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

Camellia Moses Okpodu, Wolfgang Gross, William Burkhart, 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: 5-63). We have purified the enzyme over 1000-fold using Q-Sepharose ion exchange, hydroxylapatite, and G-100 gel filtration column chromatography. The Mₐ 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 to1 or phosphatidylinositol monophosphate. Maximal PI kinase activity was recovered after renaturation of the 80-kD region of polyacrylamide gels.

The biochemical characteristics of the carrot soluble PI 4-kinase are compared with the previously reported PI 4-kinases from animals and yeast. Extensively because it catalyzes the first committed step in the synthesis of PI-4,5-P₂ (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 type II enzyme, PI 4,5-kinase, phosphorylates the D-3 and D-4 positions of the inositol ring. The type III enzyme, PI 4-kinase, phosphorylates the D-3 position of the inositol ring. 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 enzymes are stimulated by nonionic detergents and are inhibited by low levels of adenosine (Kᵢ = 20–100 μM) and ADP. They have a Kₐ 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.

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-P₂, which is the source of the second messengers IP₃ and diacylglycerol (Majerus et al., 1986; Berridge, 1987; Coté and Crain, 1993; Držbak, 1993). In addition, PI-4-P and PI-4,5-P₂ directly affect the activity of membrane enzymes (Varsányi 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-P₂ (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 type II enzyme, PI 4,5-kinase, phosphorylates the D-3 and D-4 positions of the inositol ring. The type III enzyme, PI 4-kinase, phosphorylates the D-3 position of the inositol ring. 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 enzymes are stimulated by nonionic detergents and are inhibited by low levels of adenosine (Kᵢ = 20–100 μM) and ADP. They have a Kₐ 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.

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 enzymes are stimulated by nonionic detergents and are inhibited by low levels of adenosine (Kᵢ = 20–100 μM) and ADP. They have a Kₐ 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 Kₐ for ATP (250–742 μM) than the type II PI kinase, and are relatively insensitive to adenosine (Kᵢ = 1.5 mM; Pike, 1992). The molecular mass of the type III PI kinase in a crude preparation of bovine brain was first

Abbreviations: BiP, binding protein; HSP, heat-shock proteins; HSC, heat-shock cognate; IP₃, inositol trisphosphate; LPI, lysophosphatidylinositol; MeOH, methanol; PI, phosphatidylinositol; PI-4-P, phosphatidylinositol 4 monophosphate; PI-4,5-P₂, 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, 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, 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 translocational 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 Km 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 type I1 enzymes. Garcia-Bustos et al. (1994) tested, MgCl2, or MnCl2 were added at the concentrations

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 characterization was 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-32P]ATP (7000 Ci mmol−1) was purchased from ICN (Costa Mesa, CA). Soybean phosphatidylinositol, Sepharose G-100, electrophoresis reagents, and two-dimensional pl 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 MgCl2, 0.69 mM PI, 0.2 mM sodium molybdate, 0.25% Triton X-100, and 1.3 mM ATP containing [γ-32P]ATP (0.18 μCi/nmol). PI was added to the reaction mixture as Triton X-100 unless otherwise stated. When LPI was used as a substrate, it was reconstituted in CH3Cl:MeOH (1:2). Ali-quotes 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, MgCl2 or MnCl2 were added at the concentrations
indicated; calcium was added as CaCl₂/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 CHCl₃:MeOH (1:2). Lipids were extracted according to Cho et al. (1992) and chromatographed on LK5D thin-layer plates that had dried overnight at 120°C. CHCl₃:MeOH:NH₄OH:H₂O (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 polyvinylpolypyrrolidone, 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 5,000g. The supernatant was then centrifuged for 1 h at 45,000g. The supernatant was fractionated on a Q-Sepharose column (2.5 × 25 cm) pre-equilibrated 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 MgCl₂, 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 45,000g 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 Mr 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 pro-green. 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 40,000g 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*ₚ 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, [³²P]PIP₂, 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. Mn²⁺ was more effective than Mg²⁺ at low concentrations (<5 mM); however, at high concentrations Mg²⁺ was the most effective cation. The enzyme activity in the presence of 5 to 40 mM Mn²⁺ was only 40% of that obtained with 40 mM Mg²⁺ (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 1. Purification of the soluble PI 4-kinase from carrot suspension culture cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Fold</th>
<th>Percent Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>pmol/min</td>
<td>pmol min⁻¹ mg⁻¹</td>
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<td></td>
</tr>
<tr>
<td>Crude</td>
<td>44</td>
<td>3025</td>
<td>6.8</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>8</td>
<td>788</td>
<td>98.5</td>
<td>14.5</td>
<td>26</td>
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<tr>
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<td>2400</td>
<td>343</td>
<td>16</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitant</td>
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<td>4666</td>
<td>686</td>
<td>14</td>
</tr>
<tr>
<td>Sepharose G-100</td>
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<td>320</td>
<td>7500</td>
<td>1070</td>
<td>11</td>
</tr>
</tbody>
</table>
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 $\mu$M (Fig. 7A). Using Eadie-Hostee plots the apparent $K_m$ for ATP was 463 $\mu$M (Fig. 7B). Adenosine (100 $\mu$M), which inhibits type II PI 4-kinases (Carpenter and Cantley, 1990; Pike, 1992), decreased the soluble PI 4-kinase (Fig. 8A). ADP (100 $\mu$M) had no effect on the soluble PI 4-kinase (Fig. 8B).

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 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$_2$ (C), MgCl$_2$ (A) and CaCl$_2$ (B). In A, representative results are presented as pmol [32P]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 [32P]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.

ever, 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 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).

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 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 AchlyHSP90 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 AchlyHSP90 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 pl 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 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 pl 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) (NH₄)₂SO₄ supernatant (10 µg of protein); lane 3, 0 to 65% (w/v) (NH₄)₂SO₄ pellet (40 µg of protein). The arrow denotes the migration of soluble PI 4-kinase.

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 (pl 5.6 and 6.5 as indicated by the arrows).
ylapatite fraction on two-dimensional gels, one with a pl of 5.6, and one with a pl 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 pl values from 5 to 6 with a profile similar to that shown in Figure 9b (Roychowdhury and Kapoor, 1990). pl 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 autoprophosphorylation 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\textsubscript{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\textsuperscript{-1} mg\textsuperscript{-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\textsubscript{m} for ATP was 400 \mu M as estimated by a Lineweaver-Burk plot and 463 \mu M when the data were analyzed by an Eadie-Hofstee plot. The activity was inhibited by 100 \mu M adenosine, but it was insensitive to 100 \mu M ADP and 1 mM calcium. The carrot PI 4-kinase had a pl of 5.6 and cross-reacted with antibodies to the 55-kD yeast membrane-associated PI 4-kinase that has a pl 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\textsubscript{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\textsubscript{m} for ATP (100 \mu M) (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.

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