Inositol phospholipids regulate the guanine-nucleotide-exchange factor Tiam1 by facilitating its binding to the plasma membrane and regulating GDP/GTP exchange on Rac1

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Binding of the Rac1-specific guanine-nucleotide-exchange factor, Tiam1, to the plasma membrane requires the N-terminal pleckstrin homology domain. In the present study, we show that membrane-association is mediated by binding of PtdIns(4,5)P_2 to the pleckstrin homology domain. Moreover, in 1321N1 astrocytoma cells, translocation of Tiam1 to the cytosol, following receptor-mediated stimulation of PtdIns(4,5)P_2 breakdown, correlates with decreased Rac1-GTP levels, indicating that membrane-association is required for GDP/GTP exchange on Rac1. In addition, we show that platelet-derived growth factor activates Rac1 *in vivo* by increasing PtdIns(3,4,5)P_3 concentrations, rather than the closely related lipid, PtdIns(3,4)P_2. Finally, the data demonstrate that PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 bind to the same pleckstrin homology domain in Tiam1 and that soluble inositol phosphates appear to compete with lipids for this binding. Together, these novel observations provide strong evidence that distinct phosphoinositides regulate different functions of this enzyme, indicating that local concentrations of signalling lipids and the levels of cytosolic inositol phosphates will play crucial roles in determining its activity *in vivo*.

Key words: G-protein, guanine-nucleotide-exchange factor, inositol phospholipid, pleckstrin homology domain, Rac1, Tiam1.

INTRODUCTION

PH (pleckstrin homology) domains are poorly conserved protein modules of approx. 120 amino acids that are present in a large number of diverse proteins [1–3]. A range of different ligands can bind to PH domains, including $\beta\gamma$ subunits of heterotrimeric G-proteins [4,5], protein kinase C [6,7] and phosphoinositides (reviewed in [1–3]). Indeed, phosphoinositides bind to a large number of PH domains with high affinity, but most of these interactions have a low selectivity [8]. Two exceptions to this are the PH domains of PLCδ1 (phospholipase Cδ1), and the ARF (ADP-ribosylation factor) exchange factor GRP1 (general receptor of phosphoinositides-1), which bind with high selectively to PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 respectively [8]. Analysis of several PH domains by X-ray crystallography has shown that they consist of two orthogonally arranged β-sheets, closed off at one end by an amphipathic α-helix, and exhibit a dramatic electrostatic polarization [1,2,9,10]. The PH domains of PLCδ1 [9] and GRP1 [1,2] promote selective binding of their target ligands via several specific electrostatic interactions with the phosphates in the lipid headgroup. Thus structural isomers of the ligand, or lipids with fewer phosphates, do not bind to the PH domain with high affinity, whereas phosphoinositides with additional phosphates have restricted access to the binding pocket because of steric hindrance. Binding of phosphoinositides can influence the activity of proteins in a number of different ways: facilitating their recruitment to a membrane, e.g. GRP1 [11], targeting them to a particular membrane in a constitutive manner, e.g. PLCδ1 [12], or by enhancing their catalytic activity, e.g. Tiam1 [13].

Rho-family guanine-nucleotide-exchange factors promote activation of Rho-family GTPases by facilitating the release of GDP, and thereby increasing GTP binding [14]. This family of exchange proteins all contain at least one PH domain, closely associated with the active site DH (Dbl homology) domain, suggesting that the PH domain plays an important role in its regulation. The Rac1-specific GDP/GTP-exchange protein Tiam1 contains a DH domain, a consensus myristoylation sequence, phosphorylation consensus sequences for several protein kinases, a PEST (Pro-Glu-Ser-Thr) domain, a Discs-large homology region and two PH domains, one located on each side of the DH domain. Several groups have demonstrated that inositol phospholipids regulate these exchange proteins. Indeed, PtdIns(3,4,5)P_3 enhances the catalytic activity of Vav *in vitro*, by allowing Rac1 easier access to the active site, whereas PtdIns(4,5)P_2 has the opposite effect [15]. Activation of Rac1 by Tiam1 (reviewed by Minard et al. [16]) is also stimulated by 3-phosphorylated inositol lipids *in vitro* and *in vivo* [13,17], but GDP/GTP exchange is not inhibited by a similar concentration of PtdIns(4,5)P_2 [13]. Additionally, PtdIns-(3,4,5)P_3 and PtdIns(4,5)P_2 both inhibit the exchange activity of Dbl, resulting in reduced activation of RhoA and Cdc42 [18].

While many PH-domain-containing proteins are activated by phosphoinositide-dependent recruitment to membranes, there is little evidence to support such a model for the activation of Rho-family exchange factors. Binding of PtdIns(3,4,5)P_3 or PtdIns(4,5)P_2 to Dbl may promote its binding to the plasma membrane [18], but phospholipids apparently do not affect Vav localization [19]. It has been proposed that Tiam1 catalyses guanine-nucleotide exchange on Rac1 at the plasma membrane [20], and

Abbreviations used: CamKII, Ca^{2+}/calmodulin-dependent protein kinase II; DH domain, Dbl homology domain; DiC_{16}, dipalmitoyl; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; GST, glutathione S-transferase; GRP1, general receptor of phosphoinositides-1; IGF-1, insulin-like growth factor 1; PAK, p21-activated kinase; PBD, p21 (CDC42/Rac1)-binding domain; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; PH domain, pleckstrin homology domain; PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; PLCδ1, phospholipase Cδ1; POC, perfusion open and closed; PS, phosphatidylserine; SHIP2, Src homology 2 domain containing inositol 5-phosphatase; TTBS, Tris/Tween-buffered saline.

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membrane localization of Tiam1 requires co-operation between the N-terminal PH domain and the adjacent coiled-coil domain [21]. The isolated PH domain itself is not membrane-localized [13], but since it can bind to phosphoinositides [13,22], we postulated that lipids may co-operate with an additional factor to target Tiam1 to the membrane, as proposed for other PH-domain-containing proteins [3]. Candidates for this putative auxiliary factor include $\beta\gamma$ subunits of heterotrimeric G-proteins [4], protein phosphorylation [23–25], Ras [26] and ankyrin [27].

Our results provide compelling evidence that phosphoinositides play a vital role in tethering Tiam1 to the plasma membrane. Moreover, we demonstrate that two distinct lipids regulate Tiam1 in 1321N1 astrocytoma cells; PtdIns(4,5)P_2 is required to tether Tiam1 to the plasma membrane, whereas in PDGF (platelet-derived growth factor)-stimulated cells, PtdIns(3,4,5)P_3 stimulates GDP/GTP exchange on Rac1. Soluble inositol phosphates also appear to compete with lipid ligands for binding to Tiam1 and contribute to its cytosolic translocation upon stimulation of PLC (phospholipase C)-coupled receptors. Finally, using an *in vitro* GDP/GTP-exchange assay, we show that PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 compete for the same binding site on Tiam1, indicating that the membrane distribution and relative local concentrations of these two lipids will have a significant influence on the activity state of this protein.

EXPERIMENTAL

Materials

1321N1 astrocytoma cells were obtained from the European Tissue Culture Collection. Penicillin and streptomycin were from Life Technologies. Foetal bovine serum, DMEM (Dulbecco's modified Eagle's medium), sodium orthovanadate, leupeptin, antipain, PMSF, sodium fluoride, sodium pyrophosphate, Tween 20, Triton X-100, wortmannin, anti-GST (glutathione S-transferase) antibody and glutathione–agarose beads were from Sigma. Anti-Tiam1 antibody was from Santa Cruz Biotechnology. Anti-Rac1 antibody was from Upstate Biotechnology. GDP and GTP were from Boehringer Mannheim. [^{3}H]GDP was from DuPont-NEN. Nitrocellulose filters were from Whatman. PC (phosphatidylcholine) and PS (phosphatidylserine) were from Avanti Polar Lipids. Ins(1,4)P_2, Ins(1,4,5)P_3, Ins(1,3,4)P_3, Ins(1,3,4,5)P_4, DiC_{16}–PtdIns(4,5)P_2 (where DiC_{16} is dipalmitoyl) and DiC_{16}–PtdIns(3,4,5)P_3 were from Cell Signals. Thrombin peptide (SFLLRN) was synthesized by Dr G. Bloomberg (University of Bristol, Bristol, U.K.). Plasmids for expression of GST–Rac1 and GST–PBD [p21 (CDC42/Rac1)-binding domain] of PAK (p21-activated kinase) in *Escherichia coli* were a gift from Professor A. Hall (University College, London, U.K.). Full-length SHIP2 (Src-homology-2-domain-containing inositol 5-phosphatase) was cloned by PCR from a human skeletal muscle library (Clontech), subcloned into TOPO (Invitrogen), sequenced and then subcloned into the GFP (green fluorescent protein) expression vector. The PCR primers used were: sense strand, 5′-CGGGCGGTGCTGAGCCCTGC-3′; antisense strand, 5′-TGAGTTCACAGCTTCGTGGTGCC-3′. IGF-1 (insulin-like growth factor 1) and PDGF were obtained from Sigma.

Cell culture conditions

1321N1 astrocytoma cells were maintained in DMEM supplemented with 4 mM L-glutamine, 10 % (v/v) foetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5 % CO_2. For the Rac1 activation assays and cell-fractionation experiments, cells were seeded at approx. 25 % confluency on 100-mm-diameter dishes.

Confocal microscope imaging of proteins linked to GFP

Cells grown on glass coverslips were transfected with GFP-tagged proteins for 24 h, and then were stimulated with agonists for 10 min, with or without pre-treatment with 100 nM wortmannin for 30 min. The plasmids and transfection conditions for expression of GFP-tagged C1199-Tiam1 (a truncated form of Tiam1 lacking the N-terminal PEST sequence) and the PH domain of GRP1 have been described previously [13,41]. Cells were then fixed in 3 % (w/v) paraformaldehyde in PBS for 15 min. The fixed cells were then washed four times in PBS before mounting on slides in Mowiol containing 2.5 % DABCO (1,4-diazobicyclo[2,2,2]-octane) antifade. The cells were imaged using a Leica inverted fluorescence microscope with a Hammamatsu ORCA-ER CCD (charge-coupled device) camera attached. Z series images were collected and optical sections deconvolved using Improvision OpenLab volume deconvolution software.

Microinjection of inositol phosphates

Cells were seeded on coverslips and transfected with GFP-conjugated PH domains 1 day before injection. The cells were placed in an open POC chamber (a cell culture system for perfusion open and closed, from H. Saur, Reutlingen, Germany) in Hepes-buffered DMEM without serum. The POC chamber, maintained at 37 °C, was placed on a DeltaVision microscope, and transfected cells were located using a 100PA objective. Microinjection was carried out using an Eppendorf system. The injection needle was filled with the appropriate inositol phosphate species in injection buffer (100 mM glutamic acid, 140 mM KOH, 1 mM $MgSO_4$, 1 mM dithiothreitol), pH 7.2, with citric acid. The microinjection needle was positioned above the cells using $\times$ 10 and $\times$ 40 LWD (long working distance) objectives before returning to the $\times$ 100 objective. This procedure is expected to give an initial intracellular concentration of the injected species of about 10 % of the concentration in the injection needle [28], a value which does not take account of the rate of metabolism of the injected species. It is therefore likely that the transient effects of microinjected inositol phosphates on the translocation of GFP-tagged proteins reflects the metabolism of the injected molecules (see Figure 2B). The cells were imaged with three optical sections at each time point. The first image was taken before injection and the cells were re-imaged within 20 s of injection. Images were collected at 30 s intervals for periods up to 10 min. The final images were deconvolved using the DeltaVision Softworks program.

Rac1 activation assay

The Rac1 activation assay was performed essentially as described [13,29]. The p21 PBD of PAK was expressed in *E. coli* as a GST-fusion protein, purified using glutathione–Agarose beads and used to precipitate GTP-bound Rac1 from cell lysates. After stimulation with various agonists for the indicated times, the 1321N1 astrocytoma cells were washed with 5 ml of PBS, then scraped into 0.5 ml of lysis buffer [1 % (v/v) Nonidet P40, 150 mM NaCl, 50 mM Tris/HCl, pH 7.4, 10 % (v/v) glycerol, 2.5 mM $MgCl_2$, 10 μg/ml antipain and leupeptin, 1 mM PMSF, 500 μM sodium orthovanadate, 10 mM pyrophosphate, 10 mM sodium fluoride and 1 mM dithiothreitol]. Cells were lysed by sonication (two 5 s bursts), then clarified by centrifugation at 12 000 ***g*** for 10 min. Each supernatant was incubated with 10 μg of immobilized GST–PBD for 1 h at 4 °C. Beads were collected

by centrifugation at 2000 ***g*** for 5 min, washed four times in lysis buffer, and resuspended in 30 μl of Laemmli sample buffer. The samples were resolved by SDS/PAGE on 14 % polyacrylamide gels, transferred on to PVDF membranes, and the amount of precipitated Rac1 estimated by Western blotting with an anti-Rac1 antibody.

Cell-fractionation experiment

1321N1 astrocytoma cells were stimulated with 100 μM thrombin peptide for the indicated times, washed with 5 ml of PBS, then scraped into 0.5 ml of lysis buffer (50 mM Hepes, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 10 μg/ml antipain and leupeptin, 1 mM PMSF, 500 μM sodium orthovanadate, 10 mM pyrophosphate, 10 mM sodium fluoride and 1 mM dithiothreitol). Cells were then lysed by sonication (four 5 s bursts) on ice, and centrifuged at 120 000 ***g*** for 45 min to prepare cytosol and total particulate fractions. The membrane pellet was washed twice with lysis buffer to remove cytosolic proteins. The protein concentration of each fraction was estimated by the method of Bradford [30]. Equal amounts (by protein content) of cytosol and pellet fractions were resolved by SDS/PAGE on 6 % polyacrylamide gels, transferred on to PVDF membranes and probed with an anti-Tiam1 antibody.

SDS/PAGE and Western blot analysis

SDS/PAGE was performed on 6 % or 14 % polyacrylamide gels and proteins transferred on to PVDF membranes (Millipore) for 1 h at 14 V using a semi-dry transfer unit. Membranes were blocked overnight with 5 % (w/v) non-fat dried milk in TTBS (Tris/Tween-buffered saline). For Tiam1 blots, antibody was used at a dilution of 1:2000 in TTBS containing 1 % (w/v) BSA, for 1 h. Anti-Rac1 antibody was used at a dilution of 1:1000 in TTBS containing 3 % (w/v) non-fat dried milk. Membranes were then incubated for 1 h with the appropriate horseradish-peroxidase-conjugated secondary antibody (Pierce) diluted 1:5000, before development using an ECL® (enhanced chemiluminescence) kit (Amersham Biosciences).

Radiolabelling of inositol phospholipids

1321N1 astrocytoma cells were cultured in 24-well dishes under inositol-depleting conditions and labelled with [2-^{3}H]inositol for two days as described previously [31]. Labelled cells were washed twice with 2 ml of incubation buffer and pre-incubated at 37 °C for 30–60 min in 250 μl of buffer, before further incubation for 5 min with either 10 μM wortmannin or DMSO vehicle. Cells pre-treated with wortmannin were either fixed (zero time control) or incubated with 100 μM thrombin peptide, or vehicle, for 5–300 s. Reactions were stopped by the addition of 500 μl of ice-cold 1 M trichloroacetic acid. Extracts were processed for measurement of [^{3}H]inositol lipids and inositol phosphates as described in [31].

[^{3}H]GDP-binding assay and GDP/GTP-exchange assay

C1199-Tiam1, with an N-terminal His_6 tag, was expressed in Sf9 cells and purified using Talon metal affinity resin (Clontech) in 25 mM Tris buffer, pH 8.0, containing 0.5 μM 2-mercaptoethanol and 100 mM NaCl. Tiam1 was eluted from the beads using 100 mM imidazole and dialysed before freezing. GST–Rac1 was expressed in *E. coli*, and purified using glutathione–Agarose beads in 100 mM Tris buffer, pH 8.0, containing 250 mM NaCl and 0.1 mM dithiothreitol. GST–Rac1 was eluted from the beads with 10 mM glutathione, dialysed and frozen.

The Tiam1 GDP/GTP-exchange assay was carried out as described previously [13,24]. Purified GST–Rac1 (120 pmol) was pre-loaded with 6 nmol of [^{3}H]GDP (specific radioactivity 3000 d.p.m./pmol) in 60 μl of binding buffer. An 8 μl volume of the pre-loaded GTPase was added to 32 μl of exchange mixture, which contained 5 pmol of Tiam1 or BSA, 1 mM GTP and 0.5 mM GDP in exchange buffer. At the indicated times, 8 μl aliquots were pipetted into 1 ml of stopping buffer (50 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$ and 50 mM NaCl), and [^{3}H]GDP bound to Rac1 analysed by filtering through nitrocellulose. Inositol phospholipids were added as PC/PS vesicles (final concentrations of 233 μM and 90 μM respectively) to determine their effect on Tiam1 GDP/GTP-exchange. Assays were carried out with vesicles containing 10 μM DiC_{16}–PtdIns(3,4,5)P_3, 10 μM, 100 μM or 1000 μM DiC_{16}–PtdIns(4,5)P_2, or with DiC_{16}–PtdIns(3,4,5)P_3 containing 10–1000μM DiC_{16}–PtdIns(4,5)P_2. Finally, in some experiments, Tiam1 (5 pmol) was pre-phosphorylated with 0.1 unit of CamKII (Ca^{2+}/calmodulin-dependent protein kinase II) as described in [24], and GDP/GTP-exchange activity was measured in the presence of PC/PS vesicles with or without 1000μM DiC_{16}–PtdIns(4,5)P_2. The amount of [^{3}H]GDP bound to Rac1 in 8 μl of reaction mixture at zero time was counted as 100 %. The percentage of GDP released is the percentage of this [^{3}H]GDP that is released from Rac1 during the assay.

RESULTS

The N-terminal PH domain of Tiam1 is an important element for localizing Tiam1 to the plasma membrane [13,21], but little is known about the mechanisms involved. We have established previously that this domain can bind to polyphosphorylated inositol lipids, in the rank order PtdIns(3,4,5)P_3 > PtdIns(3,4)P_2 > PtdIns(4,5)P_2, and that binding of 3-phosphorylated inositol lipids stimulates the GDP/GTP-exchange activity of Tiam1 [13]. In the present study, we investigated the roles of these phospholipids in regulating the cellular distribution and catalytic activity of Tiam1.

Astrocytoma cells were transfected with GFP-tagged C1199-Tiam1 or GFP–GRP1-PH, a protein that is recruited to the plasma membrane by increases in the cellular levels of PtdIns(3,4,5)P_3 [11]. Quiescent astrocytoma cells contain approx. 0.5 pmol/mg of protein of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, and these levels are increased 10–20-fold by stimulating the cells with insulin, or are decreased approx. 80 % by pre-treating the cells with 100 nM wortmannin [32]. Stimulation of astrocytoma cells with 1 μg/ml IGF-1 induced translocation of GFP–GRP1-PH to the plasma membrane, and this was reversed by addition of the PI 3-kinase (phosphoinositide 3-kinase) inhibitor wortmannin (Figure 1), reflecting the changes in cellular PtdIns(3,4,5)P_3 levels [32]. Tiam1 was detected in both the membrane and cytosol of resting cells, and its expression induced a flattened phenotype with extensive membrane ruffling as described previously [13]. In contrast with the PH domain of GRP1, Tiam1 localization was not affected by either 100 nM wortmannin or 1 μg/ml IGF-1 (Figure 1). Therefore, since changes in the cellular level of 3-phosphorylated inositol lipids dramatically alter the localization of GRP1, but do not significantly affect the cellular localization of Tiam1, it seems very unlikely that PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 are required for membrane-association of Tiam1.

Stimulation of astrocytoma cells with thrombin peptide (SFLLRN) produced a rapid and transient decrease in PtdIns(4,5)P_2 levels, and a corresponding increase in the cytoplasmic concentration of its cognate headgroup, Ins(1,4,5)P_3 (Figure 2A) [32]. Therefore we transfected astrocytoma cells with GFP–C1199-Tiam1, or GFP–PLCδ1-PH [which binds strongly and

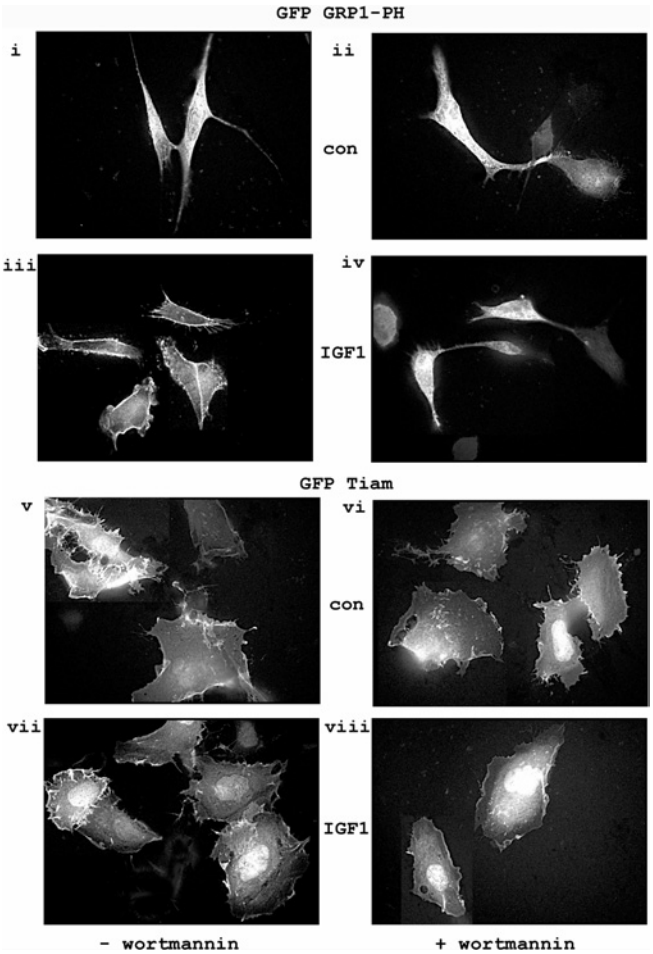

Figure 1 PI 3-kinase products regulate membrane association of GFP–GRP1, but not GFP–Tiam1

Transfected 1321N1 astrocytoma cells were fixed with paraformaldehyde, mounted with Mowiol, and imaged and deconvolved with Openlab software, as described in the Experimental section. Cells were transfected with GFP–GRP1 (i–iv) or GFP–C1199-Tiam1 (v–viii). Cells were left unstimulated [i, ii, v and vi; control (con)], stimulated with 1 μM IGF-1 for 10 min (iii, iv, vii and viii), and/or pre-treated with 100 nM wortmannin for 30 min (ii, iv, vi and viii), before being fixed. Results are representative of three different experiments.

specifically to Ins(1,4,5)P_3 and PtdIns(4,5)P_2] [1–3,8], and stimulated the cells with thrombin peptide to investigate whether this lipid is involved in membrane-association of these proteins. In unstimulated cells, C1199-Tiam1 and PLCδ1-PH remained associated with the plasma membrane over the time course of the experiment (Figure 2). In contrast, addition of 100 μM thrombin peptide induced a rapid (within 15 s) translocation of both C1199-Tiam1 and PLCδ1-PH from the membrane to the cytosol (Figure 2). Relocalization of these proteins was transient, however, since they began to re-associate with the membrane within 2 min of addition of the agonist, and had the same cellular distribution as unstimulated cells after 5 min (Figure 2). This time course of relocalization correlated very closely to the decrease in PtdIns(4,5)P_2 levels/increase in Ins(1,4,5)P_3 concentrations that are produced by this agonist (Figure 2), indicating that one or both of these events might be involved.

Stimulation of astrocytoma cells with thrombin peptide after a 5 min pre-treatment with 10 μM wortmannin resulted in a transient increase in Ins(1,4,5)P_3 concentrations and a sustained decrease in PtdIns(4,5)P_2 levels (Figure 2). This is due to inhibition by the relatively high concentration of wortmannin of a phosphoinositide 4-kinase [33], which effectively blocks resynthesis of hormone-sensitive pools of PtdIns(4)P and PtdIns(4,5)P_2 after thrombin peptide stimulation of phospholipase C. While pre-treatment with 10 μM wortmannin alone had no effect on the cellular distribution of GFP–C1199-Tiam1 or GFP–PLCδ1-PH (results not shown), both proteins were transiently relocated to the cytosol by thrombin peptide in the presence of 10 μM wortmannin (Figure 2). Thus these relocation events correlate with the increase in Ins(1,4,5)P_3 concentration, and not the decrease in PtdIns(4,5)P_2 levels, demonstrating that production of this inositol phosphate, and/or one of its metabolites, stimulates translocation. Significantly, carbachol, which can also stimulate phospholipase C in astrocytoma cells had no detectable effect on the localization of these proteins (results not shown), presumably reflecting the fact that carbachol induces a sustained increase in Ins(1,4,5)P_3 concentration that never exceeds a small fraction of the maximum achieved by thrombin peptide [32].

We then microinjected various synthetic inositol phosphates into transfected astrocytoma cells to ascertain how they affect the cellular distribution of Tiam1 and the PH domain of PLCδ1. The results (Figure 2) indicate that microinjection of 100 μM Ins(1,4,5)P_3 (estimated concentration if distributed throughout the cytosol is 10 μM) into astrocytoma cells causes GFP–PLCδ1-PH to relocate to the cytosol. This translocation is not induced by microinjection of 100 μM Ins(1,3,4)P_3 or Ins(1,4)P_2 (Figure 2). Therefore, since PLCδ1-PH binds specifically to PtdIns(4,5)P_2 [8], and translocation is only induced by Ins(1,4,5)P_3 (Figure 2), a product of the enzymic hydrolysis of PtdIns(4,5)P_2 by PLC, it seems likely that these two ligands compete for the same binding site in the PH domain, as indicated by previous work on this topic. Therefore, when the cellular concentration of Ins(1,4,5)P_3 rises, the PH domain binds to the headgroup instead of PtdIns(4,5)P_2, resulting in its translocation to the cytosol.

Similarly, microinjection of inositol phosphates causes the relocalization of Tiam1 to the cytosol. Translocation was induced by Ins(1,3,4)P_3 (results not shown), Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 (Figure 2), in agreement with the more promiscuous lipid-binding characteristics of the protein that were defined previously. Moreover, this relocalization is not due to increased intracellular Ca^{2+} concentrations, since ionomycin treatment induces relocalization of Tiam1 to the plasma membrane (results not shown) [25], nor did muscarinic receptor stimulation, which induces a robust Ca^{2+} signal, cause Tiam1 translocation (results not shown). Therefore, since inositol phosphates can interfere with lipid binding by competing for the same site in the PH domain [1–3,8], these data provide evidence that a phospholipid(s), most likely PtdIns(4,5)P_2, participates in tethering Tiam1 to the plasma membrane. This hypothesis originates from the observation that wortmannin does not affect the subcellular distribution of Tiam1 (Figure 1), and the fact that Tiam1 only shows a modest preference for binding to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 over PtdIns(4,5)P_2 [13,22], yet there is approx. 1000-fold more PtdIns(4,5)P_2 in unstimulated astrocytoma cells.

Western blotting of lysates indicates that astrocytoma cells contain endogenous Tiam1 (Figure 3), consistent with the observation that brain is a rich source of this protein [34]. Therefore astrocytoma cells were stimulated with thrombin peptide, and fractionated into cytosol and membrane components, to ascertain whether intensive stimulation of PLC alters the subcellular distribution of endogenous Tiam1. In unstimulated astrocytoma cells, Tiam1 was present in both cytosol and membranes (Figure 3). Addition of thrombin peptide caused a transient increase in cytosolic Tiam1, with a corresponding decrease in membrane-associated protein (Figure 3). This relocalization was observed 30 s

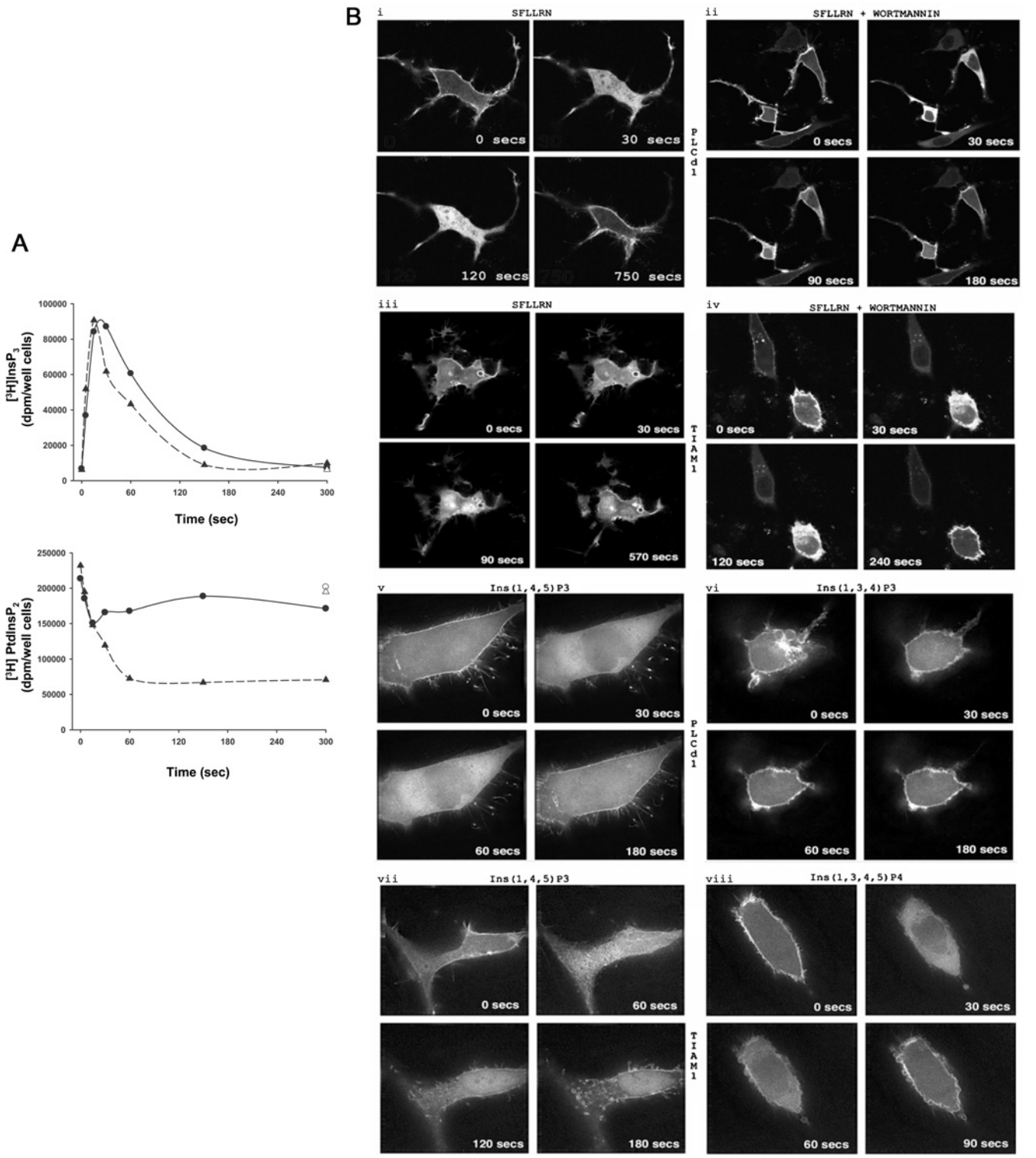

Figure 2 PtdIns(4,5)P_2 and soluble inositol phosphates regulate membrane association of GFP–PLCδ1 and GFP–Tiam1

(**A**) 1321N1 astrocytoma cells pre-labelled to steady state with [^{3}H]inositol were treated with DMSO vehicle (0.1 %, v/v) (circles) or wortmannin (10 μM) (triangles) for 5 min before further incubation without (open symbols) or with (closed symbols) the thrombin receptor activating peptide, SFLLRN (100 μM), for the times indicated. The [^{3}H]inositol metabolites shown were then extracted, separated and quantified as described in the Experimental section. (**B**) Cells were seeded on coverslips and transfected with the indicated GFP-conjugated PH domain 1 day before injection, which was performed as described in the Experimental section. The final images were collected on a DeltaVision microscope and deconvolved using the DeltaVision Softworks program. i, GFP–PLCδ1-transfected 1321N1 astrocytoma cells stimulated with thrombin peptide, SFLLRN (100 μM), for the times indicated; ii, GFP–PLCδ1-transfected 1321N1 astrocytoma cells stimulated with thrombin peptide, SFLLRN (100 μM), for the times indicated in the presence of wortmannin (10 μM); iii, GFP–Tiam1-transfected 1321N1 astrocytoma cells stimulated with thrombin peptide, SFLLRN (100 μM), for the times indicated; iv, GFP–Tiam1-transfected 1321N1 astrocytoma cells stimulated with thrombin peptide, SFLLRN (100 μM), for the times indicated in the presence of wortmannin (10 μM); v, GFP–PLCδ1-transfected 1321N1 astrocytoma cells injected with Ins(1,4,5)P_3 (100 μM) for the times indicated; vi, GFP–PLCδ1-transfected 1321N1 astrocytoma cells injected with Ins(1,3,4)P_3 (100 μM) for the times indicated; vii, GFP–Tiam1-transfected 1321N1 astrocytoma cells injected with Ins(1,4,5)P_3 (100 μM) for the times indicated; viii, GFP–Tiam1-transfected 1321N1 astrocytoma cells injected with Ins(1,3,4,5)P_4 (100 μM) for the times indicated.

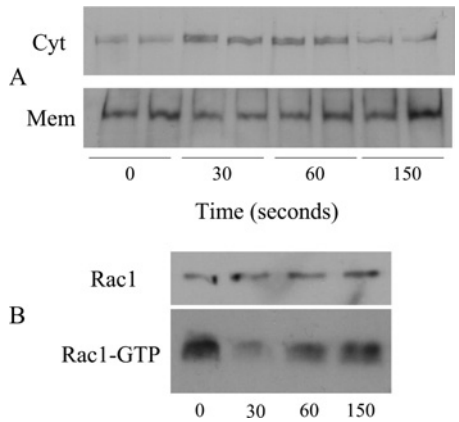

Figure 3 Thrombin peptide induces translocation of endogenous Tiam1 to the cytosol, and decreased Rac1-GTP levels in 1321N1 astrocytoma cells

1321N1 astrocytoma cells were stimulated with 100 μM thrombin peptide for the indicated times. (**A**) Cells were fractionated into cytosol (Cyt) and total membrane (Mem) fractions, then 10 μg of each fraction was probed with an anti-Tiam1 antibody. (**B**) GTP-bound Rac1 was affinity-precipitated with GST–PBD, as described in the Experimental section, and the amount of precipitated Rac1 was assessed by Western blotting (Rac1-GTP). Lysates (10 μg) were also probed with the anti-Rac1 antibody to check total Rac1 levels (Rac1). Results are representative of three independent experiments.

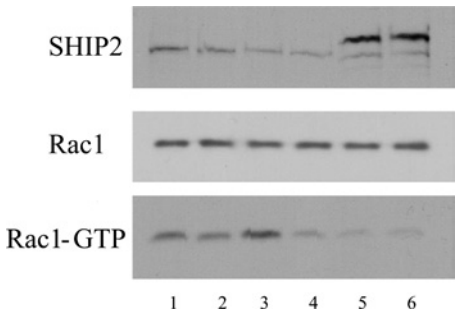

Figure 4 PDGF increases Rac1-GTP levels by increasing PtdIns(3,4,5)P_3 concentrations in 1321N1 astrocytoma cells

1321N1 astrocytoma cells were pre-incubated with 100 nM wortmannin for 30 min (lanes 2 and 4), or transfected with SHIP2 (lanes 5 and 6). Cells were then treated with 50 ng/ml PDGF (lanes 3, 4 and 6) or vehicle (lanes 1, 2 and 5) for 5 min. GTP-bound Rac1 was affinity-precipitated with GST–PBD, as described in the Experimental section, and the amount of precipitated Rac1 was assessed by Western blotting (Rac1-GTP). Lysates (10 μg) were also probed with the appropriate antibodies to check the total Rac1 levels (Rac1), and SHIP2 expression (SHIP2). Results are representative of three independent experiments.

after the addition of thrombin peptide, maintained at 60 s, and returned to basal levels after 150 s (Figure 3). Thus Tiam1 relocalization in response to thrombin peptide (Figure 2) is not an artifact of expressing a GFP-tagged protein, since it is also a property of the endogenous protein (Figure 3).

If endogenous membrane-associated Tiam1 regulates Rac1, it would be expected that translocation of Tiam1 to the cytosol would result in reduced Rac1-GTP. To examine this, astrocytoma cells were stimulated with thrombin peptide, and Rac1-GTP levels were determined using the PBD of PAK to precipitate GTP-bound Rac1 from these cells. Unstimulated cells contain a small amount of Rac1-GTP, which decreased upon addition of thrombin peptide (Figure 3). Indeed, Rac1-GTP was almost undetectable 30 s after the addition of thrombin peptide, but began to recover after 60 s and had almost returned to basal levels after 150 s (Figure 3). The time course of relocalization of GFP–Tiam1 (Figure 2) and endogenous Tiam1 (Figure 3) closely parallels the changes in Rac1-GTP levels in these cells (Figure 3) compatible with the idea that PtdIns(4,5)P_2-dependent membrane-anchoring of Tiam1 and/or related Rac1-activating proteins, is essential for efficient stimulation of this GTPase.

In earlier experiments, we have established that Tiam1 is stimulated by PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 *in vitro*, and by 3-phosphorylated inositol lipids *in vivo* [13]. Therefore we stimulated astrocytoma cells with PDGF in the presence and absence of wortmannin pre-treatment, or in cells overexpressing the 5-phosphatase SHIP2, to ascertain which 3-phosphorylated inositol lipid is involved in agonist-stimulated Rac1 activation. As expected [13], addition of 50 ng/ml PDGF for 5 min induced a modest increase in Rac1-GTP (Figure 4). This increase was totally inhibited by pre-treating with 100 nM wortmannin, or by overexpressing SHIP2 (Figure 4). Since SHIP2 removes the 5-phosphate from PtdIns(3,4,5)P_3, converting it into PtdIns(3,4)P_2 [35], these data clearly imply that PDGF-stimulated Rac1 activation in these cells is mediated by an increase in PtdIns(3,4,5)P_3 concentrations.

The data presented above suggest that PtdIns(4,5)P_2 is involved in tethering Tiam1 to the plasma membrane (Figure 2), while several groups, including ourselves, have noted that PtdIns(3,4,5)P_3 stimulates its exchange activity *in vivo* [13,36]. Moreover, the N-terminal PH domain is involved in binding to both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 [13,22], suggesting that these two ligands compete for the same binding site. We therefore used the *in vitro* GDP/GTP-exchange assay to investigate whether increasing concentrations of PtdIns(4,5)P_2 affect the PtdIns(3,4,5)-P_3-dependent stimulation of Tiam1. As expected [13], 10 μM PtdIns(3,4,5)P_3 induced a 2-fold increase in GDP/GTP-exchange activity, while PtdIns(4,5)P_2 (10–1000 μM) had no effect on basal Tiam1 activity (Figure 5). Significantly, the PtdIns(3,4,5)P_3 effect was decreased by approx. 50 % by the inclusion of 10 μM PtdIns(4,5)P_2, and totally eliminated by the addition of 100 μM PtdIns(4,5)P_2 (Figure 5). Phosphorylation of Tiam1 by CamKII also resulted in a 2-fold increase in exchange activity, consistent with previous results [24], and this stimulation was not affected by inclusion of 1000 μM PtdIns(4,5)P_2 (Figure 5). Therefore the two phospholipids compete for binding at the same site, and their binding affinities are within an order of magnitude, since the PtdIns(3,4,5)P_3 effect is totally inhibited by addition of a 10-fold excess of PtdIns(4,5)P_2 (Figure 5). These data are consistent with the binding profile determined by fat Western blotting [13], which indicates that Tiam1 has a limited preference for PtdIns(3,4,5)P_3 over PtdIns(4,5)P_2. Therefore, since PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 compete for the same binding site on Tiam1, the membrane distribution and relative local concentrations of these two lipids will have a significant influence on the activity status of this protein.

DISCUSSION

The data presented here provide compelling evidence that inositol phospholipids play a vital role in regulating the catalytic activity and subcellular localization of Tiam1. Significantly, these roles are apparently carried out by two distinct lipids: PtdIns(4,5)P_2 mediates binding of Tiam1 to the plasma membrane in astrocytoma cells (Figure 2), whereas PtdIns(3,4,5)P_3 stimulates GDP/GTP exchange on Rac1 *in vivo* (Figure 4). Moreover, since these lipids both bind to the N-terminal PH domain of Tiam1 [13,22], the local concentrations of these phosphoinositides will play a crucial role in determining the biological activity of this enzyme.

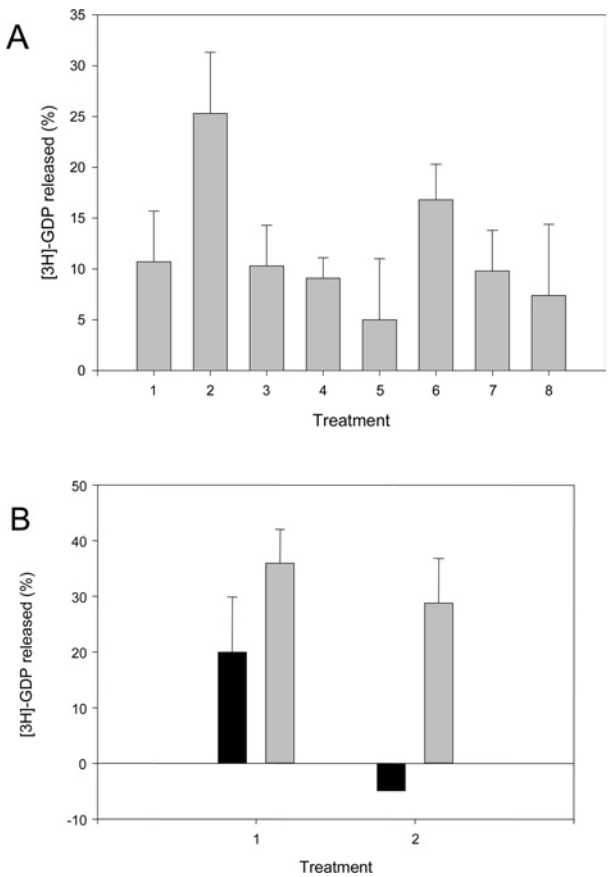

Figure 5 PtdIns(3,4,5)P_3-dependent Tiam1 stimulation *in vitro* is inhibited by inclusion of PtdIns(4,5)P_2

Nucleotide-exchange reactions were carried out as described in the Experimental section, using [^{3}H]GDP-loaded GST–Rac1 as a substrate, for 25 min. Tiam1 GDP/GTP-exchange assays were carried out in the presence of PC/PS vesicles (final concentrations of 233 μM and 90 μM respectively), containing various concentrations of phosphoinositide(s). (**A**) Assays were carried out with no phosphoinositide (treatment 1), 10 μM PtdIns(3,4,5)P_3 (treatment 2), 10 μM PtdIns(4,5)P_2 (treatment 3), 100 μM PtdIns(4,5)P_2 (treatment 4), 1000 μM PtdIns(4,5)P_2 (treatment 5), 10 μM PtdIns(3,4,5)P_3 + 10 μM PtdIns(4,5)P_2 (treatment 6), 10 μM PtdIns(3,4,5)P_3 + 100 μM PtdIns(4,5)P_2 (treatment 7), or 10 μM PtdIns(3,4,5)P_3 + 1000 μM PtdIns(4,5)P_2 (treatment 8). Experiments were also performed using vesicles containing 10 μM PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. (**B**) The GDP/GTP-exchange activity of CamKII-phosphorylated, and non-phosphorylated Tiam1, was determined in the absence (treatment 1), or presence (treatment 2), of 1000 μM PtdIns(4,5)P_2. GDP released (%) is the percentage of [^{3}H]GDP that is released from [^{3}H]GDP-Rac1 during the assay. Results are means $\pm$ S.E.M. for four independent experiments.

Binding of PtdIns(4,5)P_2 to the PLCδ1-PH domain targets this protein to the plasma membrane [12,37], its site of action. Our data indicate that a phosphoinositide also tethers Tiam1 to the plasma membrane. This is suggested by the fact that thrombin peptide, which temporarily decreases PtdIns(4,5)P_2 levels by 50% (Figure 2), induces Tiam1 and the PLCδ1-PH domain to relocate transiently from the plasma membrane to the cytosol of astrocytoma cells (Figures 2 and 3). Interestingly, these relocalization events correlate with increased Ins(1,4,5)P_3 levels in the cytosol, rather than decreased PtdIns(4,5)P_2 at the plasma membrane (Figure 2), as reported by similar studies involving the PLCδ1-PH domain [38,39]. Our data suggest, however, that only the very large increase in Ins(1,4,5)P_3 concentration induced by thrombin peptide is capable of inducing the translocation of Tiam1. The smaller increase in Ins(1,4,5)P_3 concentration stimulated by activation of muscarinic receptors did not displace Tiam1 from the plasma membrane, implying that relatively extreme levels of phospholipase C activity are required for this effect. Inositol phosphates also compete with PtdIns(3,4,5)P_3 for binding to the PH domain of the cytosolic regulator of adenylate cyclase [40], resulting in its relocation to the cytosol. Moreover, since PLCδ1-PH relocalization is induced by hydrolysing approx. 50% of the labelled cellular pool of PtdIns(4,5)P_2 to Ins(1,4,5)P_3 (Figure 2), this motif must bind preferentially to Ins(1,4,5)P_3 *in vivo*. Indeed, since thrombin peptide stimulation increases Ins(1,4,5)P_3 concentrations approx. 15-fold (Figure 2) from 2 μM to 30 μM, and PtdIns(4,5)P_2 levels in these cells are of the order of 20 μM [32], PLCδ1-PH must bind to the headgroup with an affinity that is several fold higher than that for the lipid itself. Although this differs from the findings of Kavran et al. [8], who reported that PLCδ1-PH binds to Ins(1,4,5)P_3 and PtdIns(4,5)P_2 with similar affinity, they agree with the surface plasmon resonance data of Hirose et al. [38], which demonstrates a 30-fold higher affinity for Ins(1,4,5)P_3. One possibility is that PLCδ1-PH may bind Ins(1,4,5)P_3 better than PtdIns(4,5)P_2 simply because the inositol phosphate has better access to the binding pocket. Ins(1,4,5)P_3 binding could function as an important down-regulation mechanism in PLCδ1 signalling, by sequestering the enzyme away from its substrate, PtdIns(4,5)P_2.

Microinjection of inositol lipids into astrocytoma cells also induces relocalization of Tiam1 and PLCδ1-PH to the cytosol (Figure 2), providing further evidence that phosphoinositides tether these proteins to the plasma membrane. PLCδ1-PH was translocated by microinjection of Ins(1,4,5)P_3, but not by Ins(1,3,4)P_3 or Ins(1,4)P_2 (Figure 2). This demonstrates that this PH domain binds specifically to Ins(1,4,5)P_3 or PtdIns(4,5)P_2, confirming earlier studies [8,37]. In contrast, Tiam1 relocalization was induced by Ins(1,4,5)P_3, Ins(1,3,4)P_3 or Ins(1,3,4,5)P_4 (Figure 2). This suggests that Tiam1 can bind to the three corresponding lipids; PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, consistent with data from previous lipid-binding studies [13,22]. However, it seems unlikely that either PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 regulates Tiam1 translocation, since its subcellular distribution is not affected by wortmannin treatment or insulin stimulation (Figure 1). Furthermore, since Tiam1 only shows a modest preference for binding to PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 over PtdIns(4,5)P_2 [13,22], and there is approx. 1000-fold more PtdIns(4,5)P_2 in unstimulated astrocytoma cells than either of the 3-phosphorylated lipids, it seems likely that the latter is the targeting ligand *in vivo*. Although Tiam1 contains two PH domains, phosphoinositide binding is mediated by the N-terminal PH domain only [13,22]. Therefore, since the N-terminal PH domain binds to PtdIns(3,4)P_2, PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 [13,22], the microinjected inositol phosphates presumably interfere with lipid binding to this domain. Thus, like most PH domains [8], the Tiam1 N-terminal PH domain binds to phosphoinositides with a relatively low selectivity. This is consistent with the fact that this motif does not conform to the model designed to predict PH domains that bind specifically to PtdIns(3,4,5)P_3 [1,2]. Furthermore, unlike GRP1-PH [11] or PLCδ1-PH [12], binding of phosphoinositides is required, but is not sufficient to target the isolated Tiam1 N-terminal PH domain to the plasma membrane [13], suggesting that this interaction does not have a particularly high affinity. Therefore binding of phosphoinositides to the N-terminal PH domain (Figure 2) [13,22] probably co-operates with binding of another factor to the adjacent coiled-coil domain [21,36] to target this protein to the membrane. This putative auxiliary factor could involve $\beta\gamma$ subunits of heterotrimeric G-proteins [4], protein phosphorylation [23–25], Ras [26] or ankyrin [27].

Phosphoinositides also play an important role in regulating Tiam1 GDP/GTP-exchange activity towards Rac1. Tiam1 GDP/GTP-exchange activity is stimulated by 3-phosphorylated inositol lipids *in vivo* [13,17,36], although it was not clear whether PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 caused this [13]. By over-expressing the 5-phosphatase SHIP2 in astrocytoma cells, we established that PDGF increases Rac1-GTP levels by increasing PtdIns(3,4,5)P_3 concentrations in these cells (Figure 4). This is an important observation, since a recent study has indicated that PtdIns(3,4)P_2 can also act as an important second messenger [41]. The GDP/GTP-exchange activity of Vav [15] and Dbl [18] is inhibited by 10–20 μM PtdIns(4,5)P_2 *in vitro*. Our data indicate that PtdIns(4,5)P_2 (10–1000 μM) (Figure 5) does not affect the exchange activity of Tiam1 directly (Figure 2). On the other hand, PtdIns(3,4,5)P_3 stimulates the GDP/GTP-exchange activity of Tiam1 (Figure 5) [13] and Vav [15], but inhibits that of Dbl [18]. Thus each of these exchange factors is differentially regulated by phosphoinositides, indicating that they are likely to be controlled by subtly different mechanisms and suggesting that they may regulate different cellular functions. Significantly, PtdIns(4,5)P_2 does exert an important effect on Tiam1 GDP/GTP-exchange activity *in vivo*, since thrombin peptide treatment produces a dramatic transient relocalization of Tiam1 to the cytosol and a corresponding decrease in Rac1-GTP (Figure 3). Targeting of Dbl to the plasma membrane may also involve phosphoinositides, although further work will be required to confirm this and to ascertain which lipid is involved [18]. Therefore Tiam1 and related GDP/GTP-exchange factors probably catalyse GDP release from Rho GTPases at the plasma membrane, as proposed by Robbe et al. [20], and one function of phosphoinositides is to recruit the protein to its site of action. This is consistent with the fact that the N-terminal PH domain can be largely replaced by the myristoylated membrane localization domain of c-Src [42].

The PtdIns(3,4,5)P_3 stimulatory effect on Tiam1 is inhibited by including PtdIns(4,5)P_2 in the *in vitro* exchange assay (Figure 5). This not only indicates that these lipids compete at the same binding site, but also demonstrates that activation of Tiam1 is functionally distinct from its translocation to plasma membranes. Stimulation is decreased approx. 50% by equimolar amounts of PtdIns(4,5)P_2, and is completely inhibited by a 10–100-fold excess of this lipid (Figure 5). This agrees with binding studies that indicate that Tiam1 only binds to PtdIns(3,4,5)P_3 approx. 3–5-fold better than PtdIns(4,5)P_2 [13,22]. Therefore, since insulin- or PDGF-stimulated cells typically contain a 50–100-fold excess of PtdIns(4,5)P_2, it is not immediately apparent how PtdIns(3,4,5)P_3 can activate Vav [15] or Tiam1 [13,36] signalling to Rac1 *in vivo*. This apparent contradiction may be explained by two possibilities. First, the *in vitro* binding experiments may underestimate the preference of the Tiam1 N-terminal PH domain for PtdIns(3,4,5)P_3. Alternatively, PI 3-kinase may be stimulated only in select locations *in vivo*, resulting in local regions of the plasma membrane that are highly enriched in PtdIns(3,4,5)P_3, and depleted in PtdIns(4,5)P_2, allowing GTP-loading of Rac1 to take place.

Tiam1 is regulated by protein phosphorylation [23–25], as well as by phosphoinositides. CamKII stimulates its GDP/GTP-exchange activity [24] (Figure 5), and promotes translocation to the plasma membrane [25]. Significantly, the CamKII stimulatory effect is not affected by 10–1000 μM PtdIns(4,5)P_2 (Figure 5). Thus when PtdIns(4,5)P_2 tethers Tiam1 at the plasma membrane, it sustains a low GDP/GTP-exchange activity (Figure 3) that can either be enhanced by CamKII phosphorylation or phosphorylation of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Although these activation mechanisms both result in stimulation of Tiam1, the biological consequences of these different mechanisms are not yet clear. An attractive hypothesis is that they stimulate different biological processes: thus PtdIns(3,4,5)P_3 may stimulate the cytoskeletal-associated functions of Rac1, while protein phosphorylation may enhance the transcriptional activation functions of Rac1 [43]. Therefore Tiam1 and related exchange factors probably integrate a range of diverse signals to control the activity state of the various biological processes that are regulated by Rho-family GTPases.

We thank Dr J. Collard for providing the DNA construct encoding GST–C1199-Tiam1 and Professor A. Hall for constructs expressing GST–Rac1 and GST–PBD from PAK in *E. coli*.

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Received 1 June 2004/2 July 2004; accepted 9 July 2004
Published as BJ Immediate Publication 9 July 2004, DOI 10.1042/BJ20040916