Biochem. J. (1992) 284, 39–45 (Printed in Great Britain)

Purification and characterization of human erythrocyte phosphatidylinositol 4-kinase

Phosphatidylinositol 4-kinase and phosphatidylinositol 3-monophosphate 4-kinase are distinct enzymes

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PtdIns 4-kinase has been purified 83000-fold from human erythrocyte membranes. The major protein detected by SDS/PAGE is of molecular mass 56 kDa, and enzymic activity can be renatured from this band of the gel. The characteristics of this enzyme are similar to other type II PtdIns kinases previously described: PtdIns presented in Triton X-100 micelles is preferred as a substrate over PtdIns vesicles, the enzyme possesses a relatively low K_m for ATP (20 μM), and adenosine is an effective inhibitor. A monoclonal antibody raised against bovine brain type II PtdIns 4-kinase is an effective inhibitor of the purified enzyme. PtdIns(4,5)P_2 inhibits by approx. 50% when added in equimolar amounts with PtdIns; PtdIns4P has little effect on activity. A PtdIns3P 4-kinase activity has also been detected in erythrocyte lysates. Approximately two-thirds of this activity is in the cytosolic fraction and one-third in the membrane fraction. No PtdIns3P 4-kinase activity could be detected in the purified type II PtdIns 4-kinase preparation, nor could this activity be detected in a bovine brain type III PtdIns 4-kinase preparation. The monoclonal antibody that inhibits the type II PtdIns 4-kinase does not affect the PtdIns3P 4-kinase activity in the membrane fraction. The cytosolic PtdIns3P 4-kinase can be efficiently recovered from a 60%-satd.-$(NH_4)_2SO_4$ precipitate that is virtually free of PtdIns 4-kinase activity. We conclude that PtdIns3P 4-kinase is a new enzyme distinct from previously characterized PtdIns 4-kinases, and that this enzyme prefers PtdIns3P over PtdIns as a substrate.

INTRODUCTION

A variety of polyphosphoinositides are produced *in vivo* by sequential phosphorylation of phosphatidylinositol at distinct sites on the inositol ring [1,2]. Several of these lipids are known to provide signals in response to growth factors and hormones. Most notably, PtdIns(4,5)P_2 is the precursor for the regulator of intracellular Ca^{2+} release, Ins(1,4,5)P_3 [3]. This lipid may also affect actin-filament growth by directly interacting with actin-binding proteins [4]. Polyphosphoinositides phosphorylated at the D-3 position of the inositol ring have also been implicated as second messengers. PtdIns 3-kinase was discovered because of its co-purification with activated protein-tyrosine kinases [5–7], and PtdIns3P has been detected as a low abundance lipid in intact cells [7,8]. Subsequently the cellular levels of a series of lipids phosphorylated at the D-3 position of the inositol ring [PtdIns-(3,4)P_2, PtdIns(3,4,5)P_3 and putatively PtdIns(3,5)P_2] have been found to be elevated in cells stimulated by growth factors and hormones, especially those that activate cellular protein-tyrosine kinases [9]. Stimulation of the PtdIns 3-kinase appears to be an essential event for transformation by oncogenes that involve protein-tyrosine kinase activation [9,10].

The pathways for production of these lipids have not been fully characterized, and only a few of the enzymes have been purified. At least two distinct PtdIns 4-kinases have been described (named type II and type III to distinguish them from PtdIns 3-kinase, originally named type I) [11,12]. The type II PtdIns 4-kinase appears to be nearly ubiquitous in mammalian cells, and this type of enzyme has been purified to homogeneity from bovine uterus [13] and A431 cells [14]. This enzyme has a subunit molecular mass of approx. 56 kDa [13,14], is tightly associated with the plasma membrane and migrates in detergent as a monomer [12]. The type III PtdIns 4-kinase was originally described in bovine brain [12]. It has a much higher K_m for ATP than the type II enzyme, and, unlike the type II enzyme, is not significantly inhibited by adenosine. An enzyme of similar characteristics has been purified from rat brain [15]. A monoclonal antibody that inhibits the type II PtdIns 4-kinase from a variety of tissues and species fails to inhibit the type III enzyme [16].

At least two distinct PtdIns4P 5-kinases, type I and type II, exist in the erythrocyte [17]. The type II enzyme exists in both the cytosol and membrane fraction and can be extracted from the membrane with high salt; it has been purified to homogeneity and been shown to have a 53 kDa catalytic subunit [18]. This enzyme will not significantly phosphorylate PtdIns. The type I PtdIns4P 5-kinase appears to be a distinct gene product, but has not been purified to homogeneity [17].

A PtdIns 3-kinase has been purified to homogeneity from rat liver [19]. Surprisingly, this enzyme will phosphorylate the D-3 position of PtdIns, PtdIns4P or PtdIns(4,5)P_2 almost equally well, although the substrate preference is very sensitive to assay

Abbreviations used: PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine.

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conditions. This enzyme could account for the production *in vivo* of PtdIns(3,4)P_2 by phosphorylation of PtdIns4P at the D-3 position [20]. However, an enzymic activity was recently discovered in platelets, which phosphorylates PtdIns3P at the D-4 position of the inositol ring to produce PtdIns(3,4)P_2 [21]. This activity had not been previously characterized because of the limited availability of PtdIns3P as a substrate.

Herein, PtdIns 4-kinase and PtdIns3P 4-kinase activities from the human red cell are characterized. The PtdIns 4-kinase has been purified to near homogeneity and shown to be a type II PtdIns kinase. The purified enzyme failed to phosphorylate PtdIns3P, but a separable PtdIns3P 4-kinase activity was detected in a cytosolic fraction lacking significant PtdIns 4-kinase activity. Thus, PtdIns3P 4-kinase and PtdIns 4-kinase are distinct enzymes in the erythrocyte.

EXPERIMENTAL

Materials

[^{3}H]PtdIns, [^{3}H]PtdIns4P, [^{3}H]PtdIns(4,5)P_2 and [γ-^{32}P]ATP were purchased from New England Nuclear. PtdIns and phosphatidylserine (PtdSer) were purchased from Avanti. PtdIns4P and PtdIns(4,5)P_2 were from Sigma. All the reagents for SDS/PAGE were from Bio-Rad. Silica gel 60 t.l.c. plates were from Merck. DEAE-Sephacel, sulphopropyl (SP)-Sephadex and all other reagents were from Sigma.

Purified 4C5G monoclonal antibodies and partially purified bovine brain type III PtdIns 4-kinase were prepared as previously described [16].

Assay of PtdIns kinase activity

PtdIns kinase activity was routinely assayed at 25 °C in 20 ml of a reaction mixture containing 225 μM-PtdIns, 50 mM-Hepes, pH 7.5, 1 mM-EGTA, 0.1 % Triton X-100 (PtdIns was dried with nitrogen and sonicated for 10 min in a bath sonicator together with buffer and detergent), 100 μM-[γ-^{32}P]ATP (2000 Ci/mol) and 10 mM-$MgCl_2$. The reaction was started by adding the enzyme and was terminated by adding 75 μl of 1 M-HCl and 150 μl of chloroform/methanol (1:1, v/v). The tubes were vortex-mixed for 30 s, centrifuged, and the organic phase was extracted and spotted on to 0.2 mm-thick oxalate-pretreated silica gel 60 plates (Merck). Plates were developed in chloroform/methanol/water/37 % NH_3 (60:47:11:2, by vol.). The reaction products were detected by autoradiography and identified by comparison with unlabelled standard phospholipids made visible by iodine vapour. Radioactivity in phospholipids was determined by cutting the appropriate piece of the t.l.c. plate and counting it in liquid-scintillation mixture.

Purification of PtdIns kinase from human erythrocyte membranes

Erythrocytes were obtained from 1 unit or less of fresh blood, and membranes prepared as described previously [18]. The membranes were then extracted by resuspending them with lysis buffer (5 mM-sodium phosphate, 1 mM-EDTA, 25 μM-ammonium vanadate, 1 mM-dithiothreitol, pH 7.2) to which 1 M-NaCl was added. After a 10 min incubation on ice, the membranes were centrifuged at 27000 *g* for 20 min. The resulting pellet was washed with buffer A (50 mM-Tris, pH 8, 20 % glycerol, 1 mM-dithiothreitol, 1 mM-EGTA, 0.2 mM-vanadate, 0.2 mM-phenylmethanesulphonyl fluoride and 10 μg each of aprotinin, soybean trypsin inhibitor and leupeptin/ml) and then solubilized in 10 vol. of solubilization buffer (buffer A plus 0.1 % Triton X-100). After a 15 min incubation on ice, the membranes were centrifuged at 27000 *g* for 20 min.

The detergent extract was applied to a 200 ml DEAE-Sephacel column equilibrated with solubilization buffer. The flow-through was collected, and then the column was eluted with 250 ml of the same buffer containing 1 M-NaCl; 50 ml fractions were collected from both the flow-through and the eluate, and assayed under standard conditions as described above.

The fractions from the flow-through containing most of the PtdIns 4-kinase activity were pooled, dialysed against buffer B (25 mM-Mes, pH 6, 20 % glycerol, 10 mM-$MgCl_2$, 1 mM-EGTA, 0.2 mM-ammonium vanadate, 1 mM-dithiothreitol, 0.1 % Triton X-100 and proteinase inhibitors as in buffer A), and loaded on a 50 ml SP-Sephadex column equilibrated with the same buffer. The column was eluted with a linear gradient of 0–0.4 M-NaCl in buffer B; 5 ml fractions were collected and PtdIns 4-kinase activity was assayed.

SDS/PAGE

The protein from the column fractions was precipitated with 5 % trichloroacetic acid and the pellet resuspended in 4-fold-concentrated Laemmli sample buffer. SDS/PAGE was performed on a 10 %-acrylamide separating gel and a 4 % stacking gel by the Laemmli method [22]. Proteins were detected by silver staining [23].

Renaturation of PtdIns kinase activity from SDS/PAGE

Samples containing SP-Sephadex-purified PtdIns kinase were incubated in SDS sample buffer (1 % SDS, 5 % β-mercaptoethanol, 10 % glycerol, 20 mM-Tris, pH 6.8) for 5 min at 50 °C. The SDS/PAGE gels were left overnight before use. The electrophoresis was performed as described above, at 4 °C, at low current (no more than 20 mA). The gel was sliced into 24 pieces (0.5 cm each), and each piece was homogenized and left to incubate for 16 h at 4 °C with 0.25 ml of renaturation buffer, containing 225 μM-PtdIns, 400 μM-phosphatidylcholine (PtdCho) and 0.1 % Triton X-100. PtdIns kinase activity was assayed by addition of [γ-^{32}P]ATP as described above.

Preparation of [^{32}P]PtdIns3P

[^{32}P]PtdIns3P was made by incubating PtdIns with [γ-^{32}P]ATP and purified PtdIns 3-kinase as previously described [19]. The product was extracted from the reaction mixture with chloroform/methanol/1 M-HCl as described above and stored at −70 °C. In some cases [^{32}P]PtdIns3P was purified from PtdIns by a neomycin affinity column as described by Schacht [24].

PtdIns3P 4-kinase assay

Unless otherwise described, PtdIns3P 4-kinase was assayed at 37 °C for 15 min in 20 ml of a reaction mixture containing [^{32}P]PtdIns3P (0.5–1 μM), [^{3}H]PtdIns4P (0.5–1 μM), 9 μM-PtdIns, 8 μM-PtdSer, 500 μM-ATP and 10 mM-$MgCl_2$, in 50 mM-Hepes buffer (pH 8)/1 mM-EGTA (lipids were dried under nitrogen and sonicated for 10 min in a bath sonicator). The reaction was started by adding enzyme and was terminated with chloroform/methanol/HCl. The lipids were extracted and identified as previously described [11]. In brief, the lipids were deacylated by incubation with methylamine at 54 °C for 40 min; the derived glycerophosphoinositol phosphates were applied to a strong anion-exchange h.p.l.c. (Whatman Partisil 10 SAX) column and eluted with a gradient of ammonium formate [11].

In one experiment the deacylated lipids were deglycerated as described previously [11], and the resulting inositol phosphates analysed by h.p.l.c.

Protein determination

Protein was determined by the Lowry method [25]. Triton X-100 was removed by precipitating protein in 5 % trichloroacetic acid.

Table 1. Purification of PtdIns 4-kinase

	Protein (mg)	Activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)
Cell lysate	24171	136	0.006	–	100
Membranes	1432	120	0.056	14	88
Triton extract	110	250	2.3	383	180
DEAE-Sephacel	1.3	160	120	20000	120
SP-Sephadex	0.02	10	500	83300	7

RESULTS

Purification of PtdIns 4-kinase from erythrocytes

PtdIns 4-kinase activity resides primarily in the particulate fraction of fresh human erythrocyte lysates. After incubation of erythrocyte membranes with 1 M-NaCl, most of the PtdIns 4-kinase activity remains with the membrane fraction (Table 1). PtdIns4*P* 5-kinase activity is extracted from the membranes by this procedure [18].

PtdIns 4-kinase is efficiently solubilized when the membranes are treated with a buffer containing 0.1% Triton X-100 (Table 1). From this step on, the enzymic activity is entirely dependent on exogenously added PtdIns (results not shown).

The detergent extraction yields an increase in the total activity, due either to the removal of an inhibitor present in the membranes or to the more efficient utilization of the exogenous PtdIns after solubilization. Further purification is obtained by ion-exchange chromatography on DEAE-Sephacel at pH 8. At this pH, most of the PtdIns 4-kinase fails to bind to the column and is recovered in the flow-through. Conversely, most of the proteins from the solubilized membranes are retained on the column and can be eluted with 1 M-NaCl (Fig. 1). When the peak fractions, containing 60–70% of the activity loaded on the column, are pooled, dialysed and applied to a SP-Sephadex cation-exchange column, PtdIns 4-kinase is eluted as a sharp peak with a 0–0.4 M-NaCl gradient (Fig. 2). An 83000-fold purification to a specific activity of 500 nmol/min per mg is obtained by this procedure.

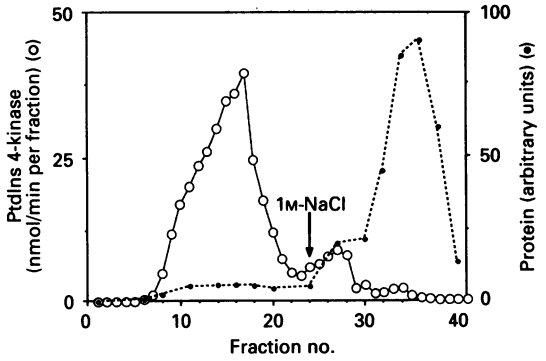

Fig. 1. DEAE-Sephacel chromatography of PtdIns 4-kinase

The Triton-X-100-solubilized fraction from erythrocyte membranes was added to a DEAE-Sephacel column as described in the Experimental section; 50 ml fractions were collected. The arrow indicates the beginning of elution with 1 M-NaCl. PtdIns 4-kinase activity (○) and protein concentration (●), expressed in relative units, were determined as described in the Experimental section. The data presented are from a representative experiment.

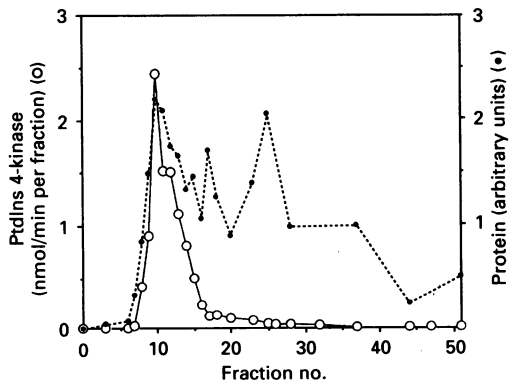

Fig. 2. SP-Sephadex chromatography of PtdIns 4-kinase

The peak fractions of PtdIns 4-kinase activity from the DEAE-Sephacel column were concentration and added to an SP-Sephadex column. The column was eluted with a linear NaCl gradient as described in the Experimental section: 5 ml fractions were collected, and PtdIns 4-kinase activity was assayed on dialysed fractions under standard conditions. The data presented are from a representative experiment.

A 56 kDa peptide contains the PtdIns 4-kinase activity

The protein content of the peak fractions from the columns is summarized in Fig. 3(*a*). Most of the major erythrocyte membrane proteins, including band 3, are removed by the DEAE-Sephacel column (lane 2 versus lane 1). Among the proteins detected in the peak of PtdIns 4-kinase activity from the DEAE-Sephacel column is a major band at 56 kDa. This protein is about 50% of the protein observed by silver staining of SDS/PAGE of the activity peak eluted from the SP-Sephadex column (Fig. 3*a* lane 3, and Fig. 3*b*). The 56 kDa peptide has been found to be co-eluted with the PtdIns 4-kinase activity from five different SP-Sephadex columns (results not shown).

Proof that the 56 kDa protein is the PtdIns 4-kinase was provided by renaturing the enzymic activity from this region of the SDS gel (Fig. 3*c*). The SP-Sephadex-purified PtdIns 4-kinase was separated on SDS/PAGE, the gel was cut into sections and PtdIns 4-kinase activity was renatured as described in the Experimental section. Adjacent lanes loaded with the same fraction and with molecular-mass markers were stained to allow alignment of the proteins with the activity. About 10% of the loaded activity was recovered in a single peak coinciding with the 56 kDa peptide (Fig. 3*c*). A small shoulder of activity was also detected in a higher-mobility fraction, suggesting that the faint band(s) detected below the 56 kDa peptide (Fig. 3*a*, lane 3) may be proteolytic fragments of the 56 kDa peptide. These results show that a single peptide at 56000 kDa contains the PtdIns 4-kinase activity. This size is similar to that of the PtdIns 4-kinases previously purified from bovine uterus [13] and A431 cells [14].

By h.p.l.c. analysis of the glycerophosphoinositol phosphate derivative, the PtdIns*P* generated *in vitro* by the purified erythrocyte enzyme is shown to be PtdIns4*P*, indicating that the enzyme is authentically a PtdIns 4-kinase (results not shown).

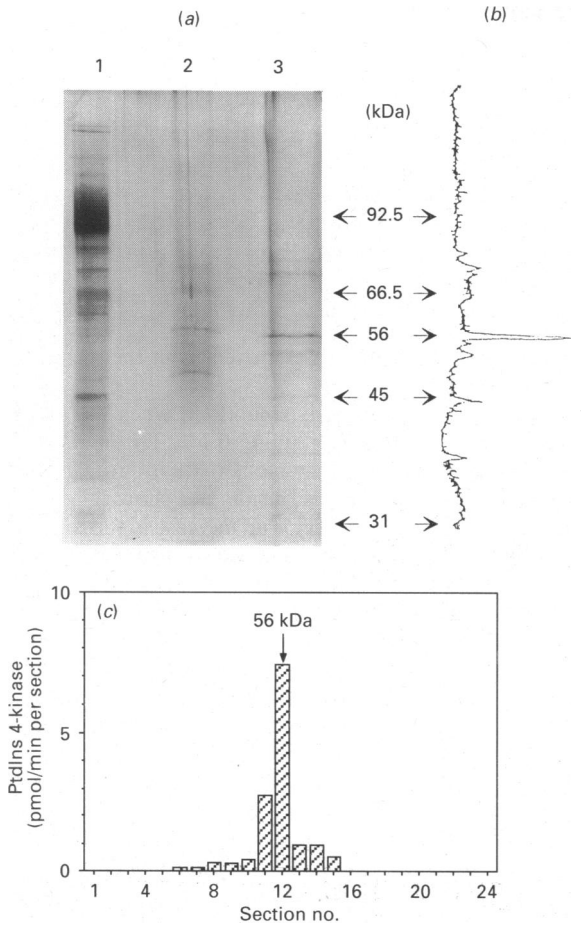

Fig. 3. SDS/PAGE analysis of PtdIns kinase from purification steps, and renaturation of PtdIns 4-kinase activity after SDS/PAGE

(*a*) Lanes 1, 2 and 3 contain respectively Triton X-100-solubilized membranes (50 μg of protein; activity 75 pmol/min), the peak fraction from the DEAE-Sephacel column (0.5 μg of protein; 60 pmol/min) and the peak fraction from the SP-Sephadex column (0.5 μg of protein; 260 pmol/min). (*b*) Laser-densitometer profile of lane 3. (*c*) A lane of the SDS gel containing an amount of the SP-Sephadex-purified enzyme equivalent to that in lane 3 was cut into sections, and PtdIns 4-kinase activity was renatured as described in the Experimental section. The activity peak contained the major 56 kDa band observed in lane 3. Approx. 10% of the total PtdIns 4-kinase activity applied to the gel was renatured in this peak.

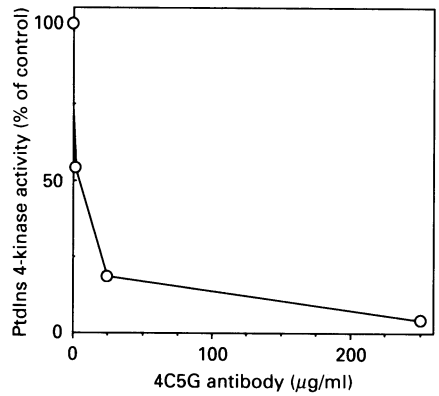

Fig. 4. Antibody 4C5G inhibits PtdIns 4-kinase of erythrocyte membranes

Purified human erythrocyte PtdIns 4-kinase was incubated with 4C5G antibody (μg/ml) as described in the Experimental section. Then PtdIns kinase was assayed under standard conditions as described in the text.

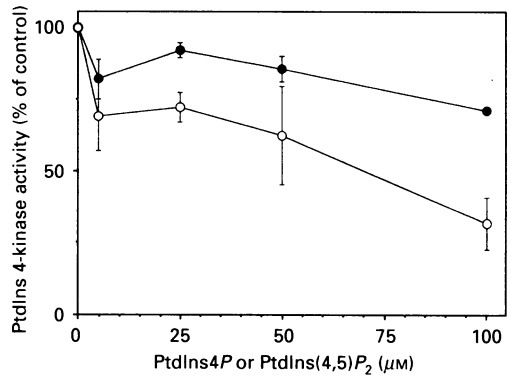

Fig. 5. Effects of PtdIns4*P* and PtdIns(4,5)P_2 on PtdIns 4-kinase

SP-Sephadex-purified PtdIns 4-kinase was assayed under standard conditions as described in the Experimental section, except that the assay contained 100 μg of PtdIns/ml, and the indicated amount of either PtdIns4*P* (○) or PtdIns(4,5)P_2 (●). The data are means ± S.E.M. of three experiments.

PtdIns 4-kinase purified from human erythrocytes is type II PtdIns kinase

The ATP-dependence, adenosine inhibition and detergent activation of this enzyme are all consistent with previous observations made with type II PtdIns kinases [11–14]. The K_m for ATP is 20 μM, and the concentration giving 50% inhibition is 75 μM (assayed at 50 μM-ATP) (results not shown). The purified enzyme readily phosphorylates PtdIns, which is present in a lipid/non-ionic-detergent mixed micelle. In absence of the detergent, the activity of the purified enzyme is decreased more than 700-fold with respect to standard conditions (results not shown). In the presence of 0.1% Triton, the apparent K_m for PtdIns was 50 μM (results not shown).

Furthermore, PtdIns 4-kinase activity purified from human erythrocytes was recognized by a highly specific inhibitory monoclonal antibody (4C5G) raised against the bovine brain type II PtdIns 4-kinase [16]. The human erythrocyte PtdIns 4-kinase activity was inhibited at similar titres to those inhibiting the bovine brain type II enzyme. The 4C5G antibody failed to inhibit both type III PtdIns 4-kinase partially purified from bovine brain, and PtdIns 3-kinase purified from a human myeloid cell line (Fig. 4).

The effects of PtdIns4*P* and PtdIns(4,5)P_2 were also investigated. Mixed micelles were prepared with 0.1% Triton X-100, 100 μM-PtdIns and 5–100 μM of either of the lipids. PtdIns4*P*, the lipid product of PtdIns 4-kinase, does not affect the activity, but PtdIns(4,5)P_2 causes greater than 45% inhibition when added in equal mass with PtdIns (Fig. 5).

Erythrocyte type II PtdIns 4-kinase does not significantly phosphorylate the D-4 position of PtdIns3*P*

The ability of the purified PtdIns 4-kinase to utilize PtdIns3*P* as a substrate was investigated. [^{32}P]PtdIns3*P* was synthesized and purified from PtdIns on a neomycin column as described in the Experimental section. [^{3}H]PtdIns was added, and the two lipids were sonicated with either PtdCho and 0.1% Triton X-100 (optimal conditions for type II PtdIns 4-kinase) or PtdSer without detergent (optimal conditions for type I PtdIns 3-kinase). Unlabelled ATP was added, and the percentage conversion of the radioactive lipids into products was assayed by measuring ^{3}H and ^{32}P contents of reactants and products from h.p.l.c. profiles before and after the incubation with enzyme (Table 2). Although

Table 2. Phosphoinositide substrate specificity of type II and type III PtdIns 4-kinases

Either the purified human erythrocyte type II PtdIns 4-kinase or the partially purified bovine brain type III PtdIns 4-kinase was incubated at 37 °C for 60 min in the presence of 0.5 mM-ATP, 10 mM-$MgCl_2$ and a mixture of [^{32}P]PtdIns3*P* (668000 d.p.m.; 1 μM final concn.) and [^{3}H]PtdIns (578000 d.p.m.; 1 μM final concn.). The lipids were prepared as follows: [^{3}H]PtdIns and neomycin-purified [^{32}P]PtdIns3*P* were sonicated in Hepes/EGTA buffer as described in the Experimental section either in the presence of PtdCho and Triton X-100 (*) or in the presence of only PtdSer with no detergent (**). The final concentrations of PtdCho or PtdSer when present were 8 μM. The final concentration of Triton X-100 when present was 0.1 %. The results shown are from one representative experiment among three. No [^{32}P]PtdIns(3,4)P_2 was detectably produced in any experiment with either enzyme.

			Conversion (%)			
			Type II PtdIns 4-kinase		Type III PtdIns 4-kinase	
Substrate	Product	Conditions...	*	**	*	**
[^{3}H]PtdIns	[^{3}H]PtdIns4*P*		12	6.7	0.7	20
[^{32}P]PtdIns3*P*	[^{32}P]PtdIns(3,4)P_2		< 0.5	< 0.5	< 0.5	< 0.5

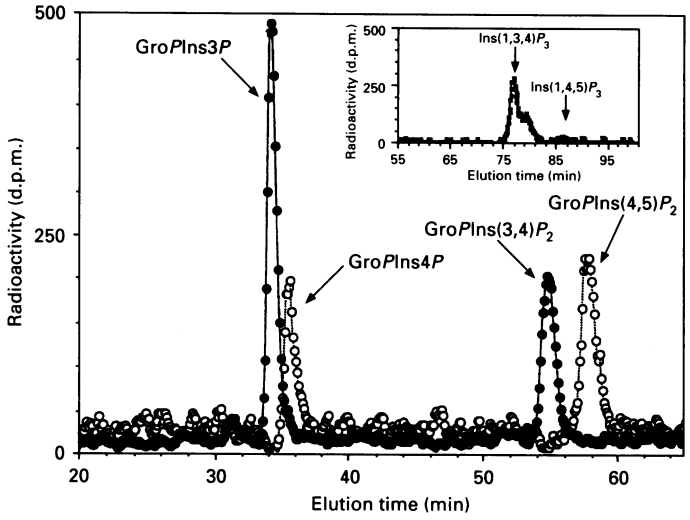

Fig. 6. PtdIns3*P* 4-kinase and PtdIns4*P* 5-kinase activities in total erythrocyte lysate

Total lysate from erythrocytes was incubated with a sonicated mixture of [^{32}P]PtdIns3*P* and [^{3}H]PtdIns4*P* and unlabelled ATP as described in the Experimental section. At the end of the assay, the lipids were extracted, deacylated and analysed by h.p.l.c. The Figure shows the products of incubation with lysate {2211 d.p.m. in the [^{3}H]PtdIns4*P* peak, 3400 d.p.m. in the [^{3}H]PtdIns(4,5)P_2 peak, 4532 d.p.m. in the [^{32}P]PtdIns3*P* peak and 2840 d.p.m. in the [^{32}P]PtdIns(3,4)P_2 peak}. The peaks were assigned on the basis of co-migration with standards. ○, ^{3}H; ●, ^{32}P. Abbreviation: Gro*P*Ins, glycerophosphoinositol. Inset: product of incubation of [^{32}P]PtdIns3*P* with lysate was deglycerated as described in the Experimental section. The resulting inositol phosphates were analysed by h.p.l.c.

conversion of [^{3}H]PtdIns into [^{3}H]PtdIns4*P* was observed under both conditions, no detectable conversion of [^{32}P]PtdIns3*P* into a PtdInsP_2 was observed under either condition. We conclude that the human erythrocyte type II PtdIns 4-kinase prefers PtdIns as a substrate at least 20-fold over PtdIns3*P*. Given the large excess of PtdIns over PtdIns3*P* *in vivo*, it seems unlikely that PtdIns3*P* is a physiological substrate for this enzyme.

A PtdIns3*P* 4-kinase activity is present in human erythrocytes

When a total erythrocyte lysate was incubated with a sonicated mixture of [^{3}H]PtdIns4*P*, [^{32}P]PtdIns3*P* and unlabelled ATP, 61 % of the former was converted into [^{3}H]PtdIns(4,5)P_2 and 39 % of the latter into [^{32}P]PtdIns(3,4)P_2 (Fig. 6). The structure of the

Table 3. Separation of erythrocyte PtdIns 4-kinase and PtdIns3*P* 4-kinase activities

	Activity (% of total in cell lysate)	
	PtdIns3*P* 4-kinase	PtdIns 4-kinase
Membranes	35	94
Cytosol	65	6
40 % pellet	1.0*	3.1*
60 % pellet	26*	0.04*

* % of total PtdIns3*P* 4-kinase or PtdIns 4-kinase in cell lysate that is recovered in sequential 40 %- and 60 %-satd.-$(NH_4)_2SO_4$ precipitates of the cytosolic fraction. The pellets were resuspended and the $(NH_4)_2$ SO_4 was dialysed away before assay.

putative PtdIns(3,4)P_2 was confirmed by co-migration of the deacylated material with the deacylation product of [^{32}P]-PtdIns(3,4)P_2 produced by phosphorylation of PtdIns4*P* with the purified PtdIns 3-kinase. In addition, the deglycerated material co-migrated with [^{3}H]Ins(1,3,4)P_3 under conditions that separated this isomer from Ins(1,4,5)P_3 by 10 min (Fig. 6, inset). One-third of the PtdIns3*P* 4-kinase activity was associated with the erythrocyte membranes, and the rest was recovered in the cytosol (Table 3). The cytosolic PtdIns3*P* 4-kinase activity was further purified by precipitation with $(NH_4)_2SO_4$. Most of the PtdIns3*P* 4-kinase activity was precipitated in 60 %-satd. $(NH_4)_2SO_4$. This fraction had very little PtdIns 4-kinase activity, providing further evidence that these are distinct enzymes.

Monoclonal antibody 4C5G inhibits PtdIns 4-kinase, but not PtdIns3*P* 4-kinase activity in erythrocyte membranes

To demonstrate further that the membrane-associated PtdIns3*P* 4-kinase and PtdIns 4-kinase are distinct enzymes, the relative ability of the 4C5G monoclonal antibody to inhibit these two activities was investigated. Erythrocyte membranes or the 60 % satd.-$(NH_4)_2SO_4$ cytosol fraction were incubated in the presence of either [^{3}H]PtdIns or [^{32}P]PtdIns3*P* and unlabelled ATP as described above but in the presence and absence of the antibody. The antibody inhibited conversion of [^{3}H]PtdIns into [^{3}H]PtdIns4*P* by 60 %, but did not significantly inhibit conversion of [^{32}P]PtdIns3*P* into [^{32}P]PtdIns(3,4)P_2 (Table 4).

Type III PtdIns 4-kinase does not phosphorylate PtdIns3*P*

The possibility that the PtdIns3*P* 4-kinase activity is carried out by a type III PtdIns 4-kinase was also investigated. Type III

Table 4. Antibody 4C5G inhibits PtdIns 4-kinase, but not PtdIns3*P* 4-kinase activity of human erythrocyte membranes

Either erythrocyte membranes or the 60%-satd.-$(NH_4)_2SO_4$-precipitated cytosolic fraction were incubated overnight in the presence of antibody 4C5G as described in the Experimental section. Then the lipid kinase assay was performed. PtdIns3*P* 4-kinase was assayed as described in the Experimental section. PtdIns 4-kinase was assayed in the absence of detergent either under standard conditions (*) or in the presence of [^{3}H]PtdIns (10^6 d.p.m.; 10 μM), PtdCho (50 μM) and unlabelled ATP (**).

	Activity (% of control)			
	Erythrocyte membrane PtdIns 4-kinase			
Conditions...	*	**	Erythrocyte membrane PtdIns3*P* 4-kinase	Erythrocyte cytosolic PtdIns3*P* 4-kinase
Control	100	100	100	100
4C5G (0.25 μg/ml)	7	39	109	103

PtdIns 4-kinase partially purified from bovine brain [12] was incubated with [^{3}H]PtdIns, [^{32}P]PtdIns3*P* and unlabelled ATP as described above (Table 2). No conversion of [^{32}P]PtdIns3*P* into [^{32}P]PtdIns(3,4)P_2 was detected under conditions where 20% of the [^{3}H]PtdIns was converted into [^{3}H]PtdIns4*P*. We conclude that PtdIns3*P* 4-kinase is a distinct enzyme from the PtdIns 4-kinases previously described.

DISCUSSION

The human erythrocyte membrane PtdIns 4-kinase has been purified 83000-fold to near homogeneity. A 56 kDa protein is the major protein detected by silver staining in SDS/PAGE analysis of the enzyme, and PtdIns 4-kinase activity was renatured from this band. The enzyme possesses properties of the type II family of PtdIns kinases previously described [11]: it has a relatively low K_m for ATP, is inhibited by adenosine and best utilizes PtdIns as a substrate when presented in a detergent micelle. Furthermore, the PtdIns 4-kinase purified from erythrocytes is recognized by monoclonal antibodies that specifically recognize type II PtdIns 4-kinase from several sources. These antibodies are highly specific for PtdIns 4-kinase. PtdIns(4,5)P_2 significantly inhibits the enzyme when present at equimolar concentrations with PtdIns. Given the relatively high ratio of PtdIns(4,5)P_2 to PtdIns in the erythrocyte plasma membrane (approx. 1:1) [26], this inhibition, although it was obtained *in vitro* by presenting both lipids in a mixed micelle with Triton, could be physiologically relevant in feedback regulation on this pathway.

Of particular interest is our conclusion that the type II PtdIns 4-kinase does not have significant PtdIns3*P* 4-kinase activity. PtdIns3*P* 4-kinase activity was recently detected in human platelets [21], and here we show that this activity is also present in human erythrocytes. Since the PtdIns 3-kinase is promiscuous in utilization of phosphoinositides as substrates [19], it is conceivable that a PtdIns 4-kinase might also utilize PtdIns3*P* as a substrate. However, we could not detect this activity in either the purified erythrocyte type II PtdIns 4-kinase or the partially purified bovine brain type III PtdIns 4-kinase. In fact, the $(NH_4)_2SO_4$ precipitate containing the highest PtdIns3*P* 4-kinase activity was virtually free of PtdIns 4-kinase activity (Table 3). Unlike the type II PtdIns 4-kinase, which can only be removed from the membrane by detergent solubilization, most of the PtdIns3*P* 4-kinase is cytosolic. These results raise the possibility that this enzyme, as has been proposed for the PtdIns 3-kinase [19], is activated by recruitment to the plasma membrane.

The discovery of the PtdIns3*P* 4-kinase raises the possibility that PtdIns(3,4)P_2 can be produced in the cell by two different pathways: phosphorylation of PtdIns4*P* at the 3-position by PtdIns 3-kinase [6], or phosphorylation of PtdIns3*P* at the 4-position by PtdIns3*P* 4-kinase [21]. If PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are providing distinct signals to the cell, as has been suggested [9], then the relative activity of the PtdIns 3-kinase and PtdIns3*P* 4-kinase in a given cell could affect the cellular response by affecting the ratio of these two lipids. In regard to this idea, it is interesting that our preliminary screening of tissues indicates that, although platelets and erythrocytes are high in the PtdIns3*P* 4-kinase activity, mouse 3T3 fibroblasts have very little activity, but still produce PtdIns(3,4)P_2 in response to platelet-derived growth factor. Further work is needed to determine the role of these enzymes in production of the D-3-phosphorylated phosphoinositides.

This research was supported by N.I.H. Grants GM 36624 and GM 41890 to L.C.C. A.G. was supported by fellowships from the American Heart Association and the European Organization for Research and Treatment of Cancer.

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Received 6 August 1991/21 November 1991; accepted 6 December 1991