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Phosphatidylinositol 3,4,5-trisphosphate is a substrate for the 75 kDa inositol polyphosphate 5-phosphatase and a novel 5-phosphatase which forms a complex with the p85/p110 form of phosphoinositide 3-kinase

Shaun P.Jackson, Simone M.Schoenwaelder[1], Maria Matzaris, Susan Brown and Christina A.Mitchell

Department of Medicine, Monash Medical School, Box Hill Hospital, Arnold Street, Box Hill, Victoria 3128, Australia

[1]Corresponding author

S.P.Jackson and S.M.Schoenwaelder contributed equally to this work

Agonist-stimulated production of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], is considered the primary output signal of activated phosphoinositide (PI) 3-kinase. The physiological targets of this novel phospholipid and the identity of enzymes involved in its metabolism have not yet been established. We report here the identification of two enzymes which hydrolyze the 5-position phosphate of PtdIns(3,4,5)P_3, forming phosphatidylinositol (3,4)-bisphosphate. One of these enzymes is the 75 kDa inositol polyphosphate 5-phosphatase (75 kDa 5-phosphatase), which has previously been demonstrated to metabolize inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]. We have identified a second PtdIns(3,4,5)P_3 5-phosphatase in the cytosolic fraction of platelets, which forms a complex with the p85/p110 form of PI 3-kinase. This enzyme is immunologically and chromatographically distinct from the platelet 43 kDa and 75 kDa 5-phosphatases and is unique in that it removes the 5-position phosphate from PtdIns(3,4,5)P_3, but does not metabolize PtdIns(4,5)P_2, Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4. These studies demonstrate the existence of multiple PtdIns(3,4,5)P_3 5-phosphatases within the cell.

Keywords: inositol 1,4,5-trisphosphate/5-phosphatase/phosphatidylinositol 3,4,5-trisphosphate 5-phosphatases/phosphoinositide 3-kinase

Introduction

Phosphoinositide (PI) 3-kinase plays an important role in transducing the mitogenic signals of various growth factor receptors and oncogene products (reviewed in Cantley *et al.*, 1991). More recently, PI 3-kinase has been implicated in a variety of other cellular responses, including membrane trafficking, actin reorganization, receptor internalization and glucose transport (reviewed in Panayotou and Waterfield., 1992; Fry, 1994). The molecular basis by which PI 3-kinase regulates such diverse cellular processes remains unclear. PI 3-kinase phosphorylates the D-3 position of the inositol ring of phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate [PtdIns(4)P] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], forming the putative second messengers phosphatidylinositol 3-phosphate [PtdIns(3)P], phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2] and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] (reviewed in Majerus *et al.*, 1990; Cantley *et al.*, 1991). While the pathway for formation and metabolism of these novel phospholipids remains controversial, recent evidence suggests that the formation of PtdIns(3,4,5)P_3 represents the primary output signal of activated PI 3-kinase (Stephens *et al.*, 1991; Hawkins *et al.*, 1992). However, at present little is known about the physiological targets of PtdIns(3,4,5)P_3 or the enzymes involved in PtdIns(3,4,5)P_3 metabolism.

Recent studies in neutrophils and fibroblasts have defined a pathway of phospholipid metabolism wherein PtdIns(4,5)P_2 is converted to PtdIns(3,4,5)P_3 by activated PI 3-kinase (Stephens *et al.*, 1991; Hawkins *et al.*, 1992). While the precise metabolism of PtdIns(3,4,5)P_3 has not been firmly established, it is clear that PtdIns(3,4,5)P_3 is not a substrate for phospholipase C and may perform a second messenger function *per se* (Cantley *et al.*, 1991). Growing evidence suggests that the potential signaling function of PtdIns(3,4,5)P_3 may be terminated by a PtdIns(3,4,5)P_3 5-phosphatase, generating PtdIns(3,4)P_2 (Stephens *et al.*, 1991; Hawkins *et al.*, 1992). This concept conforms with the paradigm set by inositol phosphate metabolism, wherein the calcium mobilizing function of Ins(1,4,5)P_3 is abrogated by its dephosphorylation to the inactive metabolite inositol 1,4-bisphosphate [Ins(1,4)P_2].

It has recently been demonstrated that enzymes involved in the metabolism of inositol phosphates are also able to metabolize their inositol phospholipid counterparts. For example, inositol polyphosphate 3-phosphatase hydrolyzes both Ins(1,3)P_2 and PtdIns(3)P (Caldwell *et al.*, 1991), while inositol polyphosphate 4-phosphatase hydrolyzes inositol 3,4-bisphosphate [Ins(3,4)P_2], inositol 1,3,4-trisphosphate [Ins(1,3,4)P_3] and PtdIns(3,4)P_2 (Norris and Majerus, 1994). These studies suggest a unifying concept in which inositol phosphate and inositol phospholipid second messengers are metabolized by a common set of cellular phosphatases. In support of this hypothesis, we have recently demonstrated that the major PtdIns(4,5)P_2 5-phosphatase activity in human platelets is the 75 kDa inositol polyphosphate 5-phosphatase (75 kDa 5-phosphatase) (Matzaris *et al.*, 1994).

Inositol polyphosphate 5-phosphatase enzymes have been identified in both the soluble and particulate fraction of multiple cell types (reviewed in Majerus, 1992; Shears, 1992; Verjans *et al.*, 1994a). Two soluble enzymes have been purified from the cytosolic fraction of human platelets (Connolly *et al.*, 1985; Mitchell *et al.*, 1989). The Type I enzyme has a molecular mass of 40–45 kDa and is phosphorylated and activated by protein kinase C

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(Connolly *et al.*, 1986). The Type II enzyme has a molecular mass of 75 kDa and the cDNA encoding this enzyme shares sequence homology with the gene that is defective in Lowe's oculocerebrorenal syndrome, a rare X-linked congenital disorder characterized by growth defects and mental retardation (Ross *et al.*, 1991; Attree *et al.*, 1992). A 43 kDa membrane-associated 5-phosphatase which appears to be identical to the Type I soluble enzyme has recently been purified and cloned (Erneux *et al.*, 1989; Laxminarayan *et al.*, 1992, 1994; De Smedt *et al.*, 1994; Hodgkin *et al.*, 1994; Verjans *et al.*, 1994b). This enzyme is functionally distinct from the Type II 75 kDa 5-phosphatase, however, in that it is unable to remove the 5-position phosphate from PtdIns(4,5)P_2 (Matzaris *et al.*, 1994).

In this report we have investigated the relationship between the platelet PtdIns(3,4,5)P_3 5-phosphatase and the 43 and 75 kDa 5-phosphatases. Our studies indicate that at least two enzymes are capable of hydrolyzing the 5-position phosphate from PtdIns(3,4,5)P_3, the 75 kDa 5-phosphatase and a previously unidentified 5-phosphatase, which forms a complex with the p85/p110 form of PI 3-kinase.

Results

Partial purification of platelet cytosolic PI 3-kinase

To investigate the identity of PtdIns(3,4,5)P_3 5-phosphatases our early studies centered on the development of a PtdIns(3,4,5)P_3 5-phosphatase assay, using ^{32}P-labeled PtdIns(3,4,5)P_3. Initially PI 3-kinase was purified to near homogeneity from human platelet cytosol using four sequential chromatographic steps, including Q- and S-Sepharose, hydroxyapatite and HPLC Mono S, as described under Materials and methods, to a final specific activity of 30 nmol PtdIns(3)P formed/mg/min, using PtdIns (200 μM) as a substrate. To obtain PtdIns(3,4,5)P_3 we incubated PtdIns(4,5)P_2 (50 μM) in the presence of ^{32}P-labeled ATP (50 μM, 1.0 μCi/nmol) and partially purified PI 3-kinase [30 nmol PtdIns(3)P formed/mg/min]. These assays were performed over a 60 min time course and the lipid products analyzed by thin layer chromatography (TLC). We consistently observed two labeled phospholipid products from these reactions (Figure 1). The identity of each of these lipids was established by performing strong anion exchange (SAX) HPLC analysis of each deacylated and deglycerated lipid, as described under Materials and methods. The lipid product migrating closest to the origin co-eluted with an Ins(1,3,4,5)P_4 standard, confirming its identity as PtdIns(3,4,5)P_3. The second radiolabeled lipid product co-eluted with an Ins(1,3,4)P_3 standard, establishing its identity as PtdIns (3,4)P_2 (data not shown). The simplest explanation for the formation of these two lipid products was that we had co-purified a PtdIns(3,4,5)P_3 5-phosphatase with PI 3-kinase, which hydrolyzed PtdIns(3,4,5)P_3 to form PtdIns(3,4)P_2. Consistent with this interpretation is the time course for the formation of both lipid products. At earlier time points (5 min) 90% of the radiolabeled lipid product was PtdIns(3,4,5)P_3, whereas at later time points (60 min) at least 75% of the lipid was converted to PtdIns(3,4)P_2, suggesting a time-dependent dephosphorylation of PtdIns(3,4,5)P_3.

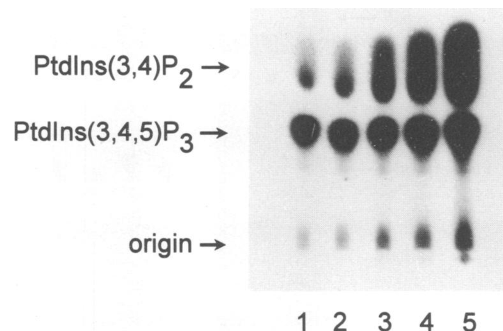


Fig. 1. Time course for PtdIns(3,4,5)P_3 formation and hydrolysis. Partially purified PI 3-kinase [30 nmol PtdIns(3)P formed/mg/min] was incubated with PtdIns(4,5)P_2 (50 μM) and phosphatidylserine (75 μM) in the presence of [^{32}P]ATP (50 μM, 1 μCi/nmol), as described under Materials and methods. Assays were performed for 5 (lane 1), 10 (lane 2), 20 (lane 3), 30 (lane 4) and 60 min (lane 5) at room temperature. Radiolabeled lipids were extracted, resolved by TLC and detected by autoradiography.

Complex formation between PI 3-kinase and PtdIns(3,4,5)P₃ 5-phosphatase

We examined each fraction eluted from Q-Sepharose, S-Sepharose, hydroxyapatite and HPLC Mono S for PtdIns(4,5)P_2 3-kinase and PtdIns(3,4,5)P_3 5-phosphatase activity. In every fraction that contained PtdIns(4,5)P_2 3-kinase activity PtdIns(3,4,5)P_3 5-phosphatase activity was also detected (data not shown). These observations were consistently observed in all PtdIns(4,5)P_2 3-kinase-containing fractions from each of the chromatographic steps from eight separate purification preparations. In contrast, when the flow-through and wash fractions from Q-Sepharose were assayed for PtdIns(4,5)P_2 3-kinase activity the generated PtdIns(3,4,5)P_3 was not dephosphorylated to PtdIns(3,4)P_2 (Figure 2A). Previous studies have demonstrated that the free (p110) catalytic subunit of PtdIns 3-kinase does not bind Q-Sepharose, whereas the heterodimeric p85/p110 complex is retained (Woscholski *et al.*, 1994). Consistent with this possibility we found that the flow-through fractions did not contain any immunodetectable p85 protein, whereas in all fractions containing PtdIns(3,4,5)P_3 5-phosphatase activity p85 was present. The PI 3-kinase activity in the flow-through fractions of Q-Sepharose was not further characterized in this study.

We quantitated the amount of PtdIns(3,4,5)P_3 5-phosphatase activity in each of the fractions eluted from hydroxyapatite and HPLC Mono S, using PtdIns(3,4,5)P_3 as a substrate as described under Materials and methods, and correlated this enzyme activity with the amount of p85 protein in each fraction. As indicated in Figure 2B, the total amount of PtdIns(3,4,5)P_3 5-phosphatase activity in each fraction from hydroxyapatite correlated closely with the amount of p85 protein. Similar results were also obtained with the fractions eluted from HPLC Mono S (data not shown). These chromatographic studies clearly demonstrate enrichment of the p85/p110 form of PI 3-kinase with the PtdIns(3,4,5)P_3 5-phosphatase. Accurate quantitation of total PtdIns(3,4,5)P_3 5-phosphatase activity in the fractions eluted from Q-Sepharose and S-Sepharose was not possible, however, due to the presence of other enzymes capable of hydrolyzing the 3-phosphorylated phosphoinositides.

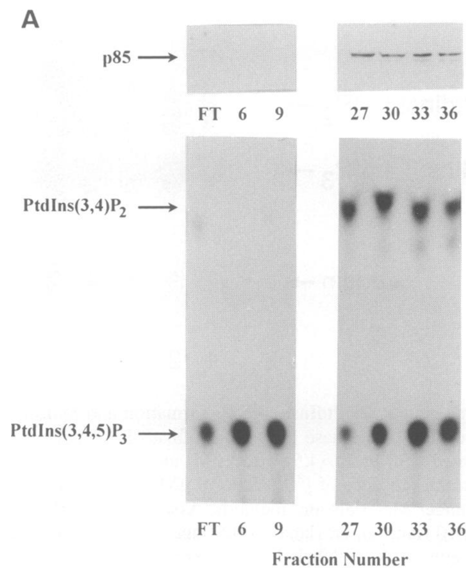


Fig. 2. Chromatography of the p85/p110 form of PI 3-kinase and the PtdIns(3,4,5)P_3 5-phosphatase. (**A**) Platelet cytosol was chromatographed on the anion exchange resin Q-Sepharose as described under Materials and methods. The flow-through, wash and eluant fractions were assayed for PtdIns(4,5)P_2 3-kinase activity and immunoblotted with antibodies against the p85 subunit of PI 3-kinase (inset), as described under Materials and methods. (**B**) The eluted fractions from hydroxyapatite were assayed for PtdIns(3,4,5)P_3 5-phosphatase activity using PtdIns(3,4,5)P_3 as the substrate, as described under Materials and methods, and immunoblotted with antibodies against the p85 subunit of PI 3-kinase (inset). These results are from one experiment, representative of three.

We attempted to separate the two enzymatic activities from the HPLC Mono S fractions using size exclusion chromatography, as described under Materials and methods. PtdIns(4,5)P_2 3-kinase activity was detected as a broad peak of activity in fractions 61–67, eluting between the 158 and 640 kDa molecular weight standards (Figure 3). As with each of the earlier chromatographic steps, PtdIns(3,4,5)P_3 5-phosphatase activity was demonstrated

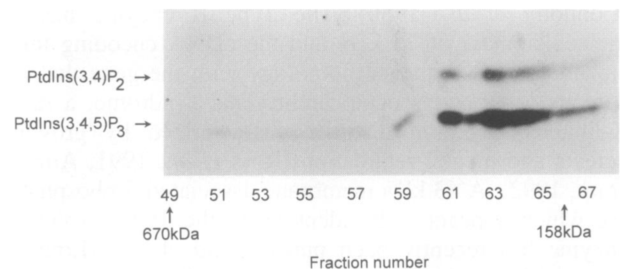


Fig. 3. HPLC gel filtration of PI 3-kinase. Partially purified PI 3-kinase [30 nmol PtdIns(3)P formed/mg/min] was loaded on a Bio-Sil SEC-250 HPLC gel filtration column (600×7.5 mm) and eluted as described under Materials and methods. Aliquots (20 μl) of each fraction (0.5 ml) were assayed for PI 3-kinase activity using PtdIns(4,5)P_2 (50 μM) as substrate in the presence of phosphatidylserine (75 μM) and [^{32}P]ATP (50 μM, 1 μCi/nmol). Assays were performed for 20 min at room temperature. Lipid products were extracted, resolved by TLC and detected by autoradiography. These results are from one experiment, representative of three.

in all fractions containing PtdIns(4,5)P_2 3-kinase activity. These results suggest one of three possible explanations regarding the cytosolic PtdIns(3,4,5)P_3 5-phosphatase activity: (i) the enzyme has identical chromatographic properties to the p85/p110 form of PI 3-kinase; (ii) it forms a complex with p85/p110; (iii) PtdIns(3,4,5)P_3 5-phosphatase activity is intrinsic to either the p85 or p110 subunit of PI 3-kinase. To investigate the possibility that these two enzymes may be part of a complex we immunoprecipitated PI 3-kinase [30 nmol PtdIns(3)P formed/mg/min] with antibodies directed against either the p85 regulatory or the p110 catalytic subunit. The immunoprecipitated PI 3-kinase phosphorylated PtdIns (4,5)P_2 (50 μM) to form PtdIns(3,4,5)P_3 (20 pmol). However, as observed with the partially purified fractions, the PtdIns(3,4,5)P_3 was subsequently dephosphorylated to PtdIns(3,4)P_2 (Figure 4A). These observations suggest that a PtdIns(3,4,5)P_3 5-phosphatase either forms a complex with PI 3-kinase or that the enzyme activity is intrinsic to the p85 or p110 subunits of PI 3-kinase.

PtdIns(3,4,5)P_3 5-phosphatase activity can be dissociated from PI 3-kinase

Recent studies have demonstrated that the p85/p110 form of PI-kinase not only has lipid kinase activity, but also intrinsic protein kinase activity, such that the p110 catalytic subunit is able to transphosphorylate residue Ser608 of the p85 regulatory subunit of the enzyme (Dhand *et al.*, 1994). This phosphorylation reaction coincides with decreased lipid kinase activity, suggesting an autoregulatory role for the p110 subunit of PI 3-kinase. To address the issue of whether PI 3-kinase contains intrinsic phosphatase activity we attempted to dissociate the PtdIns(3,4,5)P_3 5-phosphatase activity from the lipid kinase by washing p85 immunoprecipitates with high stringency buffers, as described under Materials and methods. With repeated washes we were able to eventually remove all PtdIns (3,4,5)P_3 5-phosphatase activity from the PI 3-kinase immunoprecipitates (Figure 4B). To exclude the possibility that we were denaturing the complexed PtdIns(3,4,5)P_3 5-phosphatase, rather than dissociating it from PI 3-kinase,

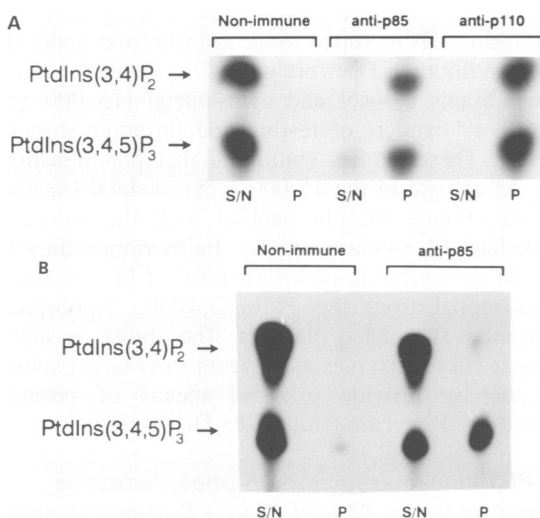


Fig. 4. Complex formation between PI 3-kinase and PtdIns(3,4,5)P_3 5-phosphatase. Immunoprecipitation reactions were performed on partially purified PI 3-kinase [30 nmol PtdIns(3)P formed/mg/min] using either non-immune serum or polyclonal antibodies directed against the p85 or p110 subunit of PI 3-kinase, as described under Materials and methods. Supernatants (S/N) were removed and the protein A pellets (P) washed four times with (**A**) 20 mM Tris, pH 7.2, 150 mM NaCl or (**B**) 10 mM Tris, pH 7.2, 1% NP-40, 0.1% SDS, 1 mM EDTA, prior to assaying for PI 3-kinase activity. All assays were performed using PtdIns(4,5)P_2 (50 μM) as substrate in the presence of phosphatidylserine (75 μM) and [^{32}P]ATP (50 μM, 1 μCi/nmol). Assays were performed for 20 min at room temperature. Lipid products were extracted, resolved by TLC and detected by autoradiography. These results are from one experiment, representative of three. Note that in the autoradiograph presented in (B) there is significant elution of PI 3-kinase activity from the immunoprecipitates washed with high stringency buffers. This extent of PI 3-kinase elution was not consistently observed in all experiments performed.

we incubated partially purified PI 3-kinase [30 nmol PtdIns(3)P formed/mg/min] with the same high stringency buffers for 1 h, removed the detergents by HPLC gel filtration and assayed for PI 3-kinase activity, as described under Materials and methods. Under these experimental conditions PI 3-kinase retained its ability to phosphorylate PtdIns(4,5)P_2 (50 μM), forming PtdIns(3,4,5)P_3 (10 pmol). However, unlike the situation with detergent-treated immunoprecipitates, the generated PtdIns(3,4,5)P_3 was subsequently dephosphorylated to PtdIns(3,4)P_2, excluding a denaturing effect of these detergents on the PI 3-kinase-associated 5-phosphatase (data not shown). In further studies we examined whether the salt or the detergent was responsible for dissociating the two enzyme activities. While washing the p85 immunoprecipitates with salt-containing buffers removed some of the complexed 5-phosphatase enzyme activity, the detergent-containing buffers were clearly more effective at dissociating the 5-phosphatase from PtdIns 3-kinase (data not shown). The observation that detergent treatment of PtdIns 3-kinase immunoprecipitates prevents PtdIns(3,4)P_2 generation excludes two other possible explanations for the production of PtdIns(3,4)P_2 in our assays. First, the 5-position phosphate does not spontaneously hydrolyze from PtdIns (3,4,5)P_3 under our assay conditions and, second, the formation of PtdIns(3,4)P_2 is not a result of contaminating PtdIns(4)P in the commercial PtdIns(4,5)P_2 substrate.

Identification of PtdIns(3,4,5)P_3 5-phosphatase activity in the cytosolic and membrane fractions of platelets

We examined the subcellular distribution of PtdIns (3,4,5)P_3 5-phosphatase activity in human platelets. Our ability to produce significant quantities of PtdIns(3,4,5)P_3 for the measurement of PtdIns(3,4,5)P_3 5-phosphatase activity was hampered by the time-dependent hydrolysis of generated PtdIns(3,4,5)P_3 by the complexed PtdIns (3,4,5)P_3 5-phosphatase. Furthermore, we could not generate large amounts of pure PtdIns(3,4,5)P_3 using the immunoprecipitates of partially purified PI 3-kinase, as has been previously reported for PtdIns(3)P (Caldwell *et al.*, 1991), as these assays consistently resulted in a low yield of PtdIns(3,4,5)P_3 (<10%) (data not shown). The reason for the limited production of PtdIns(3,4,5)P_3 relative to PtdIns(3)P in our immunoprecipitation assays remains unclear. To study the subcellular distribution of PtdIns(3,4,5)P_3 5-phosphatase we generated small amounts of PtdIns(3,4,5)P_3 (250 pmol) by phosphorylating PtdIns (4,5)P_2 (50 μM) with partially purified PI 3-kinase [30 nmol PtdIns(3)P formed/mg/min] for 5 min at room temperature, as described under Materials and methods. Under these assay conditions 90% of the radiolabeled lipid was incorporated into PtdIns(3,4,5)P_3. Incubation of this lipid with the whole-cell lysates from resting platelets resulted in rapid conversion of PtdIns(3,4,5)P_3 to PtdIns (3,4)P_2 by a highly active 5-phosphatase. In these assays <15% of the 3-position phosphate was removed from the 3-phosphorylated lipids. In contrast, >70% of PtdIns (3,4,5)P_3 was hydrolyzed by a 5-phosphatase, forming PtdIns(3,4)P_2. These observations indicate that PtdIns (3,4,5)P_3 is preferentially hydrolyzed by 5-phosphatase(s) when incubated with total cell extracts. Platelet lysates were separated into membrane and cytosolic fractions and individually assayed for PtdIns(3,4,5)P_3 5-phosphatase activity. The majority of PtdIns(3,4,5)P_3 5-phosphatase activity (78% ± 6.2, $n = 7$) (Figure 5B) was recovered in the membrane fraction of platelets, with <10% of the 3-position phosphate hydrolyzed. These results on the subcellular distribution of PtdIns(3,4,5)P_3 5-phosphatase are similar to those previously reported in human neutrophils (Stephens *et al.*, 1991).

Effect of platelet activation on the subcellular distribution of PI 3-kinase and PtdIns(3,4,5)P_3 5-phosphatase

In contrast to the platelet PtdIns(3,4,5)P_3 5-phosphatase, PI 3-kinase is predominantly located in the cytosolic fraction of resting platelets (Figures 5A and B and 6B). Previous studies have indicated that thrombin stimulation of platelets results in the redistribution of PI 3-kinase activity from the Triton X-100-soluble to the cytoskeletal fraction of platelets (Grondin *et al.*, 1991; Zhang *et al.*, 1992; Jackson *et al.*, 1994). This recruitment of PI 3-kinase into submembranous signaling complexes may juxtapose the enzyme in close proximity to its membrane lipid substrates. The demonstration that a PtdIns(3,4,5)P_3 5-phosphatase activity forms a complex with the cytosolic p85/p110 form of PI 3-kinase prompted us to examine whether the cytoskeletal translocation of PI 3-kinase coincides with translocation of PtdIns(3,4,5)P_3 5-phosphatase activity. While thrombin stimulation of platelets

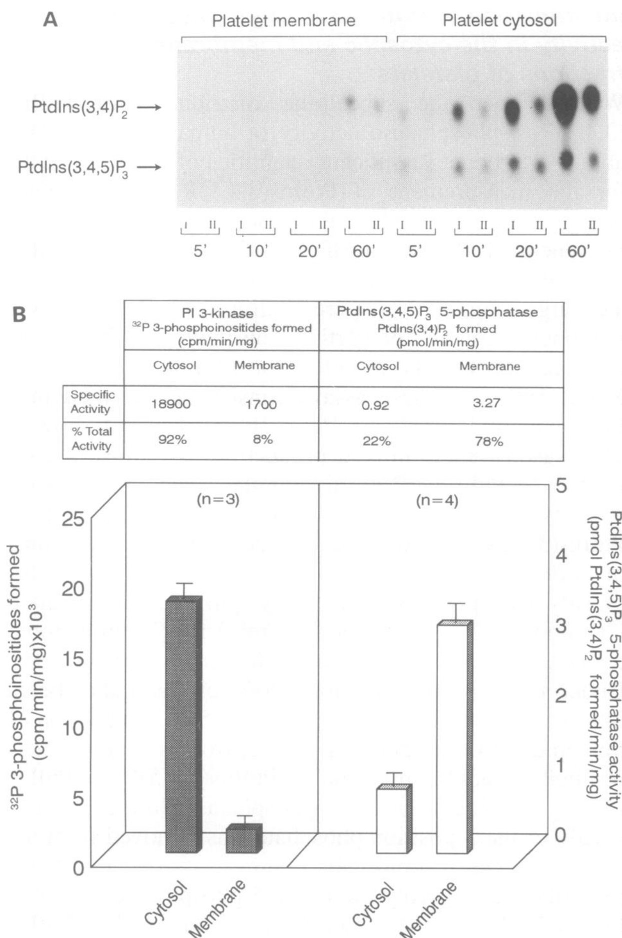


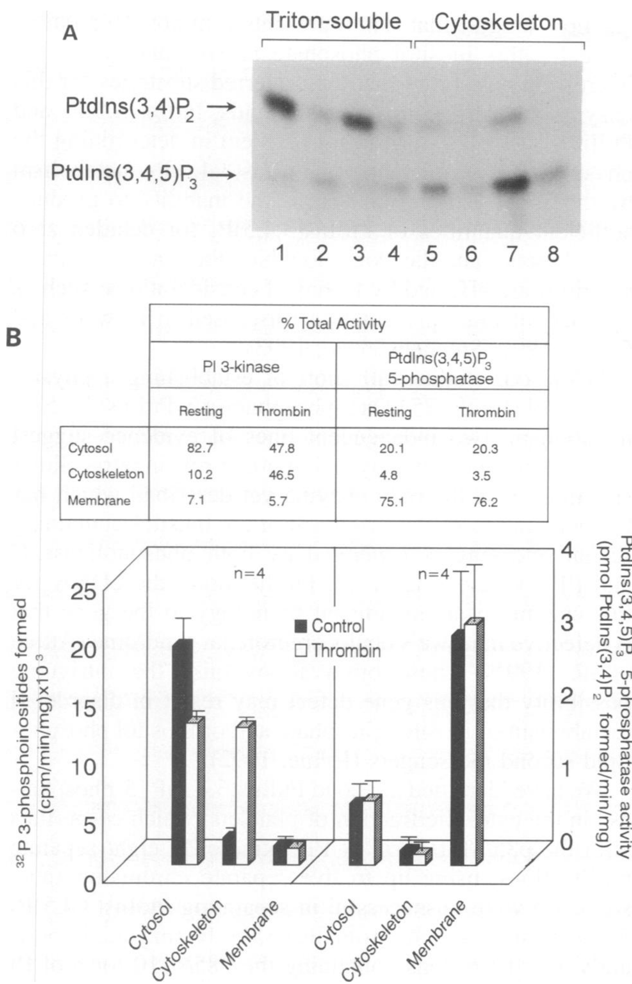
Fig. 5. Relative subcellular distribution of PI 3-kinase and PtdIns(3,4,5)P_3 5-phosphatase. (**A**) Platelet cytosolic and membrane extracts were prepared as described under Materials and methods. PI 3-kinase assays were performed on a 1:200 (I) or 1:1000 (II) dilution of each platelet fraction using PtdIns(4,5)P_2 (50 μM) as substrate. Each assay was performed at room temperature for 5, 10, 20 or 60 min in the presence of phosphatidylserine (75 μM) and [^{32}P]ATP (50 μM, 1 μCi/nmol). Radiolabeled lipids were extracted, resolved by TLC and detected by autoradiography. These results are from one experiment, representative of three. (**B**) Quantitation of PI 3-kinase and PtdIns(3,4,5)P_3 5-phosphatase activity in each of the indicated subcellular extracts was determined as described under Materials and methods. Results represent the mean ± SD of three separate experiments.

resulted in the redistribution of PtdIns(4,5)P_2 3-kinase activity from the cytosolic to the cytoskeletal fraction of platelets, there was no significant translocation of PtdIns(3,4,5)P_3 5-phosphatase activity (Figure 6A and B). This conclusion is supported by the observation that the majority of PtdIns(3,4,5)P_3 (>80%) generated by the cytoskeleton-associated PI 3-kinase in thrombin-stimulated platelets is not hydrolyzed to PtdIns(3,4)P_2 (Figure 6A, lanes 5–8), whereas in the Triton X-100-soluble (Figure 6A, lanes 1–4) or cytosolic fractions of platelets (Figure 5A) the majority of PtdIns(3,4,5)P_3 (~90%) generated by PI 3-kinase is dephosphorylated to PtdIns(3,4)P_2 (Jackson *et al.*, 1994).

Recent studies have demonstrated the existence of a G-protein-regulated form of PI 3-kinase which is immunologically and biochemically distinct from the p85/p110 tyrosine kinase-regulated enzyme (Stephens *et al.*, 1994; Zhang *et al.*, 1995). To confirm that the p85/p110 form of PI 3-kinase is recruited to the cytoskeleton under these assay conditions we performed p85 immunoblot analysis on membrane, cytosol and cytoskeletal (15 000 *g* and 100 000 *g*) extracts of resting and thrombin-stimulated platelets. These studies confirmed that p85 translocates from the cytosol to the 15 000 *g* cytoskeletal fraction of platelets (Figure 6C) in parallel with the increase in cytoskeletal PI 3-kinase activity. Furthermore, they indicate that the cytosolic p85/p110 form of PI 3-kinase can be uncoupled from the PtdIns(3,4,5)P_3 5-phosphatase in thrombin-stimulated platelets. The ability to spatially segregate these enzymes away from their target substrate may thereby provide a novel means of promoting PtdIns(3,4,5)P_3 accumulation (see Discussion).

The PI 3-kinase-associated 5-phosphatase is distinct from the 43 and 75 kDa 5-phosphatases

Two inositol polyphosphate 5-phosphatases have previously been identified in human platelets, the Type I 43 kDa 5-phosphatase and the Type II 75 kDa 5-phosphatase (Connolly *et al.*, 1985; Mitchell *et al.*, 1989). We examined the relationship between the PtdIns(3,4,5)P_3 5-phosphatase which co-purified with PI 3-kinase and the 43 and 75 kDa 5-phosphatases. As indicated in Figure 7A, the 43 kDa platelet cytosolic enzyme can be completely separated from the 75 kDa enzyme by chromatography on Q-Sepharose. PI 3-kinase elutes as a single peak between these two Ins(1,4,5)P_3 5-phosphatase activities. We pooled the Q-Sepharose fractions with the highest PI 3-kinase specific activity [0.25 nmol PtdIns(3)P formed/mg/min] and chromatographed these fractions on S-Sepharose, as described under Materials and methods. We identified a single peak of PI 3-kinase activity [6.5 nmol PtdIns(3)P formed/mg/min], which also contained PtdIns(3,4,5)P_3 5-phosphatase activity. We could not, however, detect any Ins(1,4,5)P_3 5-phosphatase activity in any of the eluted fractions (Figure 7B). The addition of a 1000-fold molar excess of Ins(1,4,5)P_3 (10 nmol) to the PtdIns(4,5)P_2 3-kinase assays failed to prevent dephosphorylation of PtdIns(3,4,5)P_3 (10 pmol). Furthermore, these same concentrations of Ins(1,4,5)P_3 had no effect on PtdIns(3,4,5)P_3 hydrolysis by the complexed 5-phosphatase when using PtdIns(3,4,5)P_3 as a substrate (data not shown). These results suggest that the PtdIns(3,4,5)P_3 5-phosphatase which associates with PI 3-kinase does not hydrolyze Ins(1,4,5)P_3 and is therefore likely to represent a novel platelet 5-phosphatase. Further evidence supporting this hypothesis was obtained by immunoblot analysis of fractions containing PtdIns(3,4,5)P_3 5-phosphatase activity with antibodies against the 43 or 75 kDa 5-phosphatases, which failed to detect either of these proteins (data not shown). Furthermore, we were unable to immunoprecipitate any PI 3-kinase activity from the partially purified fractions using antibodies against the recombinant 75 kDa 5-phosphatase (data not shown). We examined the substrate specificity of the PI 3-kinase-associated 5-phosphatase further, by incubating the highly purified PtdIns(3,4,5)P_3 5-phosphatase-containing fractions from HPLC Mono S with PtdIns(4,5)P_2 or Ins (1,3,4,5)P_4, as described under Materials and methods. No hydrolysis of either substrate was observed under these assay conditions (data not shown). Collectively



	% Total Activity			
	PI 3-kinase		PtdIns(3,4,5)P3 5-phosphatase	
	Resting	Thrombin	Resting	Thrombin
Cytosol	82.7	47.8	20.1	20.3
Cytoskeleton	10.2	46.5	4.8	3.5
Membrane	7.1	5.7	75.1	76.2

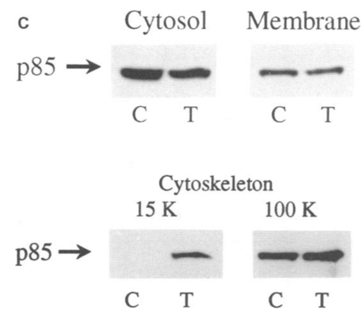


Fig. 6. Subcellular distribution of PI 3-kinase and PtdIns(3,4,5)P_3 5-phosphatase in thrombin-stimulated platelets. The Triton X-100-soluble extracts and total cytoskeleton (combined 15 000 and 100 000 *g* pellets) from resting and thrombin-stimulated platelets were prepared as described under Materials and methods. (**A**) PI 3-kinase activity in resting platelets (lanes 1, 2, 5 and 6) or following thrombin stimulation (lanes 3, 4, 7 and 8). The indicated platelet extracts at dilutions of 1:200 (lanes 1, 3, 5 and 7) and 1:1000 (lanes 2, 4, 6 and 8) were incubated with PtdIns(4,5)P_2 (50 μM) for 10 min at room temperature in the presence of phosphatidylserine (75 μM) and [^{32}P]ATP (50 μM, 1 μCi/nmol). Radiolabeled lipids were extracted, resolved by TLC and detected by autoradiography. These results are from one experiment, representative of four. (**B**) Quantitation of PI 3-kinase and PtdIns(3,4,5)P_3 5-phosphatase activity in each of the indicated subcellular fractions was determined as described under Materials and methods. The results represent the mean ± SD of four separate experiments. (**C**) The platelet cytosol, membrane, 15 000 *g* (15K) and 100 000 *g* (100K) cytoskeletal proteins were extracted from control (C) and thrombin-stimulated (T) platelets. Equal quantities of protein were subjected to 7.5% SDS–PAGE and transferred to PVDF membranes prior to immunoblotting with a polyclonal antibody against the p85 subunit of PI 3-kinase.

these results indicate that the 5-phosphatase which forms a complex with the p85/p110 form of PI 3-kinase demonstrates considerable substrate specificity for PtdIns(3,4,5)P_3.

PtdIns(3,4,5)P3 is a substrate for the 75 kDa 5-phosphatase

The predominant membrane localization of the PtdIns(3,4,5)P_3 5-phosphatase raised the possibility that this enzyme may represent the 43 kDa membrane-associated 5-phosphatase. Incubation of purified 43 kDa 5-phosphatase [9.0 nmol Ins(1,4,5)P_3 hydrolyzed/min] with PtdIns(3,4,5)P_3 (10 pmol) for up to 60 min failed to generate PtdIns(3,4)P_2 (data not shown). In contrast, PtdIns(3,4,5)P_3 (10 pmol) was hydrolyzed by the 75 kDa 5-phosphatase [0.18 nmol Ins(1,4,5)P_3 hydrolyzed/min] (Figure 8A, lane 2). To confirm that the purified 75 kDa 5-phosphatase was the enzyme responsible for PtdIns (3,4,5)P_3 metabolism, rather than a contaminating enzyme in the purified preparation, we immunoprecipitated 90% of the Ins(1,4,5)P_3 5-phosphatase activity from the purified fractions using antibodies against the recombinant 75 kDa 5-phosphatase. This reduction in Ins(1,4,5)P_3 5-phosphatase activity was associated with a corresponding 80% decrease in PtdIns(3,4,5)P_3 5-phosphatase activity, thereby confirming that the 75 kDa 5-phosphatase was indeed a PtdIns(3,4,5)P_3 5-phosphatase (data not shown).

Our inability to produce significant quantities of PtdIns(3,4,5)P_3 has precluded a detailed kinetic comparison between the 75 kDa 5-phosphatase, PI 3-kinase-associated PtdIns(3,4,5)P_3 5-phosphatase and the platelet membrane enzyme. However, we consistently noted that the rate of metabolism of PtdIns(3,4,5)P_3 by the 75 kDa 5-phosphatase appeared to be at least 5-fold less than that of the platelet membrane enzyme(s) (data not shown). This apparent rate of PtdIns(3,4,5)P_3 metabolism by the 75 kDa 5-phosphatase may be an underestimation of the actual rate, as these assays were performed in the presence of a 500-fold molar excess of PtdIns(4,5)P_2. Under these conditions this large excess of PtdIns(4,5)P_2 may compete with PtdIns(3,4,5)P_3 for the active site of the 75 kDa 5-phosphatase. Clarification of this issue will ultimately require the production of PtdIns(3,4,5)P_3 in the absence of competing substrate.

Previous studies in neutrophils have suggested that membrane-associated PtdIns(3,4,5)P_3 5-phosphatase may be distinct from PtdIns(4,5)P_2 5-phosphatase, as the activity of the latter enzyme is absolutely dependent on the presence of divalent cations (Stephens *et al.*, 1991). Our studies are consistent with these reports, in that the membrane-associated PtdIns(3,4,5)P_3 5-phosphatase in platelets was able to hydrolyze PtdIns(3,4,5)P_3 in the presence of 10 mM EDTA (Figure 8B), whereas the ability of the 75 kDa 5-phosphatase to hydrolyze PtdIns(4,5)P_2 was completely abolished by 10 mM EDTA (Matzaris *et al.*, 1994). Surprisingly, the ability of the 75 kDa

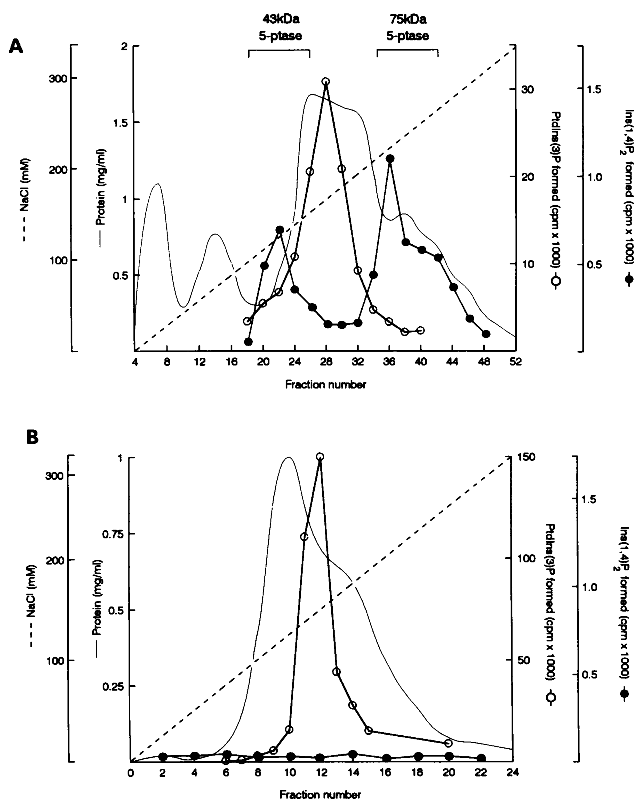


Fig. 7. Chromatographic relationship between PI 3-kinase and $Ins(1,4,5)P_3$ 5-phosphatase activity. Platelet cytosol was sequentially chromatographed on (**A**) Q-Sepharose and (**B**) S-Sepharose, as described under Materials and methods. Eluted fractions from each column were assayed for PI 3-kinase [PI(3)P formed] and $Ins(1,4,5)P_3$ 5-phosphatase activity [$Ins(1,4)P_2$ formed], as described under Materials and methods. These results are from one experiment, representative of three.

5-phosphatase to hydrolyze $PtdIns(3,4,5)P_3$ was only partially inhibited (~30%) by 10 mM EDTA (Figure 8A). These results are somewhat reminiscent of the effects of divalent cations on the substrate specificity of phospholipase C, wherein increasing calcium concentrations promote $PtdIns(4,5)P_2$ hydrolysis in preference to PtdIns or PtdIns(4)P. These observations raise the interesting possibility that changes in the cellular concentrations of divalent cations may alter the substrate preference of the 75 kDa 5-phosphatase.

Discussion

Recent evidence suggests that the major route of PtdIns $(3,4,5)P_3$ metabolism in intact cells is via dephosphorylation to $PtdIns(3,4)P_2$ by an uncharacterized 5-phosphatase (Stephens *et al.*, 1991; Hawkins *et al.*, 1992; Carter *et al.*, 1994). In this study we have demonstrated two distinct 5-phosphatases that hydrolyze $PtdIns(3,4,5)P_3$.

One of these enzymes is the 75 kDa 5-phosphatase which hydrolyzes $PtdIns(3,4,5)P_3$ forming $PtdIns(3,4)P_2$. This observation is significant in the light of recent studies demonstrating that the 75 kDa 5-phosphatase represents the major $PtdIns(4,5)P_2$ 5-phosphatase in platelets (Matzaris *et al.*, 1994; Bennett-Jefferson and Majerus, 1995). Although the 75 kDa 5-phosphatase also hydrolyzes $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$, the kinetics for the latter reaction suggest that this represents a minor degradative route for this inositol phosphate *in vivo* and raises the interesting possibility that the preferred substrates for this enzyme are the inositol phospholipids, $PtdIns(4,5)P_2$ and $PtdIns(3,4,5)P_3$. A significant problem in determining the physiological significance of $PtdIns(3,4,5)P_3$ metabolism by the 75 kDa 5-phosphatase is the inability to produce sufficient quantities of $PtdIns(3,4,5)P_3$ for detailed zero order kinetics and the extent to which the kinetics of these reactions are affected by technical considerations, such as optimal substrate presentation (discussed in Downes and Carter, 1991; Stephens *et al.*, 1993).

While our studies fall short of establishing a physiological role for the 75 kDa 5-phosphatase in $PtdIns(3,4,5)P_3$ metabolism, two independent lines of evidence suggest that this enzyme may play an important role in intracellular signaling. It is the only enzyme yet described which has the potential to metabolize each of the inositol-containing second messengers generated by both phospholipase C and PI 3-kinase (Figure 9). Furthermore, the cDNA for this enzyme bears significant homology to the gene that is defective in Lowe's oculocerebrorenal syndrome (Attree *et al.*, 1991). These observations raise the intriguing possibility that this gene defect may result in disordered metabolism of inositol phosphate and/or inositol phospholipid second messengers (Irvine, 1992).

We have identified a second $PtdIns(3,4,5)P_3$ 5-phosphatase in the cytosolic fraction of platelets, which co-purifies with the p85/p110 form of PI 3-kinase. In eight separate purifications, using up to five separate chromatographic steps, we were unsuccessful in separating $PtdIns(3,4,5)P_3$ 5-phosphatase activity from the p85/p110 enzyme. Importantly, in all fractions containing the p85/p110 form of PI 3-kinase, $PtdIns(3,4,5)P_3$ 5-phosphatase activity was also detected. Furthermore, there was a very strong correlation between the amount of p85 protein in each fraction and the total amount of $PtdIns(3,4,5)P_3$ 5-phosphatase activity. Finally, antibodies directed against either the p85 or p110 subunits of partially purified PI 3-kinase immunoprecipitated $PtdIns(3,4,5)P_3$ 5-phosphatase activity. Collectively these results strongly suggest that a $PtdIns(3,4,5)P_3$ 5-phosphatase activity forms a stable complex with the p85/p110 form of PI 3-kinase.

Two lines of evidence suggest that the $PtdIns(3,4,5)P_3$ 5-phosphatase activity in the PI 3-kinase immunoprecipitates is unlikely to be intrinsic to PtdIns 3-kinase. First, repeated washes of the PI 3-kinase immunoprecipitates with high stringency buffers eventually removed all $PtdIns(3,4,5)P_3$ 5-phosphatase activity without abolishing PI 3-kinase activity. These observations are reminiscent of previous studies demonstrating the need for high salt and detergent concentrations to separate PI 3-kinase from the viral oncogene product polyoma middle t-pp60$^{c\text{-}src}$ complex (Kaplan *et al.*, 1986; Whitman *et al.*, 1987). Second, the cytoskeletal association of the p85/p110 form of PI 3-kinase in thrombin-stimulated platelets is not associated with significant translocation of $PtdIns(3,4,5)P_3$ 5-phosphatase activity. The mechanism for this uncoupling of enzyme activities in thrombin-stimulated platelets is unclear. It is possible that the two enzymes may physically dissociate following thrombin stimulation or that only a subset of PI 3-kinase, that which is not associated with the $PtdIns(3,4,5)P_3$ 5-phosphatase, translocates to the cyto-

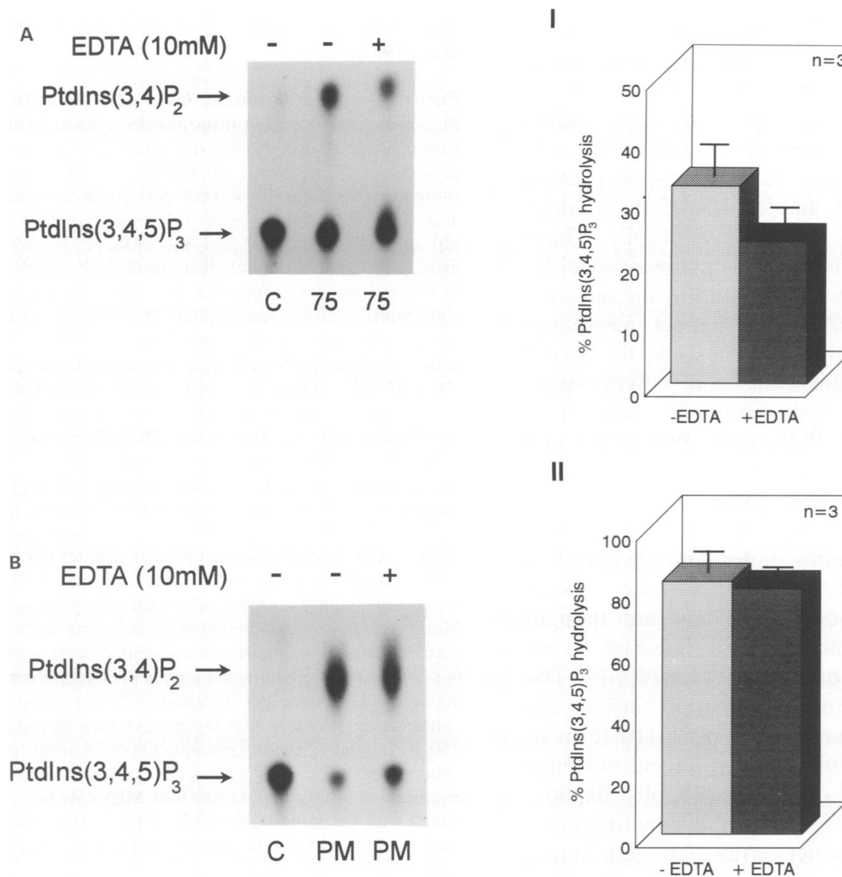


Fig. 8. The effect of EDTA on the metabolism of PtdIns(3,4,5)P_3 by platelet membrane PtdIns(3,4,5)P_3 5-phosphatase or the purified 75 kDa 5-phosphatase. (**A**) PtdIns([^{32}P]3,4,5)P_3 (10 pmol) was incubated with control buffer (lane 1), 75 kDa 5-phosphatase (75) [0.18 nmol Ins(1,4,5)P_3 hydrolyzed/min] (lane 2) or 75 kDa 5-phosphatase [0.18 nmol Ins(1,4,5)P_3 hydrolyzed/min] in the presence of 10 mM EDTA (lane 3) for 30 min at room temperature. PtdIns(3,4,5)P_3 5-phosphatase activity was quantitated as described under Materials and methods and the results are shown in histogram I, with the number of determinations and SD. (**B**) PtdIns([^{32}P]3,4,5)P_3 (10 pmol) was incubated with control buffer (lane 1), platelet membrane extract (PM) (12.5 μg) (lane 2) or platelet membrane extract (12.5 μg) in the presence of 10 mM EDTA (lane 3) for 30 min at room temperature. Lipids were extracted, resolved by TLC and detected by autoradiography, as described under Materials and methods. PtdIns(3,4,5)P_3 5-phosphatase activity was quantitated as described under Materials and methods and the results are shown in histogram II, with the number of determinations and SD.

skeleton. Recent studies have demonstrated that novel forms of PI 3-kinase exist within platelets and that these enzymes also associate with the cytoskeletal fraction of thrombin-stimulated platelets (Zhang *et al.*, 1995). The relative contribution of these novel enzymes in promoting the thrombin-stimulated increase in cytoskeletal PtdIns (4,5)P_2 3-kinase activity is an important issue to clarify, as these recent observations may partly explain the lack of a detectable increase in cytoskeletal PtdIns(3,4,5)P_3 5-phosphatase activity.

Previous purifications of PtdIns 3-kinase have not described the presence of a complexed PtdIns(3,4,5)P_3 5-phosphatase (Carpenter *et al.*, 1990; Shibasaki *et al.*, 1991). The reasons for this discrepancy with our results is not immediately apparent. However, it is possible that the association between these two enzymes is dependent on the subtype of PI 3-kinase purified or that the particular cell types previously used to purify PI 3-kinase do not express the 5-phosphatase described here. Alternatively, previous PI 3-kinase isolation procedures have used acid precipitation techniques or detergents in their purification protocols (Carpenter *et al.*, 1990; Shibasaki *et al.*, 1991), which may lead to dissociation of this enzyme complex. Future studies will be required to address this issue.

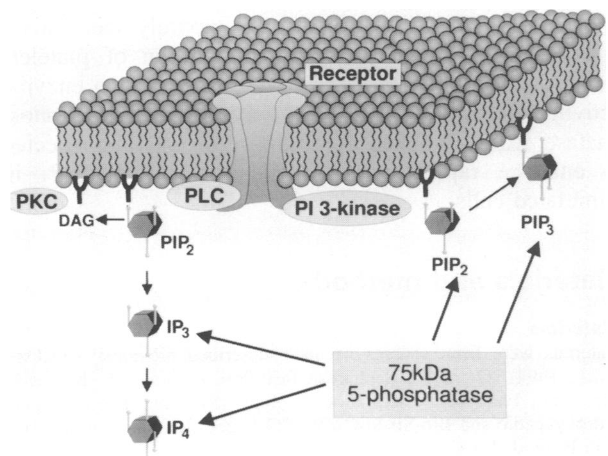


Fig. 9. Potential physiological substrates for the 75 kDa 5-phosphatase. PtdIns(4,5)P_2 and the products of its metabolism by phospholipase C and PI 3-kinase are all potential physiological substrates for the 75 kDa 5-phosphatase. PIP_2, PtdIns(4,5)P_2; PIP_3, PtdIns(3,4,5)P_3; IP_3, Ins(1,4,5)P_3; IP_4, Ins(1,3,4,5)P_4; PLC, phospholipase C; PI 3-kinase, phosphoinositide 3-kinase, PKC, protein kinase C; DAG, diacylglycerol.

The PI 3-kinase-associated 5-phosphatase appears to be unique amongst other cellular 5-phosphatases in that it selectively hydrolyzes the 5-position phosphate of PtdIns(3,4,5)P_3, but does not dephosphorylate PtdIns (4,5)P_2 or the inositol phosphates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. In direct contrast, the 43 kDa 5-phosphatase dephosphorylates Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, but does not metabolize PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. These observations suggest the existence of 'pathway-specific' signal terminating enzymes, which may allow the selective regulation of outflow signals from distinct messenger pathways. Consistent with this hypothesis is the recent identification of a membrane-associated polyphosphoinositide phosphatase which hydrolyzes phosphates from the 3-, 4- or 5-position of PtdIns(3)P, PtdIns(4)P and PtdIns(4,5)P_2, but does not metabolize Ins(1,4)P_2 or Ins(1,4,5)P_3 (Rath-Hope and Pike, 1994).

PI 3-kinase has been found to physically associate with a growing number of signaling molecules (reviewed in Stephens *et al.*, 1993; Fry, 1994). Most of these associations correlate with cell stimulation and are thought to enhance the access of activated PI 3-kinase to its physiological substrates, in particular PtdIns(4,5)P_2. The functional significance of complex formation between the PtdIns(3,4,5)P_3 5-phosphatase and the p85/p110 form of PI 3-kinase is unclear from our studies. Recent evidence indicates that the product of PtdIns(3,4,5)P_3 dephosphorylation, PtdIns(3,4)P_2, may perform a signaling function *per se*, by activating distinct forms of protein kinase C (Toker *et al.*, 1994). Therefore, the physical association of a PtdIns(3,4,5)P_3 5-phosphatase with PI 3-kinase may not simply represent a signal terminating complex, but may in fact be responsible for generating other second messengers within the cell. Alternatively, the association of these two enzymes may be important for maintaining a low level of PtdIns(3,4,5)P_3 within resting cells. The presence of PtdIns(3)P indicates that PI 3-kinase has access to phosphoinositide substrates in unstimulated cells. It is possible that PtdIns(3,4,5)P_3 generated in resting cells is rapidly metabolized by the PI 3-kinase-associated 5-phosphatase. While this hypothesis is purely speculative, it is of interest that thrombin stimulation of platelets leads to the functional dissociation of these two enzyme activities. This uncoupling of the PtdIns(3,4,5)P_3 5-phosphatase from activated PI 3-kinase would be expected to enhance rapid accumulation of PtdIns(3,4,5)P_3 in stimulated cells.

Materials and methods

Materials

Materials were from sources we have described previously (Jackson *et al.*, 1994). Q- and S-Sepharose fast flow chromatography media were from Pharmacia LKB Biotechnology (Sweden). Bio-Gel HTP-hydroxyapatite and Bio-Sil SEC 250 HPLC gel filtration columns were from BioRad (USA).

Antibodies

Anti-p85 (p85 subunit of PI 3-kinase) polyclonal antibody was from Upstate Biotechnology Inc (USA). Anti-p110 (p110 subunit of PI 3-kinase) polyclonal antibody was raised against the synthetic peptide CKMDWIFHTIKQHALN, as described by Hiles *et al.* (1992) and was a kind donation from Dr Michael Crouch (John Curtin School of Medical Research, Australia). Antibodies to the recombinant 75 kDa 5-phosphatase and the highly purified 43 kDa membrane-associated 5-phosphatase were developed as previously described (Laxminarayan *et al.*, 1993).

Purification of PI 3-kinase from platelet cytosol

PI 3-kinase was purified from platelet cytosol (obtained from the Red Cross Blood Bank, NSW, Australia) using a modified purification protocol of Carpenter *et al.* (1990). All PI 3-kinase assays and the subsequent lipid analyses were performed as described by Carpenter *et al.* (1990). Platelet cytosol (1300 mg) was dialyzed against buffer A [20 mM HEPES, pH 7.5, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF)] prior to loading on a Q-Sepharose fast flow column (180×25 mm), pre-equilibrated with the same buffer. The column was washed with 400 ml buffer A and eluted with a gradient of 0–0.5 M NaCl (750 ml). The active fractions (115 mg) were pooled and dialyzed against buffer B (20 mM MES, pH 6.7, 5 mM β-mercaptoethanol, 50 μg/ml PMSF), prior to loading on a S-Sepharose fast flow column (110×15 mm), pre-equilibrated with the same buffer. The column was washed with 100 ml buffer B and eluted with a linear gradient of 0–0.3 M NaCl (100 ml). Fractions containing PI 3-kinase activity (23 mg) were pooled, then loaded on a hydroxyapatite column (30×10 mm), pre-equilibrated with buffer C (20 mM K_2HPO_4, pH 7.0, 5 mM β-mercaptoethanol, 50 μg/ml PMSF). The column was washed with 100 ml buffer C and eluted with a 20–750 mM gradient of K_2HPO_4 (100 ml). Active fractions (5 mg) were pooled and dialyzed against buffer B, prior to loading onto HPLC Mono S 5/5, pre-equilibrated with the same buffer. The column was washed with 10 ml buffer B and eluted with a linear gradient of 0–0.3 M NaCl (20 ml). Fractions with PI 3-kinase activity (1.0 mg) were pooled and vacuum concentrated (0.5 ml) using a Micro-ProDiCon concentrator (Biomolecular Dynamics), then loaded on two Bio-Sil SEC 250 HPLC gel filtration columns (600×7.5 mm), preceded by a guard column (80×7.5 mm) connected in series. This column was pre-equilibrated with buffer D (20 mM MES, pH 6.5, 150 mM NaCl) and eluted with the same buffer (0.5 ml/min). The active fractions (0.5 ml) were pooled and vacuum concentrated to 1 ml prior to storage at –70°C.

Purification of the 43 and 75 kDa 5-phosphatases

Both the 43 kDa membrane-associated 5-phosphatase and the 75 kDa 5-phosphatase were purified as previously described (Laxminarayan *et al.*, 1993; Matzaris *et al.*, 1994).

Preparation of platelet Triton X-100 and cytoskeletal extracts

Platelets were washed as previously described (Jackson *et al.*, 1994). Washed platelet suspensions (2×10^9 platelets/ml) were stimulated with thrombin (1 U/ml) for 5 min at room temperature, with stirring. Platelets were immediately mixed with 1 vol. 10× Triton X-100 lysis buffer (200 mM Tris–HCl, pH 7.4, 10% Triton X-100, 50 mM EGTA, 4 mM leupeptin, 2 mM sodium vanadate, 250 μg/ml PMSF) to 9 vol. platelets and slowly rocked at 4°C for 60 min. Lysates were centrifuged at 1500 *g* for 10 min to remove any intact platelets. The 15 000 *g* cytoskeletal fraction was obtained by centrifugation of the total cell lysates at 15 000 *g* for 4 min. The pellet was washed four times in Triton X-100-free lysis buffer and resuspended in 1/5th the volume of the original lysate. The supernatant was centrifuged at 100 000 *g* for 3 h, washed twice with Triton X-100-free lysis buffer and finally resuspended in 1/5th the volume of the original platelet lysate (100 000 *g* cytoskeletal extract). The remaining supernatant was referred to as the Triton X-100-soluble fraction. Triton X-100-soluble and -insoluble (cytoskeletal) extracts were assayed for PI 3-kinase activity using PtdIns (4,5)P_2 as a substrate, as described below.

Preparation of platelet cytosolic and membrane extracts

Washed platelet suspensions (2×10^9 platelets/ml) were disrupted by sonication for 45 s at 4°C (Labsonic Ultrasonic Cell Disruptor; Braun Melsungen AG, Germany) and cytosolic and membrane fractions separated by centrifugation at 100 000 *g* for 60 min at 4°C. The membrane pellet was resuspended in 1 ml 1× Triton X-100 lysis buffer and membrane proteins extracted for 4 h at 4°C, while gently rocking. The 15 000 and 100 000 *g* Triton X-100-insoluble (cytoskeletal) proteins were sedimented as described above.

Detection of PI 3-kinase activity

PI 3-kinase assays of dilute platelet extracts (diluted in the final reaction mixture 1:200 or 1:1000) were performed as previously described (Susa *et al.*, 1992), with minor modifications. Using serial dilutions of platelet extracts we have previously demonstrated that this assay is within the

linear range with respect to time and protein concentration and is an accurate method for quantitating PtdIns(4,5)P_2 3-kinase activity in the presence of anionic detergents (Jackson *et al.*, 1994). All cellular extracts were diluted in kinase assay buffer (20 mM HEPES, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$) and mixed with sonicated PtdIns(4,5)P_2 (50 μM) and phosphatidylserine (75 μM) to a final reaction volume of 100 μl. [γ-^{32}P]ATP (50 μM, 1 μCi/nmol) was added to each reaction mixture and the enzyme assay incubated for 20 min at room temperature. Reactions were stopped with 100 μl 1 M HCl and phospholipids extracted with 200 μl chloroform/methanol (1:1 v/v) and 500 μl 2 M KCl. PI 3-kinase assays were performed in the linear range with respect to protein concentration and time. ^{32}P-Labeled inositol phospholipids were resolved by TLC with a 2 M acetic acid/*n*-propanol (35:65 v/v) solvent system and detected by autoradiography, as described (Carpenter *et al.*, 1990). Individual phospholipids were scraped from the TLC plate and quantitated by liquid scintillation counting. PI 3-kinase activity was quantitated on platelet extracts diluted 1:1000 by measuring the enzymatic incorporation of ^{32}P into PtdIns(4,5)P_2, forming PtdIns ([^{32}P]3,4,5)P_3, as previously described (Jackson *et al.*, 1994).

HPLC analysis of deacylated and deglycerated lipid products

The authenticity of radiolabeled phospholipids was confirmed by HPLC analysis of both deacylated and deacylated and deglycerated lipid products, as previously described (Divecha *et al.*, 1991; Stephens *et al.*, 1991).

p85 (p85 subunit of PI 3-kinase) and 43 and 75 kDa 5-phosphatase immunoblots

Platelet proteins were separated by SDS–PAGE under reducing conditions, then transferred to polyvinylidene difluoride membranes. Western blots were performed as described (Towbin *et al.*, 1979). Proteins were identified with specific primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody. Blots were developed using enhanced chemiluminescence according to the manufacturer's instructions.

Detection of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and PtdIns(4,5)P_2 5-phosphatase activity

Ins(1,[^{32}P]4,[^{32}P]5)P_3 and [^{3}H]Ins(1,3,4,5)P_4 (10 μM) hydrolysis was measured as previously described (Mitchell *et al.*, 1989). Ins(1,[^{32}P]4, [^{32}P]5)P_3 was isolated from erythrocyte ghosts as described (Downes *et al.*, 1982). PtdIns(4,5)P_2 5-phosphatase assays were performed as described (Matzaris *et al.*, 1994), using 250 μM [^{3}H]PtdIns(4,5)P_2 (3500 c.p.m./nmol) and [^{3}H]PtdIns(4,5)P_2 without added mass.

Detection of PtdIns(3,4,5)P_3 5-phosphatase activity

PtdIns([^{32}P]3,4,5)P_3 was generated by incubating partially purified PI 3-kinase [30 nmol PtdIns(3)P formed mg/min] with PtdIns(4,5)P_2 (50 μM) for 5 min at room temperature in the presence of phosphatidylserine (75 μM) and ^{32}P-labeled ATP (1 μCi/nmol). The assays were stopped with 100 μl 1 N HCl and extracted with 200 μl chloroform/methanol (1:1) and 500 μl 2 M KCl. PtdIns([^{32}P]3,4,5)P_3 (10 pmol) was dried under nitrogen and finally resuspended in 20 mM HEPES, pH 7.2, 5 mM $MgCl_2$, 1 mM EDTA. The resuspended lipid was sonicated (100 W) for 6 min on ice, prior to the addition of purified enzymes or cell extracts. Assays were performed in the presence or absence of 10 mM EDTA for the indicated times at room temperature. Hydrolysis of PtdIns([^{32}P] 3,4,5)P_3 to PtdIns([^{32}P]3,4)P_2 was determined by TLC analysis of the lipid products, as previously described (Jackson *et al.*, 1994). Individual lipids were scraped from the TLC plate and quantitated by liquid scintillation counting. PtdIns([^{32}P]3,4,5)P_3 5-phosphatase activity was expressed as pmol PtdIns([^{32}P]3,4)P_2 formed/mg/min.

Immunoprecipitation of PI 3-kinase and the 75 kDa 5-phosphatase

Protein A–Sepharose (50 μl of a 50% slurry) was incubated with either pre-immune serum (2 μl), anti-p85 (2 μl) or anti-p110 (5 μl) antibodies for 2 h at 4°C. Protein A–Sepharose was washed twice with 500 μl 20 mM Tris, pH 7.2, 150 mM NaCl. Partially purified PI 3-kinase (10 μl) [30 nmol PtdIns(3)P formed/mg/min] was incubated with the protein A–Sepharose–antibody complex for a further 2 h at 4°C. Supernatants were removed and the protein A–Sepharose pellet washed five times in either 20 mM Tris, pH 7.2, 150 mM NaCl or with a high stringency buffer containing 10 mM Tris, pH 7.2, 0.5 M NaCl, 1 mM EDTA, 1% NP-40 and 0.1% SDS. The protein A–Sepharose pellet was washed a further three times in kinase assay buffer, containing 20 mM HEPES, pH 7.2, 1.0 mM EGTA, 5 mM $MgCl_2$, and both the supernatant and protein A–Sepharose pellets assayed for PI 3-kinase activity. 75 kDa 5-phosphatase was immunoprecipitated as previously described (Matzaris *et al.* 1994).

Acknowledgements

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