Purification and Characterization of a Phosphatidylinositol Kinase from A431 Cells

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ABSTRACT: A phosphatidylinositol kinase from A431 cells has been purified to near homogeneity. Purification was achieved through the use of a combination of chromatography steps including affinity elution of the enzyme from a heparin–agarose column with PI. Characterization of the [^{32}P]PIP formed by the purified PI kinase indicates that the enzyme phosphorylates the inositol on the 4-position and is therefore a phosphatidylinositol 4-kinase. The enzyme has a subunit weight of 55 000 as estimated by SDS gel electrophoresis and appears to be active as a monomer. Studies of the hydrodynamic properties of the enzyme indicate that the PI kinase binds substantial amounts of Triton X-100 and is actually present in detergent-containing solutions as a complex with a molecular weight of approximaely 120 000. The K_m of the enzyme for PI is 16 μM and for ATP is 74 μM. The enzyme is inhibited by adenosine with an IC_{50} of 100 μM. These properties are essentially identical with those of the membrane-bound PI kinase in A431 cells which is stimulated by EGF. The data therefore suggest that the EGF-stimulated PI kinase is a 55 000-Da monomer.

Phosphatidylinositol plays a central role in the transduction of signals through cell surface receptors. A variety of hormones have been shown to stimulate the turnover of PI[1] and its phosphorylated derivatives, PIP and PIP_2 (Uhing et al., 1986; Litosch et al., 1985; Bokoch & Gilman, 1984; Berridge et al., 1984; Pike & Eakes, 1987; Murayama & Ui, 1985). Hydrolysis of PIP_2 by phospholipase C produces the two important second messengers diacylglycerol and inositol trisphosphate (Majerus et al., 1984; Nishizuka, 1984). Diacylglyerol serves to activate protein kinase C, a broad specificity, serine-threonine kinase (Koshimoto et al., 1980). Inositol trisphosphate promotes the release of calcium from intracellular stores (Berridge, 1984; Michell et al., 1981; Streb et al., 1983), thereby stimulating the activity of calcium-dependent enzymes. While the breakdown of phosphoinositides has been the subject of many recent investigations, the synthesis of the polyphosphoinositide precursors has been less well studied.

PI is converted to the monophosphorylated derivative PIP by the action of a PI kinase. Relatively little is known about this enzyme. The PI kinase phosphorylates PI with ATP as the phosphate donor. PI kinase activity has been shown to be membrane bound and has been found in numerous different membrane structures including Golgi (Jergil & Sundler, 1983), lysosomes (Collins & Wells, 1983), nuclear membranes (Smith & Wells, 1983), endoplasmic reticulum (Cockcroft et al., 1985), plasma membranes (Cockcroft et al., 1985), and coated vesicles (Campbell et al., 1985). Changes in PI kinase activity have been reported in rapidly growing cells. Increased levels of PI kinase activity have been found to be associated with early stages of hepatocarcinogenesis (Olson, 1985) and with the growth of rat mammary tumors (Sharoni et al., 1986). Both PI and PIP kinase activities have been shown to be elevated in the plasma membrane from Rous sarcoma virus infected as compared to uninfected chicken embryo fibroblasts (Sugimoto & Erikson, 1985). The latter observation suggests a link between increased PI kinase activity and increased protein tyrosine kinase activity. Although several tyrosine kinases have been reported to be associated with PI kinase activity (Whitman et al., 1985; Kaplan et al., 1986; Sugimoto et al., 1984; Macara et al., 1984; Machicao & Wieland, 1984; Sale et al., 1986), current evidence indicates that the two activities are the functions of two separate enzymes (Thompson et al., 1985; MacDonald et al., 1985; Fischer et al., 1985).

We have shown previously that EGF stimulates the production of inositol trisphosphate and also induces a rise in cellular levels of PIP in A431 cells (Pike & Eakes, 1987). The rise in PIP appears to be due to a stimulation of PI kinase activity. This increase in activity can also be demonstrated in membranes derived from EGF-treated cells (Walker & Pike, 1987). The mechanism by which EGF stimulates the activity of the PI kinase is not known. Two groups have suggested that a PI kinase might be phosphorylated on a tyrosine residue (Kaplan et al., 1987; Courtneidge & Heber, 1987); however, the evidence for this is circumstantial. To begin to address this issue directly, we sought to purify the enzyme from A431 cells, the only system in which a growth factor has been shown to increase total cellular PI kinase activity. We report here the purification to apparent homogeneity and characterization of a PI kinase from this human source.

Experimental Procedures

Materials

Bovine liver phosphatidylinositol, crude soybean phosphatidylinositol, neomycin sulfate, glyceryl controlled-pore glass beads, deuterium oxide, heparin–agarose, and reactive green–agarose were from Sigma. Ultrapure Triton X-100 and Iodo-gen were obtained from Pierce Chemical Co. The Bio-Sil TSK-250 HPLC column (21 × 600 mm) was from Bio-Rad.

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[1] Abbreviations: DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; PI, phosphatidylinositol; lyso-PI, lysophosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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$Na^{125}I$ (14.8 mCi/μg) was from Amersham. [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Tru-Count scintillation fluid was from IN/US. Phospholipase C was the generous gift of Dr. Brian Whitely (Washington University). Inositol polyphosphate 1-phosphatase the kind gift of Roger Inhorn (Washington University). All radioactive inositol phosphate standards were provided by Dr. Philip Majerus.

A431 cells were grown in roller bottles by the Washington University Tissue Culture Support Center. The yield of cells was approximately 5×10^8 cells per roller bottle.

Methods

Preparation of Neomycin–Glass Beads. Neomycin sulfate was reductively coupled to reactive porous glass beads as described by Schacht (1978). The beads were stored at 4 °C in chloroform–methanol–water (5:10:2) until needed. Once the beads were poured into columns (see below), they were kept at room temperature.

Preparation of Phosphatidylinositol. PI for affinity elution was prepared from crude soybean PI according to the method of Palmer (1981). Crude PI (125–250 mg) was dissolved in 2 mL of chloroform and passed over a 6-mL column of neomycin–glass beads. The column was washed with 12 mL of chloroform–methanol–water (5:10:2), and PI was eluted with 10 mL of the same solvent containing 0.1 M ammonium formate. Aliquots of the eluate (1.0 mL) were transferred to 12 × 75 mm glass tubes to which was added 0.5 mL of chloroform and 0.5 mL of 0.5 M HCl. The tubes were vortexed for 30 s, and the upper aqueous phase was discarded. The chloroform layer was washed once more with 0.5 mL of methanol–1 N HCl (1:1) and then dried in a Speed-Vac. The purified PI was resuspended in chloroform. The concentration of PI was estimated by determining the phosphate content of the lipid according to the method of Chen et al. (1956). The purity of the PI was determined by thin layer chromatography (Pike & Eakes, 1987). Typically, 300 mg of crude material yields 140 mg of ≥95% pure PI. The PI was stored in chloroform in an amber Reacti-Vial that was flushed with nitrogen.

Phosphatidylinositol Kinase Assay. The activity of the PI kinase was measured by quantitating the transfer of phosphate from [γ-^{32}P]ATP to PI. A 10–50-μL aliquot of PI kinase was incubated in a final volume of 100 μL containing (in final concentrations) 25 mM β-glycerophosphate, pH 7.2, 20 mM $MgCl_2$, 500 μM [γ-^{32}P]ATP [(4–6) × 10^6 cpm/assay], 100 μM PI, and 0.1% Triton X-100. Assays were begun by the addition of [γ-^{32}P]ATP and $MgCl_2$ and were incubated for 2–10 min at 30 °C. Reactions were stopped by the addition of 0.5 mL of methanol–concentrated HCl (10:1 v/v). To each tube was added 0.5 mL of water containing approximately 10000 cpm [^{3}H]PIP and 1 mL of chloroform. The tubes were then vortexed for 30 s, and the upper aqueous phase was removed and discarded. The chloroform layer was extracted first with 0.5 mL of methanol–1 N HCl (1:1 v/v) and then with 0.5 mL of methanol–0.1 N KCl (1:1 v/v). Methanol (0.75 mL) was then added to the chloroform layer, and the samples were loaded onto 5.4 × 180 mm glass columns containing approximately 150 μL of neomycin–glass beads. The samples were allowed to flow through under gravity, and the beads were washed once with 1 mL of 0.1 M ammonium formate in chloroform–methanol–water (5:10:2). PIP was eluted in 1.0 mL of the same solvent containing 0.4 M ammonium formate. The eluate was collected directly into 5-mL glass scintillation vials. Chloroform (0.5 mL) and 3 N HCl (0.5 mL) were added to each vial, and the vials were shaken or vortexed gently. The aqueous phase was discarded, and the organic phase was dried down under a stream of air. The dried samples were counted in 4 mL of Tru-Count high salt capacity scintillation fluid for both ^{3}H and ^{32}P. The recovery of each column was calculated by dividing the ^{3}H counts recovered in the eluate by the number of ^{3}H counts added to each tube before the extraction procedure. Corrections for spillover of ^{32}P into the ^{3}H window as well as calculation of column recoveries were carried out by a programmed LKB 1217 Rack Beta liquid scintillation counter using the appropriate standards. The incorporation of ^{32}P into PI was calculated by dividing the actual ^{32}P counts by the recovery calculated for that column. Recoveries ranged between 40 and 70%. Columns were regenerated between use by washing first with 1 M ammonium formate in chloroform–methanol–water (5:10:2) and then with salt-free solvent.

Purification of Phosphatidylinositol Kinase from A431 Membranes. All procedures were carried out at 4 °C unless otherwise indicated.

(*A*) *Preparation of Membranes.* A typical enzyme preparation began with five roller bottles of cells. A431 cells were scraped from the roller bottles and homogenized in 50 mM Tris-HCl, pH 7.2, 250 mM sucrose, 10 mM DTT, 1 mM EGTA, 10 mM benzamidine, 1 μg/mL leupeptin, 5 μg/mL α_2-macroglobulin, and 500 μM PMSF with 40 mL of homogenization buffer per roller bottle. The homogenate was centrifuged for 30 min at 100000*g*. The pellets were resuspended at a final concentration of 5 mg/mL protein in 50 mM HEPES, pH 7.2, containing protease inhibitors at the same concentration as in the homogenization buffer.

(*B*) *Triton X-100 Solubilization.* The membranes were solubilized by stirring for a total of 30 min in a final concentration of 0.87% Triton X-100. The detergent was added gradually from a 10% solution over 20 min. After addition of all of the detergent, the suspension was stirred for a further 10 min. The material was then centrifuged for 30 min at 100000*g*, and the supernatant which contains the PI kinase was retained.

(*C*) *Heparin–Agarose* (*Salt Elution*). The soluble fraction was applied to a 10-mL heparin–agarose column equilibrated with 50 mM HEPES and 0.25% Triton X-100, pH 7.2, and washed with 90 mL of this buffer containing 0.05 M NaCl. The PI kinase was eluted with a 200-mL gradient of 0.05–0.5 M NaCl. Fractions containing the PI kinase were pooled and dialyzed for 2 h against 4 L of 50 mM HEPES and 0.25% Triton X-100, pH 7.2.

(*D*) *Heparin–Agarose* (*Affinity Elution*). The dialyzed pool of PI kinase was applied to a 2-mL heparin–agarose column equilibrated with 50 mM HEPES and 0.25% Triton X-100, pH 7.2, and washed with 15 mL of this buffer containing 0.05 M NaCl. The PI kinase was eluted by a 5-mL pulse of this buffer containing 2 mM PI. Following the 5-mL pulse, a 20-mL gradient of the PI-containing buffer from 50 to 300 mM NaCl was applied to the column. Fractions containing the PI kinase were pooled.

(*E*) *Reactive Green–Agarose.* The eluate from the heparin column was diluted 5-fold with 50 mM HEPES, pH 7.2, 0.25% Triton X-100, and 2 mM DTT and applied directly to a 1-mL reactive green–agarose column equilibrated with 50 mM HEPES, pH 7.2, 0.25% Triton X-100, and 2 mM DTT and washed with 10 mL of this buffer containing 200 mM KCl. The PI kinase was eluted by application of two 1-mL pulses of this buffer containing 1 M KCl.

(*F*) *Gel Filtration Chromatography.* The concentrated PI kinase was loaded onto a Bio-Rad TSK-250 HPLC gel filtration column (600 × 21.5 mm) equilibrated with 50 mM

Table I: Purification of Phosphatidylinositol Kinase from A431 Cells[a]

step	total protein (μg)	total act. (nmol/min)	sp act. (nmol min^{-1} mg^{-1})	x-fold purification	yield (%)
(1) A431 membranes	217000	446	2.0	1.0	100
(2) Triton X-100 solubilization	94800	405	4.3	2.1	90.8
(3) heparin–agarose (salt elution)	16990	300	17.7	8.8	62.7
(4) heparin–agarose (PI elution)/reactive green	714	140	196	98	32
(5) TSK-250 HPLC gel filtration	39	40	1050	525	9

[a] PI kinase activity was assayed in duplicate as described under Experimental Procedures. Results are from a purification procedure that started with five roller bottles of cells.

HEPES, pH 7.2, 0.1% Triton X-100, 150 mM NaCl, and 3 mM DTT at a flow rate of 1.25 mL/min. One-minute fractions were collected. A single peak of PI kinase activity was eluted, and the fractions were pooled.

Characterization of the [^{32}P]PIP Product. Phosphatidylinositol was phosphorylated under standard conditions with the most highly purified enzyme. The [^{32}P]PIP was isolated as usual, dried down, and dissolved in 50 mM HEPES, pH 7.0, containing 10 mg/mL deoxycholate. The material was then added to 200 μL of a reaction mixture containing phospholipase C in 50 mM HEPES, pH 7.0, 1 mM $CaCl_2$, and 100 mM NaCl to yield a final concentration of deoxycholate of 1 mg/mL. The phospholipase C reaction was run for 10 min at 37 °C and was stopped by the addition of 1 mL of $CHCl_3$–methanol–concentrated HCl (100:100:0.6) plus 0.3 mL of 1 N HCl and 5 mM EGTA. Following vortexing, the inositol phosphates are found in the aqueous fraction and were separated on Dowex AG1X-8 as described previously (Inhorn et al., 1987). Greater than 90% of the input counts in [^{32}P]PIP were found in the aqueous fraction and were recovered on the Dowex column.

The [^{32}P]IP_2 from the Dowex column was collected, lyophilized, and resuspended in water. The material was then treated with inositol polyphosphate 1-phosphatase according to the method of Inhorn and Majerus (1987). The products of the reaction were then reapplied to the Dowex column and eluted as above.

Protein Assay. Protein concentrations were determined by the Pierce BCA assay method or, when DTT was used in the buffers, by the method of Lowry (Lowry et al., 1951) with prior precipitation of the protein in 0.0125% deoxycholate and 6% trichloroacetic acid. The protein concentration of the material from the gel filtration column was determined by amino acid analysis.

SDS Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970). For activity gels, 2–3 μg of purified PI kinase was incubated with SDS sample buffer for 30 min at 20 °C and run on a 10% polyacrylamide gel for 12 h at 4 °C. The lane containing the PI kinase was cut out and washed with 50 mM Tris-HCl, pH 7.2, 0.25% Triton X-100, and 2 mM DTT for 1 h at 4 °C to remove the SDS. The lane was then cut into 0.5-cm segments which were minced and incubated with 50 mM Tris, pH 7.2, 2 mM DTT, 0.25% Triton X-100, 500 mM [γ-^{32}P]ATP, 20 mM $MgCl_2$, and 100 μM phosphatidylinositol in a final volume of 500 μL for 6 h at 20 °C. Reactions were terminated by the addition of 2.25 mL of methanol–HCl (9:1). The lipids were extracted and analyzed by thin-layer chromatography as described previously (Pike & Eakes, 1987). After autoradiography, the spots corresponding to PIP on the films were scanned with an LKB densitometer.

Glycerol Density Gradients and Related Calculations. Linear 9–40% glycerol density gradients of 5 mL were prepared in buffer containing 50 mM Tris-HCl, pH 7.2, 0.25% Triton X-100, and 2 mM DTT. For D_2O gradients, all solutions were prepared with deuterium oxide in place of water. Samples were applied in 100-μL aliquots and contained both PI kinase and standard proteins. Standards included catalase ($s_{20,w}$ = 11.3 S), lactate dehydrogenase ($s_{20,w}$ = 7.3 S), malate dehydrogenase ($s_{20,w}$ = 4.3 S), ovalbumin ($s_{20,w}$ = 3.6 S), and cytochrome *c* ($s_{20,w}$ = 2.1 S). Gradients prepared in water were centrifuged for 16 h at 4 °C at 60000 rpm in an SW65 rotor. Gradients prepared in deuterium oxide were centrifuged similarly for 24 h. Fractions of 150 μL were collected and assayed for PI kinase activity as well as for marker enzymes.

The partial specific volume of a protein/detergent complex, ν, can be calculated according to

$$\bar{\nu} = \left(\frac{s\eta_{D,r_0}}{s\eta_{H,r_0}} - 1\right) \div \left(\frac{s\eta_{D,r_0}}{s\eta_{H,r_0}}\rho_{H,r_0} - \rho_{D,r_0}\right)$$

where $s\eta_{H,r_0}$ and $s\eta_{D,r_0}$ are the apparent sedimentation coefficients in H_2O and D_2O, respectively, and ρ_{H,r_0} and ρ_{D,r_0} are the densities of the 9% glycerol solutions in H_2O and D_2O, respectively.

The sedimentation coefficient of the PI kinase corrected to water at 20 °C can then be calculated:

$$s\eta_{T,M} = \frac{s_{20,w}\eta_{20,w}(1 - \bar{\nu}\rho_{T,M})}{1 - \nu\rho_{20,w}}$$

where $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density, respectively, of H_2O at 20 °C, $s\eta_{T,M}$ is the apparent sedimentation coefficient of the complex at temperature T in medium M, and $\rho_{T,M}$ is the vicosity of the solvent at temperature T in medium M.

Results

Purification of PI Kinase from A431 Membranes. A431 cells were chosen as the source for PI kinase for purification since previous studies had indicated that the enzyme was regulated by EGF in these cells (Pike & Eakes, 1987; Walker & Pike, 1987). We have previously demonstrated that PI kinase activity is predominantly associated with the particulate fraction of A431 cells. Preliminary studies indicated that the specific activity of the membrane-bound enzyme could be increased from 0.25 to 2 nmol min^{-1} mg^{-1} if the membranes were isolated from cells grown for 24–72 h past confluent. Therefore, all roller bottle cultures were allowed to grow for at least 24 h beyond the point at which they had initially become confluent. The enzyme is readily solubilized by low concentrations of Triton X-100 with optimal release of the enzyme from membranes occurring at a Triton X-100 concentration of 0.87%.

Table I shows a summary of the PI kinase purification procedure. The procedure takes advantage of the observation that addition of PI to the buffer used to elute the enzyme from the heparin–agarose column substantially alters the chromatographic behavior of the PI kinase. This is demonstrated in Figure 1. As shown in the top panel, when the heparin–agarose column is developed with a NaCl gradient, the enzyme

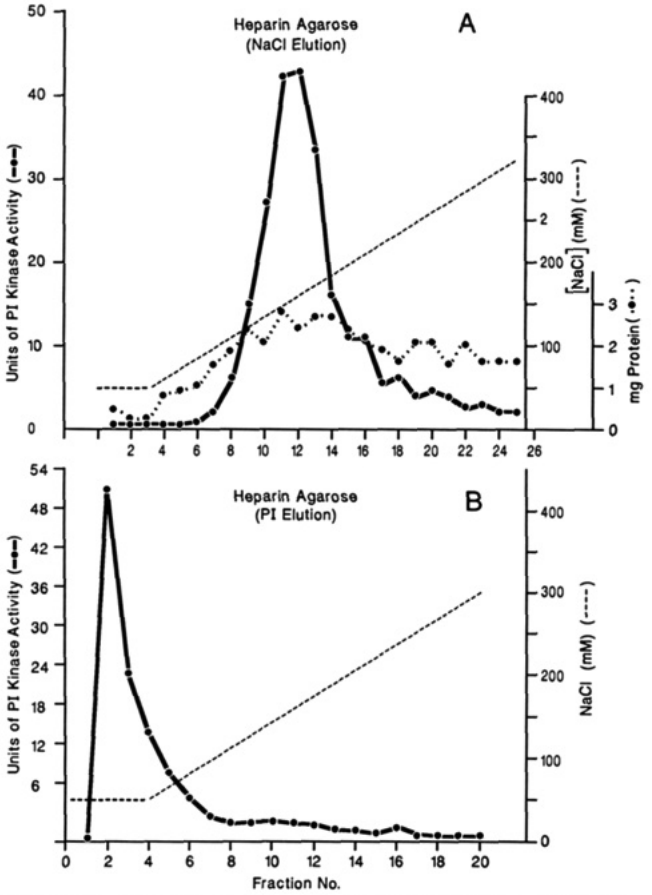

FIGURE 1: Heparin–agarose chromatography of the PI kinase. Solubilized A431 cell membranes were applied to heparin–agarose columns as described under Experimental Procedures. Column fractions were assayed for PI kinase and protein as described. (A) Profile from a 10-mL heparin–agarose column eluted with NaCl. (B) Profile from a 2-mL heparin–agarose column eluted with PI. PI kinase was eluted with a 5-mL pulse of 2 mM PI followed by a 20-mL gradient from 0.05 to 0.3 m NaCl containing 2 mM PI as described.

elutes as a single peak of activity at approximately 125 mM NaCl. Approximately a 5-fold purification is achieved in this step. Dialysis of the eluted PI kinase allows reapplication of the material to a smaller heparin–agarose column for affinity elution with PI. The bottom panel of Figure 1 shows the elution profile of the heparin–agarose column developed in buffers containing 2 mM PI. The enzyme is eluted from the column at a concentration of 50 mM NaCl in the presence of this phospholipid. If lower concentrations of PI are used in this step, the enzyme is incompletely eluted, and additional enzyme is subsequently eluted with a NaCl gradient. PI interferes with several different protein assays; consequently, it is difficult to accurately determine the concentration of the protein in the fractions eluted from this column. In addition, unless PI of very high purity is used for elution, a contaminating lipid in the preparation inhibits the activity of the PI kinase. This contaminant has not been identified but exhibits chromatographic behavior similar to that of phosphatidylcholine.

Fractions containing the PI kinase can be concentrated by chromatography on reactive–green agarose. The eluate from the second heparin–agarose column can be diluted and applied directly to this column since the PI kinase is able to bind to this resin in the presence of PI. The majority of the PI flows through the column permitting accurate quantitation of PI kinase activity and protein concentration during this step.

The final purification step is HPLC gel filtration chromatography. The PI kinase elutes as a single peak ahead of the

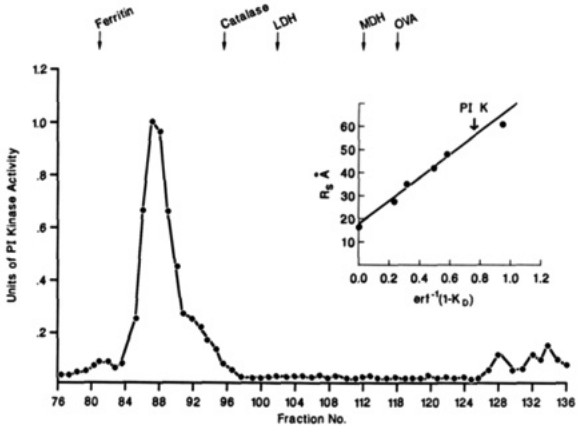

FIGURE 2: Gel filtration chromatography of PI kinase. Fractions from the heparin–agarose column eluted with PI were pooled and concentrated on reactive green–agarose. The material was applied to a TSK-250 HPLC gel filtration column and chromatographed as described under Experimental Procedures. Eluted fractions were assayed for PI kinase activity. The inset shows a plot of Stokes radius vs $erf^{-1}(1 - K_D)$ (Ackers, 1967) used to estimate the Stokes radius of the PI kinase by interpolation. Standards used were ferritin (61 Å), catalase (48 Å) (Nozaki et al., 1976), lactate dehydrogenase (42 Å), malate dehydrogenase (35.1 Å), ovalbumin (27.6 Å), and cytochrome *c* (16.4 Å). The arrow indicates the position of the purified PI kinase.

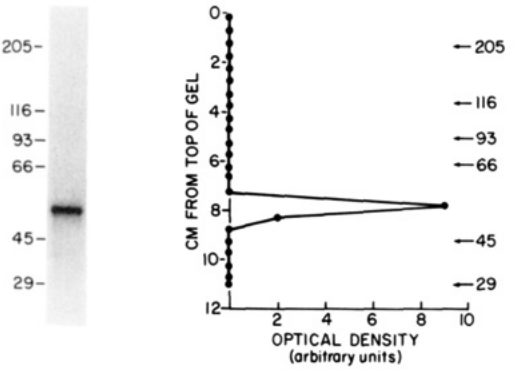

FIGURE 3: SDS–polyacrylamide gel electrophoresis of the purified PI kinase. (Left panel) An aliquot of purified PI kinase was radioiodinated and subjected to SDS–polyacrylamide gel electrophoresis followed by autoradiography. (Right panel) Approximately 2–3 μg of purified PI kinase was analyzed by SDS gel electrophoresis, and sections of the gel were assayed for enzyme activity as described under Experimental Procedures. Following autoradiography of the thin-layer plates, the autoradiograms were scanned by a densitometer. The data are plotted as arbitrary units of absorption.

catalase marker (Figure 2). The purified material can be concentrated, if desired, by adsorption to and elution from a small reactive green–agarose column. With this procedure, a 500-fold purification of the PI kinase is achieved with an overall yield of 10%. The activity of the purified PI kinase is relatively labile. Optimal preservation of activity is achieved through storage of the enzyme in buffer containing 30% glycerol and 2 mM DTT at −20 °C. Under these conditions, activity can be maintained for at least 2 weeks with minimal loss.

The purified PI kinase was radioiodinated and subjected to SDS gel electrophoresis. The autoradiogram from this analysis is shown in the left panel of Figure 3. The purified material contains a single polypeptide chain of 55 000 Da. In a parallel experiment, an aliquot of the purified PI kinase was analyzed by SDS gel electrophoresis. The lane was cut into 0.5-cm sections which were assayed for PI kinase activity as outlined under Experimental Procedures. As shown in the right panel of Figure 3, a single peak of PI kinase activity was detected

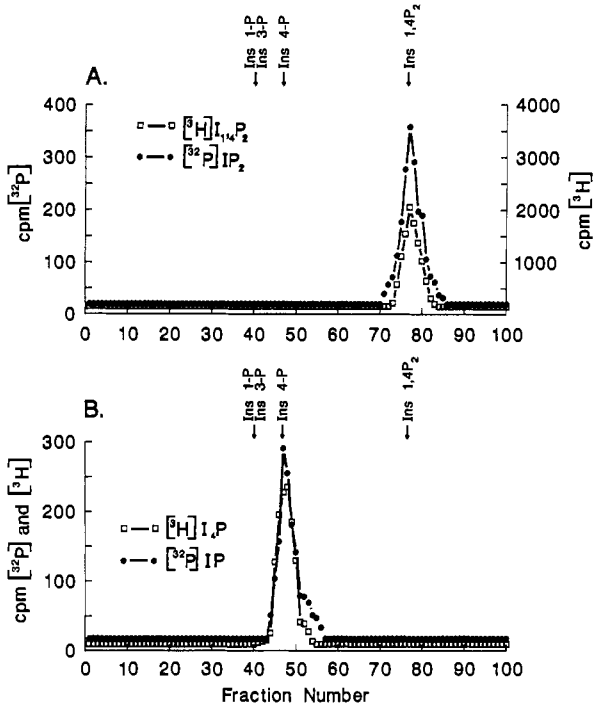

FIGURE 4: Characterization of the product formed by the purified PI kinase. (Panel A) the [^{32}P]PIP formed by the purified PI kinase was hydrolyzed with phospholipase C. Following extraction of the lipids, an aliquot of [^{3}H]inositol 1,4-bisphosphate was added to the aqueous phase, and the water-soluble compounds were chromatographed on a Dowex column as described under Experimental Procedures. Arrows indicate the elution positions of other authentic tritium-labeled inositol phosphate standards that were run on the column. (Panel B) The [^{32}P]IP_2 isolated from the above Dowex column was treated with inositol polyphosphate 1-phosphatase as described under Experimental Procedures, and the products were chromatographed on a Dowex column.

at a position that corresponds to that of the 55 000-Da band. Thus, PI kinase activity appears to be associated with the major polypeptide purified by this procedure.

The product of the reaction using the purified enzyme was characterized to determine which position on the inositol ring was phosphorylated by the PI kinase. Characterization involved hydrolysis of the [^{32}P]PIP product with phospholipase C followed by analysis of the resulting [^{32}P]IP_2 by chromatography on Dowex. As shown in Figure 4A, the ^{32}P-labeled product cochromatographed with a sample of authentic [^{3}H]inositol 1,4-bisphosphate. These data suggest that the original [^{32}P]PIP produced by the purified PI kinase had been phosphorylated at the 4-position of the inositol ring. To further confirm this hypothesis, the [^{32}P]IP_2 isolated from the Dowex column shown in Figure 4A was subjected to hydrolysis by the inositol polyphosphate 1-phosphatase. This enzyme will cleave phosphate from the 1-position of inositol 1,4-bisphosphate but not from that of inositol 1,3-bisphosphate (Inhorn & Majerus, 1987). As shown in Figure 4B, the ^{32}P-labeled IP_2 was completely converted to inositol monophosphate, indicating that the material served as a substrate for the inositol polyphosphate 1-phosphatase. This inositol monophosphate cochromatographed with [^{3}H]inositol 4-monophosphate, the dephosphorylation product of authentic [^{3}H]inositol 1,4-bisphosphate. Both the ^{3}H- and ^{32}P-labeled compounds were clearly distinct from inositol 1-monophosphate and inositol 3-monophosphate. These data confirm that the [^{32}P]IP_2 isolated in Figure 4A is [^{32}P]inositol 1,4-bisphosphate and indicate that the original [^{32}P]PIP had been labeled in the 4-position by the PI kinase. The purified PI kinase is therefore a phosphatidylinositol 4-kinase.

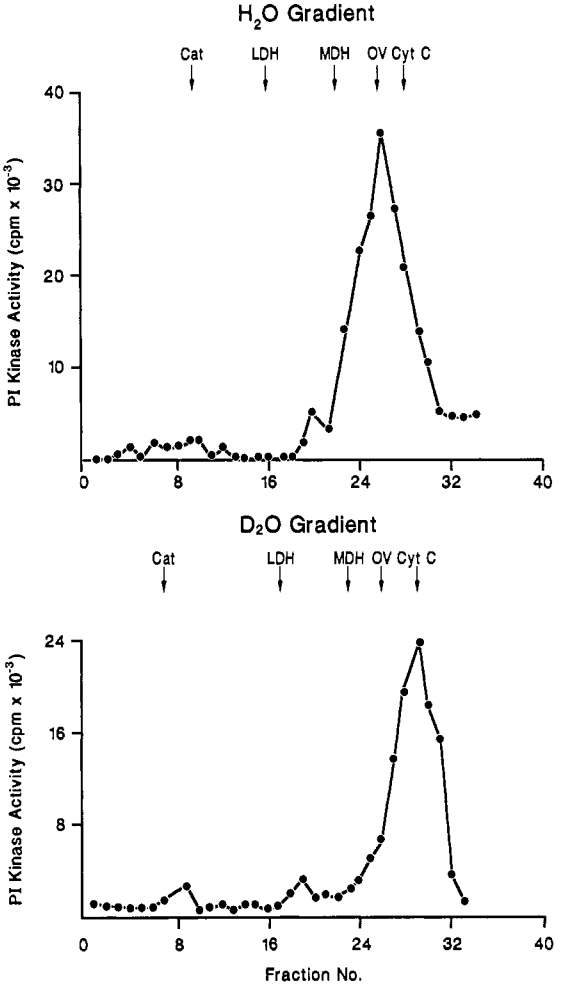

FIGURE 5: Glycerol density gradient centrifugation of the purified PI kinase. Purified PI kinase was analyzed by glycerol density gradient centrifugation in H_2O (top panel) and D_2O (bottom panel) as described under Experimental Procedures.

Physical Properties of the Purified PI Kinase. As shown in Figure 2, the PI kinase eluted from the TSK-250 column ahead of catalase and exhibited a Stokes radius of 55 Å. This would correspond to a mass of approximately 275 000 Da for a typical globular protein. The subunit size of 55 000 Da and the relatively large Stokes radius obtained by gel filtration suggested that the PI kinase was either a multimer or that it was a monomer that bound large amounts of detergent. These two possibilities can be distinguished since detergent binding increases not only the apparent size of the protein but also its partial specific volume. A protein with an abnormally high partial specific volume shifts its position relative to standard marker proteins when the protein and marker enzymes are cosedimented in solvents of different densities (Edelstein & Schachman, 1967; Sadler, 1979). The purified PI kinase was therefore analyzed by density gradient centrifugation on glycerol gradients made with either H_2O or D_2O. This procedure allows calculation of the partial specific volume of the protein/detergent complex as well as the sedimentation coefficient ($s_{20,w}$) of the complex. The molecular weight of the protein/detergent complex can then be calculated according to the Svedberg equation with these parameters in conjunction with the Stokes radius determined by gel filtration chromatography (Tanford, 1961; Clarke, 1975).

Figure 5 shows the results of the analysis of the purified PI kinase by glycerol density gradient centrifugation. In the gradient prepared in H_2O, the PI kinase sedimented to a position close to the ovalbumin standard. An apparent sed-

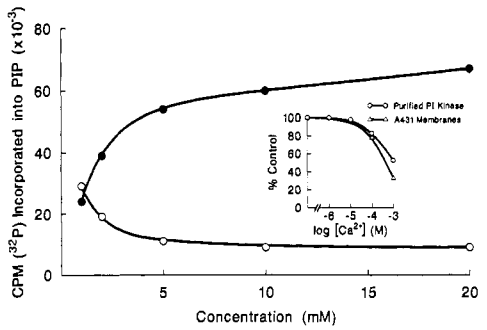

FIGURE 6: Divalent cation requirements of the purified PI kinase. Purified PI kinase was assayed in duplicate as usual except that the Mg^{2+} ions were replaced by different concentrations of either Mg^{2+} (●) or Mn^{2+} (○). The inhibitory effects of Ca^{2+} on the purified enzyme as well as A431 membranes are shown in the inset. Assays were carried out in the presence of 20 mM Mg^{2+} and increasing concentrations of Ca^{2+}.

imentation coefficient of 2.8 S can be calculated by interpolation between the standard proteins. The combination of the large Stokes radius (55 Å) and the small apparent sedimentation coefficient (2.8 S) suggests that the PI kinase binds a large amount of low-density lipid or detergent. The evidence for this is seen in the behavior of the PI kinase in the gradient prepared in D_2O. In this case, the position of the PI kinase shifted significantly so that it cosedimented with the cytochrome *c* marker and exhibited an apparent sedimentation coefficient of 1.4 S. This shift relative to the standard proteins (all with partial specific volumes between 0.72 and 0.74 cm^3/g) is consistent with a very high partial specific volume for the PI kinase due to detergent binding. Calculation of this parameter using the equations shown under Experimental Procedures yields a value of 0.83 cm^3/g for the PI kinase/Triton X-100 complex.

With this information, a value of 3.4 S is obtained for the sedimentation coefficient of the PI kinase corrected to water at 20 °C. The molecular weight of the PI kinase/Triton X-100 complex can then be calculated from the Svedberg equation, which yields a value of 120 000. These data indicate that the PI kinase is present in Triton X-100 containing solutions as a monomer with a molecular weight of 55 000 with approximately 65 000 Da of bound detergent. Since the molecular weight of a Triton X-100 micelle is about 90 000 (Helenius & Simons, 1975), it seems likely that the active species of the PI kinase is a single 55 000-Da polypeptide chain bound to essentially one Triton X-100 micelle.

Properties of the Purified PI Kinase. Figure 6 shows the divalent cation requirements of the PI kinase. Relatively low concentrations (less than 1 mM) of magnesium are required to support high enzymatic activity. Millimolar concentrations of manganese also support activity but at a level significantly below that seen with magnesium. Calcium does not support PI kinase activity. As shown in the inset, the PI kinase is inhibited by calcium when assayed in the presence of 20 mM magnesium. The K_i is approximately 100 μM. An identical value is obtained for the inhibition by calcium of the PI kinase present in A431 cell membranes.

As shown in Table II, when a variety of nucleotides were added to the PI kinase assay, ADP, AMP, and adenosine significantly inhibited PI kinase activity. GTP, GDP, CTP, and UTP failed to affect enzyme activity. Also shown in Table II are the effects of addition of various inositol phospholipids on the activity of the PI kinase. Addition of PIP, PIP_2, or lyso-PIP to PI kinase assays containing 20 μM PI lead to the inhibition of enzyme activity. None of these lipids served as phosphate acceptors when assayed at concentrations up to 100 μM with the enzyme in the absence of PI. To investigate the effects of other lipids on the activity of the PI kinase, the enzyme was assayed in the presence of a 1:1 mixture of phosphatidylinositol and either phosphatidic acid, diacylglycerol, cholesterol, phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine. None of these lipids significantly altered the ability of the enzyme to phosphorylate PI (data not shown).

Table II: Effect of Nucleotides and Inositol Phospholipids on PI Kinase Activity[a]

addition	% control	addition	% control
experiment 1		experiment 2	
50 μM ADP	60	100 μM lyso-PI	67
500 μM ADP	20	100 μM PIP	35
50 μM AMP	89	100 μM PIP_2	42
500 μM AMP	57		
50 μM GDP	95		
500 μM GDP	86		
50 μM GTP	98		
500 μM GTP	101		
50 μM UTP	92		
500 μM UTP	103		
50 μM CTP	102		
500 μM CTP	90		

[a] For experiment 1, purified PI kinase was assayed as described under Experimental Procedures except that the concentration of [γ-^{32}P]-ATP was reduced to 50 μM. Unlabeled nucleotides were added with the labeled ATP to start the reaction. For experiment 2, PI kinase was assayed in the presence of 20 μM PI, and thin-layer chromatography was utilized to isolate the phospholipids as described previously (Pike & Eakes, 1987). The added phospholipids were resuspended in 50 mM β-glycerophosphate and Triton X-100 to a final concentration of 0.1% and added with the PI substrate. All assays were performed in duplicate, and the data are presented as a percentage of the control activity observed in the absence of any addition.

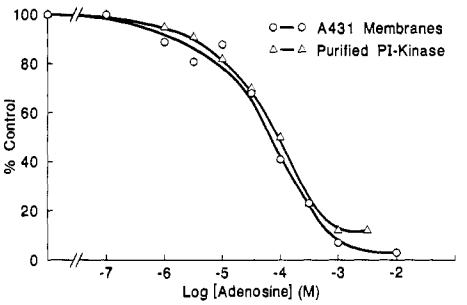

FIGURE 7: Inhibition of PI kinase by adenosine. PI kinase activity was measured in duplicate in the presence of varying concentrations of adenosine for 5 min at 30 °C as described under Experimental Procedures except that 50 μM [γ-^{32}P]ATP was used. Open circles represent assays carried out with the PI kinase from A431 membranes. Open triangles represent assays carried out with the purified PI kinase.

PI kinase activity has previously been reported to be inhibited by adenosine (Doctrow & Lowenstein, 1987). The IC_{50} values obtained have varied among different sources and have been used in an attempt to classify PI kinase activities (Endemann et al., 1987; Whitman et al., 1987). We therefore determined the K_i for inhibition of the purified PI kinase by adenosine so this value could be compared with previously reported values and also with the IC_{50} for the inhibition of the membrane-bound form of PI kinase present in A431 cells. As shown in Figure 7, adenosine is a potent inhibitor of the purified PI kinase exhibiting an IC_{50} of approximately 90 μM. Essentially identical data were obtained when the inhibition of the membrane-bound PI kinase from A431 cells was assayed in the presence of adenosine.

The kinetic parameters for the utilization of both PI and ATP by the purified PI kinase were investigated. Figure 8 shows Lineweaver–Burk plots for the utilization of PI and ATP

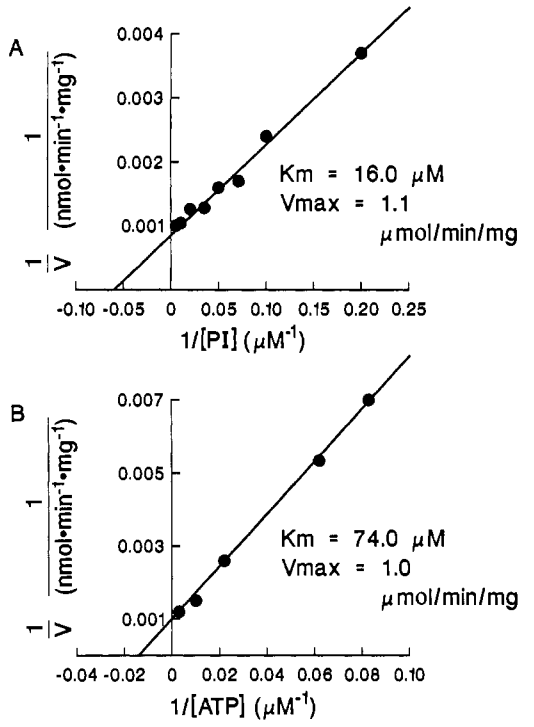

FIGURE 8: Lineweaver–Burk plots for the utilization of PI and ATP by the purified PI kinase. Purified PI kinase was assayed in duplicate for 5 min at 30 °C as described under Experimental Procedures. Activity in the presence of varying concentrations of PI is shown in the top panel. Activity in the presence of varying concentrations of ATP is shown in the bottom panel.

by the PI kinase. The analysis yields K_m values of 17 μM and 74 μM for these two substrates, respectively. Essentially identical K_m values have been obtained for the unstimulated PI kinase assayed in A431 cell membranes (Walker & Pike, 1987).

Discussion

We have purified a PI kinase to apparent homogeneity from A431 cells. The enzyme has a subunit molecular mass of 55 kDa as estimated by SDS gel electrophoresis and a specific activity of 1 μmol min^{-1} mg^{-1}. The PI kinase became quite labile following the affinity elution step, and a loss of up to one-third of the activity occurred during overnight storage at −70 °C. Therefore, the specific activity obtained here is probably somewhat less than the maximum value for this enzyme. Purification was achieved through a combination of chromatographic techniques including affinity elution of the PI kinase from a heparin–agarose column. Heparin is an inhibitor of PI kinase activity (Vogel & Hoppe, 1986), and heparin–agarose acts as a cation-exchange resin. The PI kinase was found to bind strongly to heparin–agarose but only weakly to other cation-exchange resins. It therefore seems likely that binding of the PI kinase to heparin is somewhat specific. The sulfate sugar moieties of heparin may be able to bind at the lipid binding site permitting specific competition and elution with PI.

The final purification step utilizes an HPLC gel filtration column. The bulk of the PI kinase activity elutes at a position just ahead of catalase. However, occasionally small amounts of material elute just at or behind the position of ovalbumin. This may be due to the presence of proteolytic fragments of the enzyme and/or interaction of the protein with the resin as observed in previous studies utilizing crude preparations of PI kinase. Characterization of the [^{32}P]PIP formed by the purified enzyme through enzymatic digestion of the material indicated that the PI kinase phosphorylated the inositol at the 4-position and is therefore a phosphatidylinositol 4-kinase.

Assay of purified material subjected to SDS gel electrophoresis demonstrated that PI kinase activity is present in the region of the 55-kDa band. Thus, PI kinase activity appears to be associated with the major polypeptide in this preparation. The purified PI kinase exhibited unusual hydrodynamic parameters, sedimenting near ovalbumin (M_r 43000) in a density gradient but chromatographing ahead of catalase (M_r 240000) on gel filtration columns. As described under Results, this behavior is consistent with the interpretation that the active PI kinase species is a 55000-Da monomer which binds the bulk of a micelle of Triton X-100.

The characteristics of the PI kinase that we have purified are similar to, if not identical with, those of the membrane-bound enzyme we have previously described (Pike & Eakes, 1987; Walker & Pike, 1987). Both enzymes are active in the presence of Triton X-100. The IC_{50} values for the inhibition of the PI kinase by calcium and adenosine are identical for both the membrane and the purified enzymes. In addition, the K_m values for ATP and PI estimated for both enzymes are essentially identical. Finally, in unpublished work we have found that PI kinase activity solubilized from A431 membranes exhibits the same gel filtration profile as the purified PI kinase. Thus, the purified PI kinase appears to represent the same enzyme that we have characterized in A431 cell membranes. Since we have demonstrated that the membrane-bound PI kinase is stimulated by EGF, it seems likely that the 55-kDa PI kinase that we have purified is the EGF-regulated enzyme. The mechanism by which EGF stimulates the activity of the PI kinase in A431 cells is unknown. Although the enzyme is activated in membranes prepared from cells treated with EGF, we have been unable to demonstrate a stimulation of the enzyme by EGF in isolated membrane preparations (unpublished observations). Thus, activation of the PI kinase by EGF in A431 cells may require additional cytosolic components or may be especially sensitive to proteases or phosphatases activated during cell lysis.

Previous purifications of PI kinases have yielded a 45000-Da polypeptide from bovine brain (Saltiel et al., 1987) and a 55000-Da polypeptide from porcine liver (Hou et al., 1988). Both preparations exhibited a specific activity at least 2 orders of magnitude lower than that obtained by our method. We have found that PI kinase activity is relatively labile; thus, it is possible that these two preparations contain proteolyzed and/or denatured forms of the PI kinase.

Cantley and co-workers (Endemann et al., 1987; Whitman et al., 1987) have attempted to classify PI kinases solubilized from various sources on the basis of, in part, differences in their sedimentation properties on sucrose gradients. Clearly, distinctions based on sedimentation properties may be misleading since the PI kinase purified here exhibits unusual hydrodynamic properties. In addition, the enzymatic properties of our purified enzyme are not like any of those previously categorized (Endemann et al., 1987; Whitman et al., 1987). Since PI kinase activities appear to exhibit diverse kinetic and physical properties, classification of these enzymes should await purification and characterization of the different proteins.

Two groups have recently reported a correlation between the presence of an ~85-kDa protein phosphorylated on a tyrosine in an immunoprecipitate from polyoma virus infected cells and the presence of PI kinase activity in the same immunoprecipitates (Kaplan et al., 1987; Courtneidge & Heber, 1987). Because PDGF treatment of the cells also increased the phosphorylation of this protein, Kaplan et al. (1987)

concluded that the 85-kDa protein was a PI kinase that was regulated by growth factors via phosphorylation on a tyrosine residue. This conclusion is at variance with our finding that the EGF-stimulated PI kinase from A431 cells contains a 55-kDa subunit. If the 85-kDa band is a PI kinase, it must represent a form of the enzyme that is distinct from that present in A431 cells.

As enzymes participating in the metabolism of phosphoinositides, PI kinases could play a pivotal role in the control of signal transduction via PI turnover. The fact that the PI kinase from A431 cells is stimulated by EGF provides a further indication that this enzyme has an important role in the control of cell growth. Purification of this enzyme is a significant step in the search to define the role of the PI kinase in growth regulation and also paves the way for studies designed to elucidate the mechanism by which the PI kinase is activated by EGF.

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