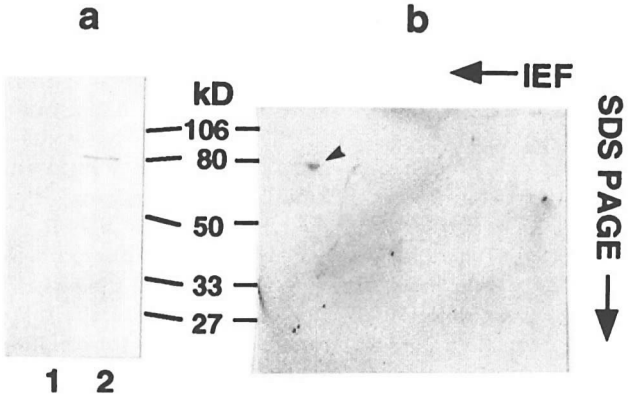

Figure 9. Western blots of the carrot soluble PI 4-kinase after Sepharose G-100 chromatography probed with antibodies to the membrane-associated yeast PI 4-kinase. a, One-dimensional gel. Lane 1, Molecular mass markers; lane 2, Sepharose G-100 fraction (15 μg of protein). b, Two-dimensional gel. The pI of the cross-reacting polypeptide was estimated to be 5.6 based on standards.

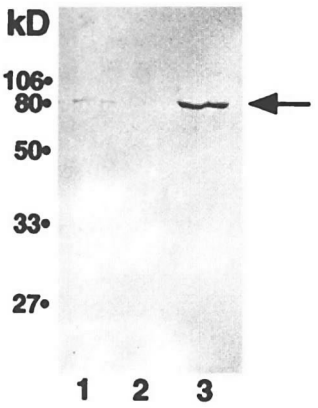

Figure 10. Western blot of the soluble PI 4-kinase (after ammonium sulfate precipitation) probed with monoclonal antibodies to HSP90. Ammonium sulfate was added to the PI 4-kinase after Sepharose G-100 chromatography. The pellet and supernatant were analyzed by SDS-PAGE. Lane 1, Sepharose G-100 fraction (10 μg protein); lane 2, 0 to 65% (w/v) $(NH_4)_2SO_4$ supernatant (10 μg of protein); lane 3, 0 to 65% (w/v) $(NH_4)_2SO_4$ pellet (40 μg of protein). The arrow denotes the migration of soluble PI 4-kinase.

that had been favorable for separating the HSC82 from the yeast soluble PI 4-kinase or from mammalian steroid receptor.

We attempted to separate the proteins by immunoprecipitation, but sufficient antibodies to the yeast PI 4-kinase were not available for immunoprecipitation. The AC19 antibodies to *Achlya* HSP90 precipitated PI 4-kinase activity as well as HSC82. The AC19 antibody is the only one available that cross-reacts with plant HSP90 (D.O. Toft, personal communication), and it may be that it recognized a highly conserved ATP binding or autophosphorylating domain on both proteins (Csermely and Kahn, 1991) and therefore both precipitated. Antibodies to both the yeast PI 4-kinase and the *Achlya* HSP90 family of proteins cross-reacted with the same 80-kD polypeptide that corresponded to PI 4-kinase activity on one-dimensional gels. On two-dimensional gels, a polypeptide with a pI of 5.6 cross-reacted with the PI 4-kinase antibody (Fig. 9b), suggesting that it is the PI 4-kinase. However, the HSP90 antibodies recognized two polypeptides from the hydrox-

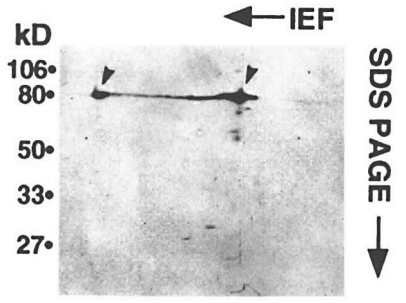

Figure 11. Western blot of the hydroxylapatite fraction separated by two-dimensional SDS-PAGE using antibodies to HSP90. The hydroxylapatite pool (30 μg) was separated by two-dimensional gel electrophoresis. Antibodies to HSP90 recognized two polypeptides at 80 kD (pI 5.6 and 6.5 as indicated by the arrows).

ylapatite fraction on two-dimensional gels, one with a pI of 5.6, and one with a pI of 6.5 (Fig. 11), indicating that either there were two isoforms of HSC82 and one co-migrated with PI 4-kinase, or that the PI 4-kinase and HSC82 share epitope homology, or both. Several isoforms of HSC82 have been reported that range in pI values from 5 to 6 with a profile similar to that shown in Figure 9b (Roychowdhury and Kapoor, 1990). pI values reported for yeast PI 4-kinases are 5.02 and 6.53 (Carman et al., 1994). Thus, the two proteins could have co-migrated on two-dimensional gels.

The fact that the AC19 antibodies immunoprecipitated PI 4-kinase activity also suggested that HSC82 and PI 4-kinase might share epitope homology. The AC19 antibody cross-reacted with another member of the plant HSP family of proteins, maize BiP (data not shown), indicating that the antibody recognized a common epitope. BiP is a member of an ER-localized HSP70 subfamily and has a conserved ATP-binding domain and an autophosphorylation site (Fontes et al., 1991). Importantly, the purified maize 70-kD BiP had no PI kinase activity and did not cross-react with antibodies to the yeast PI 4-kinase (data not shown). Because the HSCs co-purified with the PI 4-kinase and appeared to share epitope homology, we are currently selecting for cell lines with low levels of HSC82 to facilitate the PI 4-kinase purification.

DISCUSSION

PI 4-kinase was purified 1070-fold relative to the activity in the cell extract. PI 4-kinase activity co-purified with a 82-kD HSC. The M_r of the carrot PI 4-kinase was estimated to be 83,000 by gel filtration. There was some enzyme activity (<20% of the total activity) in the 45-kD region of the gel filtration profile. It is possible that several isoforms of the soluble PI kinase are present or that the activity at the lower molecular mass resulted from proteolysis.

The specific activity of the purified enzyme was 7.5 nmol phosphatidylinositol monophosphate min^{-1} mg^{-1} protein. Analysis of the product indicated that the soluble enzyme from carrot cells phosphorylated the fourth hydroxyl of the inositol group on PI. PI 4-kinase activity was enhanced by Triton X-100 and required divalent cations. The K_m for ATP was 400 μM as estimated by a Lineweaver-Burk plot and 463 μM when the data were analyzed by an Eadie-Hofstee plot. The activity was inhibited by 100 μM adenosine, but it was insensitive to 100 μM ADP and 1 mM calcium. The carrot PI 4-kinase had a pI of 5.6 and cross-reacted with antibodies to the 55-kD yeast membrane-associated PI 4-kinase that has a pI of 5.02.

Based on its biochemical characteristics, the carrot soluble PI 4-kinase does not fit clearly into either the type II or type III classification described by Pike (1992). For example, the size, K_m for ATP, and insensitivity to calcium are characteristic of the type III PI 4-kinase; however, sensitivity to adenosine is a distinguishing characteristic of the type II kinases, and based on this criterion the soluble carrot PI 4-kinase would be classified as a type II kinase. This is in contrast to the soluble PI 4-kinase from yeast (Flanagan and Thorner, 1992) that was insensitive to micromolar concentrations of adenosine (i.e. type III-like) but had a low K_m for ATP (100 μM) (i.e. type II-like). Neither the membrane-associated (Buxeda et al., 1993) or the soluble (Flanagan and Thorner, 1992) form of PI 4-kinase from yeast nor the soluble carrot PI 4-kinase (this work) fit clearly into the type II or type III categories that are currently used to classify PI 4-kinase from animal cells. When sequence information becomes available, the classification system should be re-evaluated.

Currently, little sequence information is available. The first reported deduced amino acid sequence of a putative cDNA encoding the PI 4-kinase has been retracted (Yamakawa et al., 1991, 1992). Although antibodies raised against the putative PI kinase expressed in *Escherichia coli* immunoprecipitated the enzyme activity, complete analysis of the cDNA revealed that the clone coded for a long-chain acyl CoA synthetase and not the PI 4-kinase (Yamakawa et al., 1992). Flanagan et al. (1993) reported a partial amino acid sequence for the 125-kD yeast soluble PI 4-kinase. They isolated the putative gene by PCR using oligonucleotides based on the sequence of a 21-amino acid polypeptide at the carboxy terminus of the protein. The deduced amino acid sequence of the yeast gene product was 30% identical to the carboxy terminus of the yeast PI 3-kinase (Vps34), 29% identical to the 110-kD catalytic subunit of the bovine PI 3-kinase, and shows similar identity to yeast Tor2, a 282-kD protein with homology to PI 3-kinase in the ATP-binding region near the carboxy terminus. Yeast deletion mutants were lethal and overexpression of the gene increased the soluble PI kinase activity 3-fold. Garcia-Bustos et al. (1994) used antibodies raised against the nuclear envelope of yeast to clone a gene encoding PI 4-kinase (PIK1). The cDNA gave the same deduced amino acid sequence reported by Flanagan and Thorner (1992). The protein characterized by Garcia-Bustos et al. (1994) was associated exclusively with the nucleus and the deduced sequence had a nuclear localization motif. Whether this is an isoform of the soluble PI 4-kinase reported by Flanagan and Thorner (1992) or the same enzyme remains to be determined. PI 4-kinase has been a difficult enzyme to purify and to sequence in all systems studied (Pike, 1992).

In plants, only a small portion of the total cellular PI kinase activity was soluble. In contrast, the concentration of HSP90 proteins within cells has been estimated to be 10^{-5} M (Koyasu et al., 1986). The relative abundance of the HSP90 proteins and their similarities to kinases have proven to be problematic when purifying both lipid and protein kinases (Matts and Hurst, 1989; Flanagan and Thorner, 1992). To further complicate the purification, both the carrot HSC82 and PI 4-kinase immunoprecipitated with HSP90 antibodies, and sufficient antibodies to the yeast PI 4-kinase were not available for immunoprecipitation. For these reasons, our current strategy is to search for cell lines with low HSC82 in order to obtain sufficient pure PI 4-kinase for sequencing. Although our goal was not to purify HSC82, our purification scheme proved to be effective and could be used to obtain HSC82 from other plant material, which to our knowledge has not been accomplished.

In summary, we have characterized PI 4-kinase from carrot suspension culture cells. The soluble carrot PI 4-ki-

nase differs in size and in kinetic properties from the soluble yeast PI 4-kinase (Flanagan and Thorner, 1992). Purification of the plant enzymes involved in the inositol signal transduction pathway is an essential step in understanding the regulation of the pathway.

ACKNOWLEDGMENTS

We would like to thank Dr. David Toft for his generous gift of the partially purified chicken HSP90 and the AC19 antibody to HSP90. We are grateful to Dr. George Carman for antibodies to the yeast PI 4-kinase and Dr. Robert Grossfeld for antibodies to HSP70. We thank Drs. Rebecca Boston and Jeff Gillikin for purified BiP and Drs. A. Graziani and L. Cantley for head group analysis of phosphatidylinositol monophosphate. We also acknowledge Irena Brglez for superb technical assistance.

Received October 12, 1994; accepted November 1, 1994.

LITERATURE CITED

Belunis CJ, Bae-Lee M, Kelley MJ, Carman GM (1988) Purification and characterization of phosphatidylinositol kinase from *Saccharomyces cerevisiae*. J Biol Chem **263:** 18897–18903

Berridge MJ (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. Annu Rev Biochem **56:** 159–193

Bjerrum OJ, Schafer-Nielson C (1986) Buffer systems and transfer parameters for semi-dry electroblotting with a horizontal apparatus. *In* MJ Dunn, ed, Electrophoresis '86. VCH, Weinheim, Germany, pp 315–327

Boss WF, Ruesink AW (1979) Isolation and characterization of concanavalin A labeled plasma membranes of carrot protoplasts. Plant Physiol **64:** 1005–1011

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Buckhout TJ (1984) Characterization of Ca^{2+} transport in purified endoplasmic reticulum isolated from *Lepidium sativum* L. roots. Plant Physiol **76:** 962–967

Burkhart W (1993) *In situ* proteolytic digestion performed on proteins bound to Hewlett-Packard hydrophobic sequencing columns. *In* R Angeletti, ed, Techniques in Protein Chemistry IV. Academic Press, San Diego, CA, pp 399–406

Buxeda RJ, Nickels JT Jr, Belunis CJ, Carman GM (1991) Phosphatidylinositol 4-kinase from *Saccharomyces cerevisiae*: kinetic analysis using Triton X-100/phosphatidylinositol-mixed micelles. J Biol Chem **266:** 13859–13865

Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S (1991) Oncogenes and signal transduction. Cell **64:** 261–301

Carman GM, Belunis CJ, Nickels JT (1992) Phosphatidylinositol 4-kinase from yeast. Methods Enzymol **209:** 183–189

Carman GM, Buxeda RJ, Nickles JT (1995) Phosphatidylinositol 4-kinases in *Saccharomyces cerevisiae*. *In* RW Gross, ed, Lipobiology. Jai Press, Greenwich, CT (in press)

Carpenter C, Cantley LC (1990) Phosphoinositide kinases. Biochemistry **29:** 11147–11160

Catelli MJ, Binart N, Jung-Testas I, Renoir JM, Baulieu EE, Feramisco JR, Welch WJ (1985) The common 90-kd protein component of non-transformed 8S steroid receptors is a heat-shock protein. EMBO J **4:** 3131–3135

Chen Q, Boss WF (1990) Short-term treatment with cell wall degrading enzymes increases the activity of the inositol phospholipid kinases and the vanadate-sensitive ATPase of carrot cells. Plant Physiol **94:** 1820–1829

Cho MH, Chen Q, Okpodu CM, Boss WF (1992) Separation and quantitation of [^{3}H]inositol phospholipids using thin-layer chromatography and a computerized ^{3}H imaging scanner. LC.GC **110:** 464–468

Coté GG, Crain RC (1993) Biochemistry of phosphoinositides. Annu Rev Plant Physiol Plant Mol Biol **44:** 333–356

Csermely P, Kahn CR (1991) The 90-kDa heat shock protein (hsp-90) possesses an ATP binding site and autophosphorylating activity. J Biol Chem **266:** 4943–4950

Drøbak BK (1993) Plant phosphoinositides and intracellular signalling. Plant Physiol **102:** 705–709

Endemann G, Dunn SN, Cantley LC (1987) Bovine brain contains two types of phosphatidylinositol kinases. Biochemistry **26:** 6845–6852

Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M, McCormick F, Williams LT (1992) Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signalling pathways. Cell **69:** 413–423

Flanagan CA, Schnieders EA, Emerick AW, Kunisawa R, Adom A, Thorner J (1993) Phosphatidylinositol 4-kinase: gene structure and requirement for yeast viability. Science **262:** 1444–1448

Flanagan CA, Thorner J (1992) Purification and characterization of a soluble phosphatidylinositol 4-kinase from the yeast *Saccharomyces cerevisiae*. J Biol Chem **267:** 24117–24125

Fontes EBP, Shank BB, Wrobel RL, Moose SP, O'Brian GR, Wurtzel ET, Boston RS (1991) Characterization of an immunoglobulin binding protein homolog in the maize floury-2 endosperm mutant. Plant Cell **3:** 483–496

Garcia-Bustos JF, Marini F, Stevenson I, Frei C, Hall MN (1994) PIK1, an essential phosphatidylinositol 4-kinase associated with the yeast nucleus. EMBO J **13:** 2352–2361

Graziani A, Ling LE, Endemann G, Carpenter CL, Cantley LC (1992) Purification and characterization of human erythrocyte phosphatidylinositol 4-kinase. Phosphatidylinositol 4-kinase and phosphatidylinositol 3-monophosphate 4-kinase are distinct enzymes. Biochem J **284:** 39–45

Gross W, Yang W, Boss WF (1992) Release of carrot plasma membrane-associated phosphatidylinositol kinase by phospholipase A_2 and activation by a 70-kDa protein. Biochim Biophys Acta **1134:** 73–80

Hou W-M, Zhang Z-L, Tai H-H (1988) Purification and characterization of phosphatidylinositol kinase from porcine liver microsomes. Biochim Biophys Acta **959:** 67–75

Imai A, Rebecchi MJ, Gershengorn MC (1986) Differential regulation by phosphatidylinositol 4,5-bisphosphate of pituitary plasma-membrane and cytosolic phosphatidylinositide kinases. Biochem J **240:** 341–348

Koning AJ, Rose R, Comai L (1992) Developmental expression of tomato heat-shock cognate protein 80. Plant Physiol **100:** 801–811

Koyasu S, Nishida E, Kadowaki T, Matsuzaki F, Iida K, Harada F, Kasuga M, Sakai H, Yahara I (1986) Two mammalian heat shock proteins, HSP90 and HSP100, are actin-binding proteins. Proc Natl Acad Sci USA **83:** 8054–8058

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Majerus PW, Connolly TM, Deckmyn H, Ross TS, Bross TE, Ishli H, Bansal VS, Wilson DB (1986) The metabolism of phosphoinositide-derived messenger molecules. Science **234:** 1519–1526

Matts RL, Hurst R (1989) Evidence for the association of the heme-regulated eIF-2α kinase with the 90-kDa heat shock protein in a rabbit reticulocyte lysate *in situ*. J Biol Chem **264:** 15542–15547

Memon A, Chen Q, Boss WF (1989) Inositol phospholipids activate plasma membrane ATPase in plants. Biochem Biophys Res Commun **162:** 1295–1301

Michell RH, Harwood JL, Coleman R, Hawthorne JN (1967) Characteristics of rat liver phosphatidylinositol kinase and its presence in the plasma membrane. Biochim Biophys Acta **144:** 649–658

Miyata Y, Yahara I (1992) The 90-kDa heat shock protein, HSP90, binds and protects casein kinase II from self-aggregation and enhances its kinase activity. J Biol Chem **267:** 7042–7047

Morgan SJ, Smith AD, Parker PJ (1990) Purification and characterization of bovine brain type I phosphatidylinositol kinase. Eur J Biochem **191:** 761–767

Nickels JT Jr, Buxeda RJ, Carman GM (1992) Purification and characterization, and kinetic analysis of a 55-kDa form of the

phosphatidylinositol 4-kinase from *Saccharomyces cerevisiae*. J Biol Chem **267:** 16297–16304

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021

Okpodu CM, Gross W, Boss WF (1990) Soluble phosphatidylinositol (PI) kinase is present in carrot suspension culture cells (abstract No. 362). Plant Physiol **93:** S-63

Payrastre B, van Bergen en Henegouwen PMP, Berton M, den Hartigh JC, Plantavid M, Verkleij AJ, Boonstra J (1991) Phosphoinositide kinase, diacylglycerol kinase, and phospholipase C activities associated to the cytoskeleton: effect of epidermal growth factor. J Cell Biol **115:** 121–128

Pike LJ (1992) Phosphatidylinositol 4-kinase and the role of polyphosphoinositides in cellular regulation. Endocr Rev **13:** 692–706

Porter FD, Li Y-S, Deuel TF (1988) Purification and characterization of a phosphatidylinositol 4-kinase from bovine uteri. J Biol Chem **263:** 8989–8995

Riehl RM, Sullivan WP, Vroman BT, Bauer G, Pearson GR, Toft DO (1985) Immunological evidence that the nonhormone binding component of avian steroid receptors exists in a wide range of tissues and species. Biochemistry **24:** 6586–6591

Roychowdhury HS, Kapoor M (1990) Heat shock response in *Neurospora crassa*: purification and some properties of HSP80. Biochem Cell Biol **68:** 1218–1221

Sanchez ER, Meshinchi S, Teinrungroj W, Schlesinger MJ, Toft DO, Pratt WB (1987) Relationship of the 90-kDa murine heat shock protein to the untransformed and transformed states of the L cell glucocorticoid receptor. J Biol Chem **262:** 6986–6991

Satiel AR, Fox JA, Sherline P, Sahyoun N, Cuatrecasas P (1987) Purification of phosphatidylinositol kinase from bovine brain mylein. Biochem J **241:** 759–763

Schäfer M, Behle G, Varsayni M, Heilmeyer LMG Jr (1987) Ca^{2+} regulation of 1-(3-*sn*-phosphatidyl)-1D-*myo*-inositol 4-phosphate formation and hydrolysis on sarcoplasmic-reticular Ca^{2+}-transport ATPase: a new principle of phospholipid turnover regulation. Biochem J **247:** 579–587

Smith DF, Sullivan WP, Marion TN, Zaitsu K, Madden B, McCormick DJ, Toft DO (1993) Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. Mol Cell Biol **13:** 869–876

Smith DF, Toft DO (1990) Purification of nontransformed avian progesterone receptor. *In* G Litwack, ed, Receptor Purification, Vol 2. The Humana Press, Clifton, NJ, pp 241–257

Sommarin M, Sandelius AS (1988) Phosphatidylinositol and phosphatidylinositol phosphate kinases in plant plasma membranes. Biochim Biophys Acta **958:** 268–278

Takahashi T, Naito S, Komeda Y (1992) Isolation and analysis of the expression of two genes for the 81-kilodalton heat shock proteins from *Arabidopsis*. Plant Physiol **99:** 383–390

Tan Z, Boss WF (1992) 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. Plant Physiol **100:** 2116–2120

Varsayni M, Tolle HG, Heilmeyer LMG Jr, Dawson RMC, Irvine RF (1983) Activation of sarcoplasmic reticular Ca^{2+} transport ATPase by phosphorylation of an associated phosphatidyl-inositol. EMBO J **2:** 1543–1548

Walker DH, Dougherty N, Pike LJ (1988) Purification and characterization of a phosphatidylinositol kinase from A431 cells. Biochemistry **27:** 6504–6511

Wheeler JF, Boss WF (1989) The presence of *sn*-1-palmitoyl lysophosphatidylinositol monophosphate correlates positively with the fusion-permissive state of the plasma membrane of fusogenic carrot cells grown in suspension culture. Biochim Biophys Acta **984:** 33–40

Whitman M, Kaplan D, Roberts T, Cantley L (1987) Evidence for two distinct phosphatidylinositol kinases in fibroblasts. Biochem J **247:** 165–174

Wiechelman KJ, Braun RD, Fitzpatrick JD (1988) Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. Anal Biochem **175:** 231–237

Wissing JB, Grabowski L, Drewitz E, Hanenberg A, Wylegalla C, Wagner KG (1992) Plasma membrane preparations from suspension cultured plant cells contain the enzymes for the recycling of phosphatidic acid and diacylglycerol. Plant Sci **87:** 29–37

Yamakawa A, Nishizawa M, Fujiwara KT, Kawai S, Kawasaki H, Suzuki K, Takenawa T (1991) Molecular cloning and sequencing of cDNA encoding the phosphatidylinositol kinase from rat brain. J Biol Chem **266:** 17580–17583

Yamakawa A, Nishizawa M, Fujiwara KT, Kawai S, Kawasaki H, Suzuki K, Takenawa T (1992) Molecular cloning and sequencing of cDNA encoding the phosphatidylinositol kinase from rat brain. (Additions and Corrections). J Biol Chem **267:** 25620

Yamakawa A, Takenawa T (1988) Purification and characterization of membrane-bound phosphatidylinositol kinase from rat brain. J Biol Chem **263:** 17555–17560

Yang W, Burkhart W, Cavallius J, Merrick WC, Boss WF (1993) Purification and characterization of a phosphatidylinositol 4-kinase activator in carrot cells. J Biol Chem **268:** 392–398