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Purification and Characterization of Membrane-Bound Phosphatidylinositol 4-Kinase from Mouse Brain

Sang Min Lee, Hyeog Gin Son, Young Seek Lee, Kang Suk Lee[1], Sue Goo Rhee[2] and Key Seung Cho*

Department of Biochemistry and Molecular Biology, College of Sciences, Hanyang University, Ansan 425-791
[1]Laboratory of Radiation Biology, The Atomic Energy Research Institute, Taejon 305-353, Korea
[2]Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

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Abstract: A membrane-bound phosphatidylinositol 4-kinase (PI 4-kinase) was separated in a sucrose gradient and solubilized with 1% Triton X-100 from mouse brain. The enzyme was purified 2,952-fold by various chromatographic techniques including DEAE-cellulose, PI-Sepharose and Sephacryl S-200 gel filtration. The molecular weight of PI 4-kinase was approximately 76 kDa by gel filtration and 70.8 kDa by SDS-polyacrylamide gel electrophoresis. The purified enzyme exhibited specific activity of 11.2 nmol/min/mg protein and pI value of 4.7. Kinetic analysis of the PI 4-kinase indicated apparent K_m values of 190 μM and 120 μM for phosphatidylinositol and ATP, respectively. The maximal activity of this purified enzyme was observed at pH 7.4 at an incubation temperature of 37℃. The enzyme activity was significantly activated by Mg^{2+}, Mn^{2+} and Fe^{2+}, and inhibited severely by Ca^{2+}. PI 4-kinase was proved to be pure in its immunoblot test by polyclonal antibody prepared from immunized rabbit sera. By this test, we were able to detect the existence of the same type of PI 4-kinase from other mouse organ tissues, such as liver, heart, kidney and spleen. Furthermore, similar immunoblot analysis with the same antisera recognized the different epitopes of PI 4-kinase proteins from various organs of rabbit, chinese hamster and rat.

Key words: enzyme purification, mouse brain, phosphatidylinositol 4-kinase.

In the past decade, there has been rapid progress in understanding the receptor-mediated intracellular signal transduction system in which one of the phosphoinositides, phosphatidylinositol 4,5-bisphosphate (PIP_2), is hydrolyzed to diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3) acted as second messengers to initiate the cellular signal cascade (Hokin, 1985; Berridge, 1987; Fisher and Agranoff, 1987; Nishizuka, 1988). It became clear that DG stimulates protein kinase C, whereas IP_3 elicits a rise in intracellular calcium concentration from endoplasmic reticulum and finally activates Ca^{2+}-calmodulin dependent protein kinases in numerous cellular events, such as cell growth, differentiation, secretion, contraction, metabolism, phototransduction and motility (Bell, 1986; Ikebe and Reardon, 1990).

Phosphoinositides are minor phospholipids (less than 5% phospholipids) that turn over much more rapidly than other lipids in the membrane (Mejerus *et al.*, 1985). This is the reason why much attention has recently been paid to their metabolism and function in various membranes. Phosphatidylinositol (PI) is phosphorylated to form phosphatidylinositol 4-monophosphate (PIP) catalyzed by phosphatidylinositol 4-kinase (PI 4-kinase, EC 2.7.1.67) that transfers the γ phosphate of ATP to position 4 of the inositol ring of PI. This compound is further phosphorylated (on position 5) by phosphatidylinositol 5-phosphate kinase (PIP 5-kinase) to form PIP_2, which in animal cells can generate second messengers by the action of PI-specific phospholipase C (Wilson *et al.*, 1984; Katan and Parker, 1987; Ryu *et al.*, 1987).

Recent reports on the purification and characterization of PI 4-kinase from rat brain (Van Dongen *et al.*, 1984; Yamakawa and Takenawa, 1988), bovine uteri (Porter *et al.*, 1988), bovine brain myelin (Saltiel *et al.*, 1987), porcine liver (Hou *et al.*, 1988), human erythrocytes (Jenkins *et al.*, 1991; Wetzker *et al.*, 1991; Graziani *et al.*, 1992) and yeasts (Buxeda *et al.*, 1991; Flanagan and Thorner, 1992; Nickels *et al.*, 1992) have demonstrated that these enzymes are believed to reside in the membrane fractions because nonionic detergents are required for solubilization, except one form of erythrocyte PI 4-kinase cytosolic. The molecular weights of

*To whom correspondence should be addressed.
Tel: 82-345-400-5511, Fax: 82-345-419-1760

these enzymes from various mammalian sources were 45~80 kDa, but heterogeneity and multi-subunit complexity have been demonstrated in human erythrocytes and yeast cells (Wetzker *et al.*, 1991; Flanagan and Thorner, 1992).

To elucidate the role of PI 4-kinase in signal transduction, we report here the purification and characterization of membrane-bound PI 4-kinase which represents the first such enzyme to be purified from the membrane fraction of mouse brain.

Materials and Methods

Materials

Phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), ATP, ethyleneglycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), Sephacryl S-200, phenylmethanesulfonylfluoride (PMSF) and Tris were obtained from Sigma. [γ-^{32}P] ATP and L-α-[myo-inositol-2-^{3}H(N)]PI were purchased from Amersham. TLC plate, dithiothreitol (DTT) and all the solvents used were from Merck. Acrylamide, standard proteins, immunoblotting prestained marker, nitrocellulose and Biobeads SM-2 were obtained from Biorad. DEAE-Cellulose and epoxy-activated sepharose were from Whatman and Pharmacia, respectively.

Phosphatidylinositol 4-kinase assay

For the measurement of specific activity during purification of PI 4-kinase, unilamellar vesicles of substrate, ^{3}H-PI were prepared as described by Hofmann and Majerus (1982a), and assays were performed in a 200 μl reaction mixture containing 0.5 mM ^{3}H-PI (20,000~32,000 cpm) or 1 mM ^{32}P-ATP (1 μCi), 10 mM $MgCl_2$, 1 mM EGTA, 0.2% (w/v) Triton X-100, 3 mM ATP (in case of ^{3}H-PI used as substrate), 50 mM Tris-HCl, pH 7.4 and 2 μg enzyme. All assays were run at 37℃ for 10 min and terminated as described by Hofmann and Majerus (1982b). The resulting lipid containing organic solvent phase was dried under N_2 gas stream and dissolved in 200 μl of chloroform : methanol : 12 N HCl (200 : 100 : 1, v/v). The lipids were spotted onto TLC plate and developed with solvent system, chloroform : acetone : methanol : glacial acetic acid : water (40 : 15 : 13 : 12 : 8, v/v). The separation of labelled products was visualized by I_2 vapour or autoradiography and quantitated by liquid scintillation counting as described by Cho *et al.* (1977).

Purification of phosphatidylinositol 4-kinase

Step 1. Preparation of mouse brain extracts: Two hundred grams of mouse brain were freshly obtained from 400 mice (weight, 30± 1.29 g) and were homogenized in a teflon homogenizer with 400 ml of 50 mM Tris-HCl buffer, pH 7.4 containing 0.1 M EGTA, 0.2 mM PMSF, 0.1 mM DTT and 0.32 M sucrose (buffer A). The homogenates were centrifuged at 600×g for 10 min. The supernatant was recentrifuged at 27,500×g for 10 min and precipitate obtained was again homogenized with 2.4 l of 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EGTA, 0.1 mM DTT, 0.2 mM PMSF and 0.8 M sucrose (buffer B). The supernatant obtained by centrifugation at 27,500×g for 1 h was diluted to 1 : 3 ratio with 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EGTA, 0.1 mM DTT and 0.2 mM PMSF (buffer C), and was centrifuged at 27,500×g for 20 min. The precipitate (Ppt 2) was homogenized in 120 ml of 50 mM Tris-HCl buffer, pH 7.4 containing 75 mM KCl, 0.1 mM EGTA, 1 mM DTT, 0.2 mM PMSF and 1% Triton X-100 (buffer D) and was centrifuged at 100,000×g for 30 min. The clear supernatant was used for next purification by column chromatography.

Step 2. Ion exchange chromatography on DEAE-cellulose: The supernatant (120 ml) from Step 1 was poured onto DEAE-cellulose column (1.8×20 cm) preequilibrated with 1 l of buffer D and washed with 200 ml of the same buffer to remove unbound proteins. The column was eluted by 500 ml of buffer D with NaCl gradient (0.0~1.0 M) at a flow rate of 0.73 ml/min. The PI 4-kinase active fractions (Fig. 1A) eluted at 0.4~0.5 M NaCl were pooled (50 ml), concentrated by 60% ammonium sulfate saturation and dialyzed to remove salt overnight with buffer E.

Step 3. PI-Sepharose affinity chromatography: PI-sepharose materials were prepared from PI (40 mg) vesicles coupled with epoxy-activated sepharose (5 g) shaking for 16 h at 37℃ and blocked uncoupled group with 3.5 ml of ethanolamine by incubation at 50℃ for 4 h. PI-sepharose was poured into column (1×25 cm) and equilibrated with 3 bed volumes of 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EGTA and 2 mM DTT (buffer E). Enzyme solution from step 2 was treated with Biobeads SM-2 resin to remove Triton X-100 (Holloway, 1973) before loading onto the column. The column was washed with 30 ml of buffer E and was eluted with buffer E containing 0.5% Triton X-100 and PI 0.5 mg/ml (buffer F) at a flow rate of 0.3 ml/min. The enzyme active fractions (6 ml) from tube No. 38-43 were concentrated to 1 ml in the speed vac. concentrator (Sarvant, model SVC-200H) and used for next step purification.

Step 4. Sephacryl S-200 gel filtration chromatography: The enzyme solution from step 3 was applied onto Sephacryl S-200 column (1.6×120 cm) preequilibrated with 50 mM Tris-HCl buffer, pH 7.4 containing

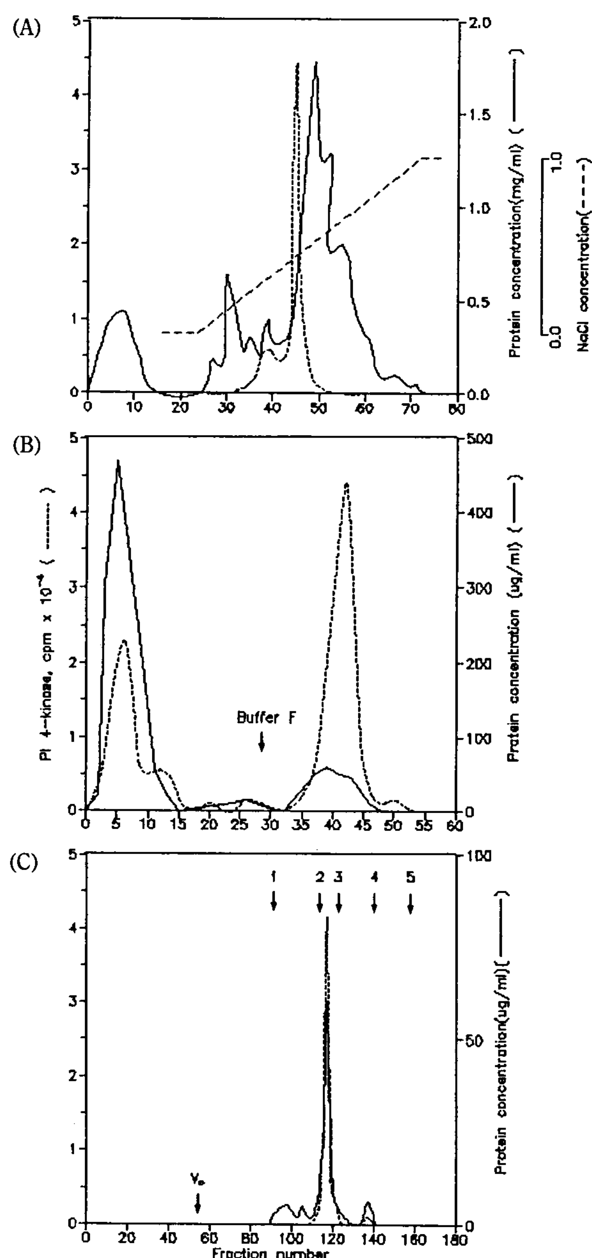

Fig. 1. Purification of PI 4-kinase from 1% Triton X-100 extracts of mouse brain on column chromatography. A membrane-bound PI 4-kinase was isolated in sucrose gradient and solubilized in 50 mM Tris-HCl buffer, pH 7.4 containing 1% Triton X-100 from 200 g of mouse brain in step 1 as described. (A) 120 ml of solublized protein was applied onto DEAE-cellulose column (1.8×20 cm) previously equilibrated with 1 l of buffer D and eluted by 500 ml of buffer D with NaCl gradient (0.0~1.0 M) at a flow rate of 0.73 ml/min. Enzyme active fractions (-----) 50 ml eluted at 0.4~0.5 M NaCl were pooled and concentrated by 60% ammonium sulfate and dialyzed overnight. (B) Enzyme solution from step 2 was treated with Biobeads SM-2 resin to remove Triton X-100 and was loaded onto PI-Sepharose column (1×25 cm) and eluted with buffer F at a flow rate of 0.3 ml/min. The enzyme active fractions (6 ml) from tube No. 38~43 were concentrated to 1 ml. (C) One ml of enzyme solution was applied onto Sephacryl S-200 column (1.6×20 cm) preequilibrated with buffer G and eluted at flow rate of 10 ml/h by same buffer. The enzyme active fractions (8 ml) from tube No. 114-117 were pooled and concentrated by speed vac. concentrator and stored at −20℃.

1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 0.5% Triton X-100 and 10 % glycerol (buffer G) and eluted at flow rate of 10 ml/h by buffer G. The fractions of enzyme activity (tube No. 114-117) were pooled and concentrated by speed vac. concentrator and stored at −20℃. For determination of the native molecular weight of purified kinase, blue dextran was used to measure the void volume. The column was calibrated with bovine liver catalase (245 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.2 kDa) as markers of known molecular weight (Fig. 1C).

SDS-polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis was performed by the method of Laemmli (Laemmli, 1970) in 10% slab gels containing sodium dodecylsulfate and stained by Coomassie blue and silver staining (Wray *et al.*, 1981).

Immunoblot analysis

Antisera against the purified PI 4-kinase from mouse brain were prepared in New Zealand White rabbits. Electrophoretic transfer of protein from slab gels to nitrocellulose sheets and subsequent immunoblot using anti-rabbit IgG conjugated peroxidase were as described (Towbin *et al.*, 1979).

Identification of PIP formation

TLC was performed on commercially pre-coated 20 ×5 cm, 0.25 mm silica gel plate, using chloroform : acetone : methanol : glacial acetic acid : water (40 : 15 : 13 : 12 : 8, v/v) as the solvent and detected the bands under I_2 vapour. For the identification of ^{32}P-PIP formation from PI and γ-^{32}P-ATP, the plate was dried, covered with plastic wrap and autoradiographed at −60℃ for 24 h.

Other methods

Isoelectric focusing was performed in Phast Gel IEF gel (39×47×0.35 mm, pH range 3 to 9) on a Phast system (Pharmacia, USA) (Lee *et al.*, 1995). Proteins were determined by the method of Lowry *et al.* (1951). Radioactivity was measured as described by Cho and Proulx (1973).

Results

Purification of PI 4-kinase from mouse brain membrane fractions

A membrane-bound PI 4-kinase was isolated first from mouse brain homogenates in buffer A and B containing different concentrations of sucrose and then diluted at a ratio 1 : 3 with buffer C. Precipitate was

Table 1. Purification of PI 4-kinase from the mouse brain tissues

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Pufication fold	Yield (%)
Homogenate	12,365	3.26	–	–	–
Fraction (Ppt 2)	1,100	4.18	0.0038	1	100
Soluble fraction	440	3.52	0.008	2.1	84.2
DEAE-cellulose	200	3.30	0.0165	4.3	79.0
60% Ammonium sulfate	22	1.04	0.0472	12.4	24.9
PI-sepharose	0.24	0.90	3.75	986.8	21.5
Gel filtration	0.07	0.785	11.217	2,952	18.8

obtained by centrifugation at 27,500×*g* for 20 min in which a high enzyme activity was recovered (Ppt 2) (Table 1). PI 4-kinase was solubilized by treatment of buffer D containing 1% Triton X-100 and was used for further purification by DEAE-Cellulose ion-exchange chromatography. The enzyme activity was eluted at a salt concentration between 0.4~0.5 M NaCl in 500 ml of buffer D (Fig. 1A). The enzyme active fractions pooled were concentrated by 60% ammonium sulfate saturation and dialyzed in buffer E overnight. This enzyme solution was treated with Biobeads SM-2 resin to remove Triton X-100 (Holloway, 1973) and was loaded onto the PI-Sepharose affinity column preequilibrated with 3 bed volumes of buffer E and then washed an unbound protein from resin with the same buffer. The major peak of unbound protein was eluted at a front phase which has still enzyme activity, but the major kinase activity was eluted at a flow rate 0.3 ml/min by buffer F (Fig. 1B). The 6 ml of solution pooled was concentrated to 1 ml in a speed vac. concentrator and applied onto Sephacryl S-200 gel filtration column prepared by preequilibration of column with 5 bed volumes of buffer G. PI 4-kinase activity eluted as a single sharp peak (Fig. 1C) was correlated to the band with the molecular weight of 76 kDa compairing with a void volumes of marker proteins. The final product was purified 2,952-fold based on an activity in Ppt2 and has a specific activity of 11.2 nmol/min/mg protein with an overall yield of 18.8% (Table 1).

SDS-polyacrylamide gel and immunoblot analysis

The main peak fractions of Sephacryl S-200 gel filtration (114-117) were analyzed on SDS-PAGE and immunoblot. As shown in Fig. 2A, purified PI 4-kinase exhibited one protein band corresponding to a molecular weight of 70.8 kDa on SDS-polyacrylamide gel stained with coomassie blue and silver reagent. Immunoblot of the SDS-PAGE mini gel was carried out by using anti-PI 4-kinase antibody that recognize PI 4-kinase polypeptide (Fig. 2B). The molecular weight of the purified enzyme determined by SDS-PAGE and immunoblot analysis was 70.8 kDa as compared to marker proteins known molecular weight (Fig. 2C).

Results shown in Fig. 3 are the immunoblots of PI 4-kinase from various organ tissues of brain, liver, kidney, heart and spleen in mouse and other animals. In Fig. 3A, all the PI-kinase from various organs of mouse show a single band of the same molecular weight of 70.8 kDa, which seemed to be due to the homology in polypeptides of PI 4-kinase. Interestingly, similar immunoblot analysis with the same antisera recognized the different epitopes of PI 4-kinases from various organs of rabbit (a), chinese hamster (b) and rat (c). PI 4-kinases from different sources of those animals showed protein bands corresponding to molecular weights of 46 kDa, 40 kDa and 80 kDa, respectively (Fig. 3B).

Identification of PIP formation

PI-kinase activities were determined under optimal conditions as described in "Experimental procedures". As shown in Fig. 4 of autoradiogram (A) and I_2 vapour staining (B), the crude Triton X-100 extracts phosphorylated PI to PIP and PIP_2 (Fig. 4Aa and Ba), but the purified enzyme phosphorylated only PI to PIP and did not phosphorylated PIP to PIP_2 (Fig. 4Ab and Bb). The result from the TLC autoradiogram has demonstrated clearly that the purified PI 4-kinase catalyzed only phosphorylation of PI to PIP.

Enzyme kinetics of purified PI 4-kinase

The purified PI 4-kinase was used to determine the kinetic parameters of this enzyme for PI and ATP as substrates. The effect of increasing concentrations of PI and ATP on PI 4-kinase is shown in Fig. 5A for PI and Fig. 5B for ATP, in which enzyme activity showed typical saturation kinetics at concentrations of PI and ATP above 200 μM and 100 μM, respectively. Analysis of a linear double-reciprocal plots of these data revealed an apparent K_m of 190 μM, a V_{max} of 0.93 nmol/min/mg protein for PI, and Km of 120 μM,

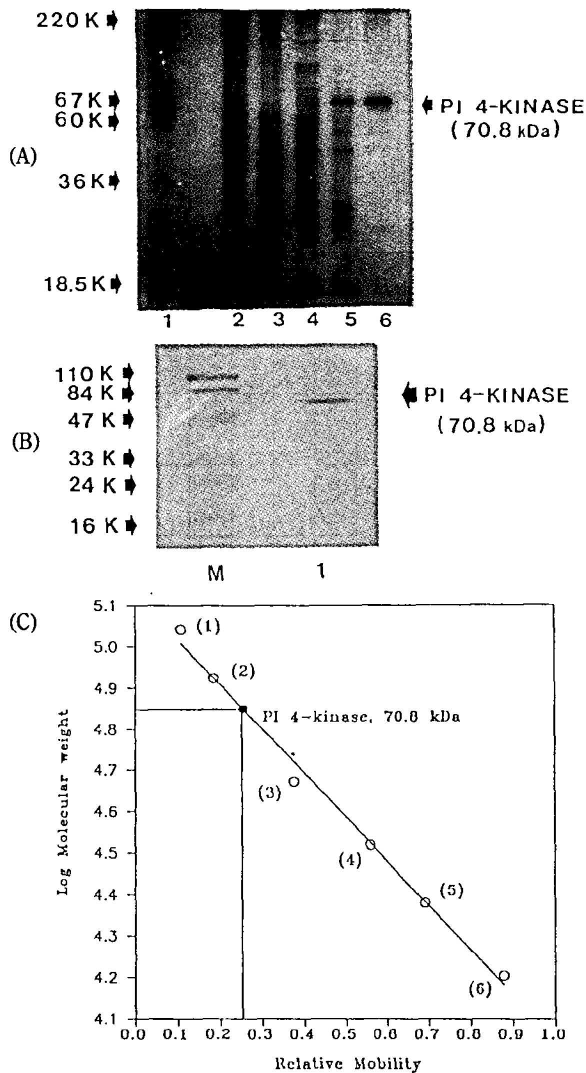

Fig. 2. Determination of molecular weight of PI 4-kinase by SDS-PAGE and Immunoblot analysis. (A) 10% SDS-PAGE gel was run on the vertical electrophoresis unit at 15 mA for 11 h. The samples loaded on the gel were: Lane 1, molecular weight markers, (1) Horse spleen ferritin, half unit (220 kDa), (2) Bovine serum albumin (67 kDa), (3) Beef liver catalase, subunit (60 kDa), (4) Beef heart lactate dehydrogenase, subunit (36 kDa), (5) Horse spleen ferritin, subunit (18.5 kDa). Lane 2, homogenate fraction; lane 3, solubilized fraction; lane 4, DEAE-cellulose fraction; lane 5, PI-sepharose fraction; lane 6, gel filtration fraction. (B) Immunoblot analysis of PI 4-kinase. Protein was electrophoresed in a mini gel, blotted to Zeta-probe and probed with rabbit antibody against PI 4-kinase protein. Lane M, Biorad's pre-stained molecular marker: (1) rabbit muscle phosphorylase B (110 kDa), (2) bovine serum albumin (84 kDa), (3) hen egg white albumin (47 kDa), (4) bovine carbonic anhydrase (33 kDa), (5) soybean trypsin inhibitor (24 kDa), (6) hen egg white lysozyme (16 kDa). lane 1, purified PI 4-kinase from mouse brain. (C) Determination of molecular weight of PI 4-kinase by immunoblot. Molecular markers were shown in (B).

a V_{max} of 0.71 nmol/min/mg protein for ATP.

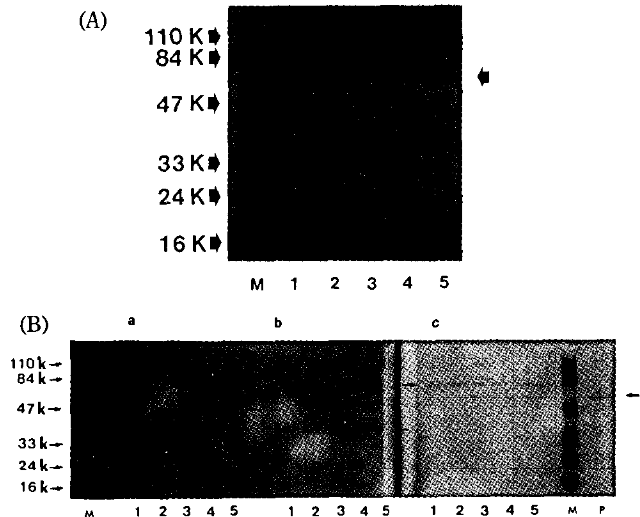

Fig. 3. SDS-PAGE and Immunoblots of various PI 4-kinase from different organs of mouse (A) and different species of rabbit, chinese hamster and rat (B). (A) Triton X-100 extracts solubilized from mouse organs were electrophoresed in a 9% mini gel and immunoblotted with anti-PI 4-kinase antibody. Lane M, Biorad's pre-strained molecular weight markers as shown in Fig. 2B; lane 1, brain; lane 2, liver; lane 3, kidney; lane 4, heart; lane 5, spleen. (B) Triton X-100 extracts solubilized from different organs of various species of rabbit (a), chinese hamster (b), and rat (c) were electrophoresed in a 9% mini gel and immunoblotted with anti-PI 4-kinase antibody. Lane M, Biorad's pre-stained molecular weight markers; lane 1, brain; lane 2, liver; lane 3, kidney; lane 4, heart; lane 5, spleen; lane P, purified PI 4-kinase from mouse brain.

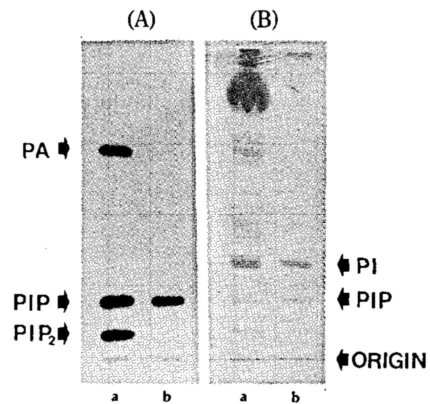

Fig. 4. Formation of PIP by purified PI 4-kinase. Identification of products of PI 4-Kinase from fractions of Triton X-100 extract (100 μg protein) and final stage of gel filtration (2 μg protein) was carried out in a total volume of 200 μg containing 50 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM PI, 1 mM γ-^{32}P-ATP (1 μCi) and 0.2% Triton X-100 at 37℃ for 10 min. TLC was performed at the conditions as described under experimental procedures. Plate A, Autoradiogram, Lane Aa, products from Triton X-100 extract; lane, Ab, product from gel filtration fraction. Plate B, TLC plate stained with I2 vapour. Lane Ba, products from Triton X-100 extract; lane Bb, products from gel filtration fraction.

Effectors of PI 4-kinase activity

The effect of pH on the PI phosphorylation by γ-^{32}P-ATP in the presence of Mg^{2+} and Mn^{2+} is shown in Fig. 6, in which the PI 4-kinase was active at the

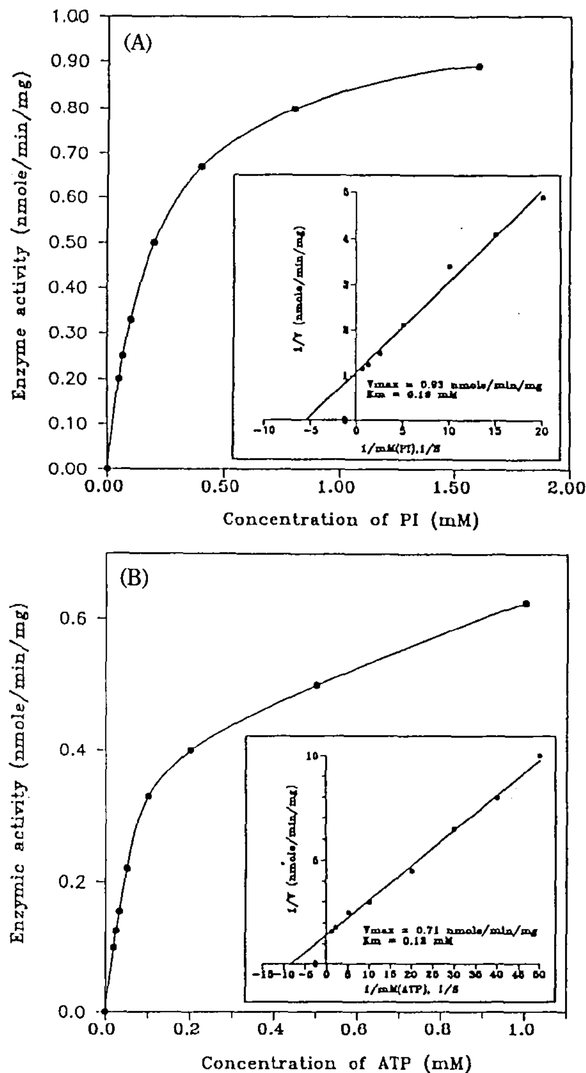

Fig. 5. Relationship between reaction velocity of PI 4-kinase and substrate concentration. Initial reaction velocities of purified PI 4-kinase at various concentrations of each of substrate were measured at a fixed concentration of the other substrate and the data obtained were plotted in Lineweaver-Burk double-reciprocal format. Panel A, relationship of reaction rate to PI concentrations; panel B, relationship of reaction rate to ATP concentrations. Other conditions were described in Materials and Methods.

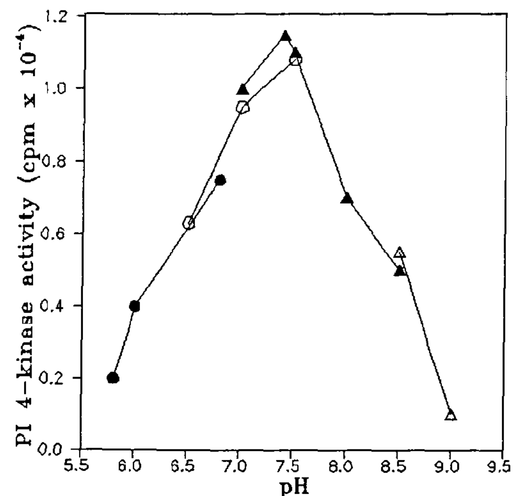

Fig. 6. Effect of pH on PI 4-kinase activity. The reaction mixture contained 1 mM [γ-^{32}P]ATP (1 μCi), 20 mM $MgCl_2$, 1 mM EGTA, 0.2% (w/v) Triton X-100, 1 mM PI and 2 μg enzyme in a total volume of 200 μl with various buffers, pH range 5.8~6.8 (●-●) of 50 mM MES, pH range 6.5~7.5 (○-○) of 50 mM PIPES, pH range of 7.0~8.5 (▲-▲) of 50 mM Tris-HCl, pH range 8.5~9.0 (△-△) of 50 mM borate-NaOH buffer. All other conditions were as described in the text.

physiological pH range of 7.3~7.5 and decreased sharply beyond this pH range. The rate of phosphorylation of γ-^{32}P-ATP into PI measured as a function of concentrations of divalent cations using small unilamella PI vesicles as substrate is shown in Fig. 7. This enzyme was activated significantly at 8 mM Mg^{2+}, 5 mM Mn^{2+} and 5 mM Fe^{2+} with the increasing rate of 450%, 400 % and 400%, respectively. Calcium ion, however, acted as the most potent inhibitor as well as other cations tested, Zn^{2+}, Co^{2+}, Cd^{2+} and Hg^{2+} also inhibited the enzyme activity (result not shown). In order to examine the correlation between stimulatory cations and inhibitory Ca^{2+}, enzyme activity was measured in increasing concentrations of Ca^{2+} in the presence of individual 8 mM Mg^{2+} and 4 mM Mn^{2+}. As shown in Fig. 8, enzyme activation by Mg^{2+} at 8 mM was not influenced at all until increasing to 8 mM Ca^{2+} and inhibited significantly thereafter. On the other hand, a stimulatory effect by Mn^{2+} at 4 mM was strictly inhibited in the increasing concentrations of Ca^{2+}. For these purposes, the enzyme was dialyzed to remove the EGTA and other salts.

Discussion

This is the first purification and characterization of a phosphatidylinositol-specific 4-kinase (PI 4-kinase) from mouse brain. PI 4-kinase is an integral membrane protein and requires non-ionic detergent, such as Triton X-100, for solubilization. In this study, the membrane-bound PI 4-kinase was seperated under the concentration of 0.32 M sucrose and 0.8 M sucrose before treatment of Triton X-100. The yield of these steps (Ppt 2) was usually more than 120% calculated on the basis of the total activity in homogenates. This result suggests that some kind of intrinsic inhibitor may be removed in these steps.

Purification of PI 4-kinase was achieved by conventional three chromatography steps of the 1% Triton X-100 solubilized enzyme. The final step of purification was performed by Sephacryl S-200 gel filtration. The overall recovery of these procedures was 18.8%, and purification was 2,950 fold from sucrose fractionated

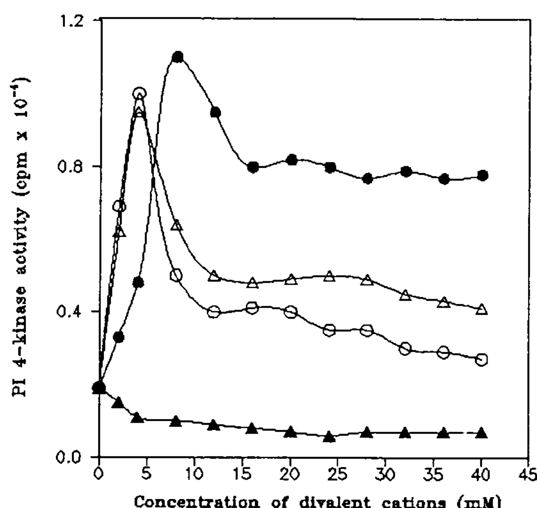

Fig. 7. Effect of divalent cations on PI 4-kinase activity. The PI 4-kinase activity was estimated in the presence of different concentrations of cations. To determine the cationic effect, the enzyme was dialyzed against 50 mM Tris-HCl buffer pH 7.4, containing 1 mM DTT, 10% glycerol and 0.5% Triton X-100 to remove EDTA and other salt. All other conditions were same as described in Materials and Methods. ●-●: Mg^{2+}, ○-○: Mn^{2+}, ▲-▲: Ca^{2+}, △-△: Fe^{2+}.

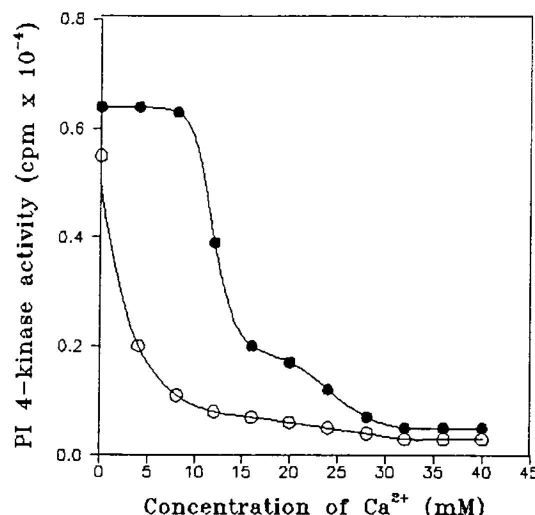

Fig. 8. Effect of Ca^{2+} on PI 4-kinase in the presence of optimal concentration of Mg^{2+} and Mn^{2+}. The PI 4-kinase activity was measured in the increasing concentrations of Ca^{2+} with dialyzed 1 μg enzyme. All other conditions were same as described in in Materials and Methods. ●-●: 8 mM Mg^{2+}, ○-○: 4 mM Mn^{2+}.

membrane fraction (Ppt 2) to yield a specific activity of 11.2 nmol/min/mg protein (Table 1). Eluted fractions from Sephacryl S-200 gel revealed that PI 4-kinase activity was correlated with a molecular weight of 76 kDa (Fig. 1C). SDS-PAGE analysis of this fraction had a molecular weight of 70.8 kDa detected by Coomassie brilliant blue and Silver staining (Fig. 2A). Moreover, only one band was detected with a molecular weight of 70.8 kDa by immunoblot analysis (Fig. 2B, 2C). This enzyme differs in molecular size from the PI 4-kinase purified from rat brain (Van Dongen *et al.*, 1984; Yamakawa and Takenawa, 1988), bovine uterus (Porter *et al.*, 1988), bovine brain myelin (Saltiel *et al.*, 1987), porcine liver (Hou *et al.*, 1988) and human erythrocytes (Jenkins *et al.*, 1991; Wetzker *et al.*, 1991; Graziani *et al.*, 1992). The purified PI 4-kinase from rat brain reported a molecular weight of 76 kDa estimated by SDS-PAGE and 80 kDa by gel filtration. Other PI 4-kinases from various mammalian sources were reported to have a molecular weight of 45~60 kDa, smaller than that of rat brain.

On immunoblot analysis, anti-PI 4-kinase antibody cross-reacted with PI 4-kinases of the same molecular weight of 70.8 kDa from various organ tissues of brain, liver, kidney, heart and spleen in mouse (Fig. 3A). On the other hand, the same antibody also cross-reacted with PI 4-kinases of different molecular weight from different species of rabbit, chinese hamster and rat (Fig. 3B). These results reveal that each animal has a unique PI 4-kinase with identical polypeptide and same the molecular weight even in different organ tissues. Moreover, this antibody is non-specific enough to recognize the different epitopes of PI 4-kinase proteins from different species of animals. From these results, rabbit, chinese hamster and rat have PI 4-kinase with molecular weight of 47 kDa, 40 kDa and 80 kDa, respectively (Fig. 3B). Since the anti-PI 4-kinase antibody is immunocross-reactive between different species, it suggests that the PI 4-kinases have some homology in polypeptide and are somewhat conserved.

The protein purified from mouse brain was identified as PI 4-kinase using two criteria. First, the purified enzyme phosphorylated PI exclusively and not PIP (Fig. 4Ab). This strict substrate specificity is the same as that reported for purified PI kinases from rat brain (Yamakawa and Takenawa, 1988), bovine uterus (Porter *et al.*, 1988) and human erythrocytes (Jenkins *et al.*, 1991; Wetzker *et al.*, 1991). Triton X-100 crude extract phosphorylated PI and also PIP as shown in Fig. 4Aa and Fig. 4Ba. Second, enzymatic activity could be found to comigrate in SDS-PAGE gels with silver-stained protein band and to cross-react with anti-PI 4-kinase antibody (Fig. 2). On the other hand, the identity of ^{32}P-labelled product comigrated with authentic PIP was established using thin layer chromatography detected by autoradiography and I_2 vapour staining (Fig. 4Ab and Fig. 4Bb).

The purified PI 4-kinase from mouse brain has K_m values of 190 μM and 120 μM, and V_{max} values of 0.93 nmol/min/mg protein and 0.71 nmol/min/mg protein for PI and ATP, respectively, which are similar to the kinetic constants from rat brain (Yamakawa and

Takenawa, 1988), but these values are much higher than that from bovine uterus (Porter *et al.*, 1988), porcine liver microsomes (Hou *et al.*, 1988), and human erythrocytes (Wetzker *et al.*, 1991; Graziani *et al.*, 1992). On the basis of molecular weight, kinetic constants and activation by Triton X-100 (not shown), this purified enzyme from mouse brain is similar to the type 1 PI kinase characterized by Endemann *et al.* (1987).

The cation requirement of the enzyme was investigated with several divalent cations. Individual Mg^{2+}, Mn^{2+} and Fe^{2} activated significantly the PI 4-kinase activity with the rate increasing 450%, 400% and 400% at non-physiological concentrations of 8 mM, 5 mM and 5 mM, respectively. At concentrations below 5 mM, Mn^{2+} and Fe^{2+} were more effective than Mg^{2+} under the same conditions. On the other hand, Ca^{2+} inhibited completely the enzyme activity at all concentrations used. The most important thing is that activation of enzyme by Mg^{2+} at 8 mM in increasing concentrations of Ca^{2+} until 8 mM was not affected at all as shown in Fig. 8. On the contrary, enzyme activation by 5 mM Mn^{2+} was abolished completely in the presence of Ca^{2+}. These results indicate potentially that the enzyme possesses a different interacting Mg^{2+} and Mn^{2+} binding sites on it and would be very sensitive to changes in those cation concentrations. The concentration of cytosolic free Mg^{2+} has been estimated to be 0.37 mM (Corkey *et al.*, 1986) and is known to be relatively stable in cells. Considering the fact that Mn^{2+} is more effective than Mg^{2+} in a lower concentration and is competitive on binding with Ca^{2+}, and also Ca^{2+} levels in cytoplasm change rapidly in response to physiological events, one might speculate that an interaction of Mg^{2+} or Mn^{2+} and Ca^{2+} with active site of protein may regulate the activity of PI 4-kinase.

In the metabolic hydrolysis of phosphoinosides, PIP_2 is a good substrate for PI-PLC to produce second messengers, DG and IP_3 in a receptor-mediated signal transduction. In a previous report (Hwang *et al.*, 1988), we observed that selectivity of substrates, such as ^3H-PI and ^3H-PIP_2 in the hydrolysis by mouse brain cytosolic PI-PLC are possibly regulated by the concentrations of Ca^{2+} and ATP at a physiological pH. The enzymatic hydrolysis of PIP_2 was 8 times higher than that of PI without Ca^{2+}, and activated 100% in the presence of 1 mM ATP. However, hydrolysis of PI depends on the concentrations of Ca^{2+} and decreases significantly in the presence of over 10 μM ATP. The previous and recent data strongly support that both PI-kinase and PI-PLC are probably regulated by the concentrations of Ca^{2+}, Mg^{2+} or Mn^{2+} and ATP under physiological conditions.

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