Regulation of the Rac1-specific exchange factor Tiam1 involves both phosphoinositide 3-kinase-dependent and -independent components

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The small GTPase Rac1 is involved in regulating membrane ruffling, gene transcription, cell-cycle progression and cell transformation, and some of these events are blocked by inhibitors of phosphoinositide 3-kinase (PI 3-kinase). Moreover, Rac1 can be activated by several guanine nucleotide exchange factors, which facilitate the release of GDP. We therefore investigated the ability of PI 3-kinase lipid products to regulate Tiam1, a Rac1-specific exchange factor. Tiam1 bound to polyphosphorylated inositol lipids in the rank order $\text{PtdIns}(3,4,5)\text{P}_3 > \text{PtdIns}(3,4)\text{P}_2 \gg \text{PtdIns}(4,5)\text{P}_2$, and this binding could be attributed to the N-terminal pleckstrin-homology (N-PH) domain. Both $\text{PtdIns}(3,4,5)\text{P}_3$ and $\text{PtdIns}(3,4)\text{P}_2$ enhanced Tiam1 guanine nu-

cleotide exchange activity *in vitro*, but $\text{PtdIns}(4,5)\text{P}_2$ had no effect. Co-expression of a constitutively active PI 3-kinase with Tiam1 increased the amount of GTP-bound Rac1 *in vivo*, a response which required the N-PH domain of Tiam1. Ectopic expression of Tiam1 caused membrane ruffling in Swiss 3T3 cells that was characterized by wortmannin-sensitive and -insensitive components, which required the N-PH domain and the C-terminal PH domain of Tiam1 respectively. These results reveal novel facets of Tiam1-dependent regulation of Rac1 function.

Key words: membrane ruffling, phosphatidylinositol 3,4,5-trisphosphate, pleckstrin-homology domains, Rac1.

INTRODUCTION

The Rho family of small GTPases regulate a wide range of key cellular processes. In fibroblasts, Rac1 plays a critical role in membrane ruffling and lamellipodia formation [1,2], Cdc42 is important in the formation of actin microspikes and filopodia [2], and Rho is involved in the formation of actin stress fibres and focal adhesions [2–4]. Rho proteins also promote gene transcription: Rac1 and Cdc42 stimulate Jun kinase and p38 mitogen-activated protein kinase [5–8], leading to activation of c-jun, ATF-2 and Elk-1 transcription factors, whereas Rac1, Cdc42 and RhoA can stimulate the serum response factor and nuclear factor κ B transcription factors [9,10]. In addition, Rho-family GTPases are important regulatory factors in cell-cycle progression [11], enhance Ras-triggered transformation of NIH3T3 fibroblasts [12,13] and are required in the NADPH oxidase-mediated phagocytic response in neutrophils [14].

Rho-family GTPases cycle between GTP-bound (active) and GDP-bound (inactive) states, and their activity state is influenced by three groups of regulatory proteins. Rho-GDI proteins interact with Rho GTPases, inhibiting GDP dissociation [15] and GTP hydrolysis [16], as well as contributing to retention of the GTPases in the cytosol [17]. A number of Rho-GTPase-activating proteins ('Rho-GAPs') have also been identified: these proteins enhance the intrinsic GTPase activity of Rho proteins, probably by extending an arginine finger into the active site of the GTPase [18]. Finally a large family of guanine nucleotide exchange factors has recently been discovered [19] that promote binding of GTP to Rho-family GTPases by facilitating the release of GDP.

All Rho-family exchange factors contain a Dbl homology (DH) domain and at least one pleckstrin homology (PH) domain. This family of proteins is only weakly conserved within the DH domain (10–30% identity) which catalyses $\text{GDP} \leftrightarrow \text{GTP}$ exchange, and minimally conserved outside this domain. The PH domain(s) may play an important role in the subcellular targeting or activation of these proteins, probably through interaction with lipids [20,21] and/or $\beta\gamma$ subunits of heterotrimeric G-proteins ($\text{G}\beta\gamma$) [22]. In addition several exchange factors contain SH2 (Src homology 2), SH3 or zinc-finger domains [19], which may also mediate protein–protein or protein–lipid interactions.

It is becoming clear that a number of different factors are involved in the regulation of Rho exchange factors. The Rho/Rac GTP exchange factors Vav [23] and Vav-2 [24] are both phosphorylated by Lck (an src-family tyrosine kinase) on tyrosine, stimulating GDP/GTP nucleotide exchange on Rac1 and RhoA-like GTPases respectively. Moreover, the Rac1-specific exchange protein Tiam1 is phosphorylated by several different agonists in Swiss 3T3 fibroblasts, via activation of protein kinase C (PKC) and Ca^{2+} /calmodulin-dependent protein kinase II (CamKII) [25–27], and phosphorylation of Tiam1 by CamKII enhances its nucleotide exchange activity towards Rac1 [27]. On the other hand, the Rho-specific exchange protein p115 directly associates with, and is activated by, $\text{G}_{13}\alpha$ [28], whereas Dbl associates with $\text{G}\beta\gamma$ subunits, although this does not stimulate its GDP/GTP exchange activity *in vitro* [22].

The fact that all Rho exchange factors possess closely associated PH and DH domains, suggests that intercommunication between these motifs is likely to be important in their regulation. Therefore, since inositol phospholipids can bind to PH domains

Abbreviations used: CamKII, Ca^{2+} /calmodulin-dependent protein kinase II; C-PH, C-terminal pleckstrin-homology domain; Δ C-PH-Tiam1, Tiam1 (a Rac1-specific exchange factor) with C-PH removed; GFP, green fluorescent protein; GST, glutathione S-transferase; N-PH, N-terminal PH domain; Δ N-PH-Tiam1, Tiam1 with N-PH removed; PBD, p21 (Cdc42/Rac1)-binding domain; PDGF, platelet-derived growth factor; Δ -PH-Tiam1, Tiam1 with both PH domains removed; PKC, protein kinase C; PI 3-kinase, phosphoinositide 3-kinase; SAL-PtdIns(3,4,5) P_3 , sn-1-stearoyl-2-arachidonoyl L -PtdIns(3,4,5) P_3 ; SAD-PtdIns(3,4,5) P_3 , sn-1-stearoyl-2-arachidonoyl D -PtdIns(3,4,5) P_3 ; DH, Dbl homology; ECL, enhanced chemiluminescence; Sf9, *Spodoptera frugiperda* (fall armyworm) cells; PLC, phospholipase C; GTP[S], guanosine 5'-[γ -thio]triphosphate; ARNO, ADP-ribosylation factor nucleotide-binding site opener; PAK, p21-activated kinase.

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[20,21], these lipids could influence the activity or localization of some Rho exchange proteins, and this could be one mechanism by which PtdIns(3,4,5) P_3 plays its central role in Rac1-mediated membrane ruffling [29]. Indeed, PtdIns(3,4) P_2 and PtdIns(3,4,5)- P_3 activate, and PtdIns(4,5) P_2 inhibits, Vav *in vitro*, whereas PtdIns(3,4,5) P_3 enhances Lck-catalysed phosphorylation of Vav [21]. However, little is known concerning the regulation of Rho exchange proteins by inositol phospholipids in the cell. We show here that phosphoinositide 3-kinase (PI 3-kinase) lipid products can activate the Rac1-specific exchange factor Tiam1 both *in vitro* and *in vivo*, via a mechanism that requires the N-terminal PH (N-PH) domain. This mechanism is masked in full-length Tiam1, which can also be activated by a PI 3-kinase-independent process that requires the C-terminal PH (C-PH) domain.

EXPERIMENTAL

Materials

HEK-293 cells were obtained from the American Type Culture Collection. Penicillin and streptomycin were from Life Technologies, Inc. Foetal bovine serum, modified Eagle's medium, sodium orthovanadate, leupeptin, antipain, PMSF, NaF, sodium pyrophosphate, Tween-20, Triton X-100, wortmannin, antibody to glutathione S-transferase (GST), GSH-agarose beads and fatty-acid-free BSA were obtained from Sigma. Antibody to Tiam1 was from Santa Cruz. Antibody to Rac1 was from Upstate Biotechnology. GDP and GTP were from Boehringer-Mannheim. [^{3} H]GDP was from DuPont-NEN. Nitrocellulose filters were from Whatman. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidic acid were from Avanti Polar Lipids. Dipalmitoyl PtdIns(3,4,5) P_3 , dipalmitoyl PtdIns(3,4) P_2 and all other synthetic phosphoinositides were from Echelon Research Laboratories (Salt Lake City, UT, U.S.A.). *sn*-1-Stearoyl-2-arachidonoyl D- and L-isomers of PtdIns(3,4,5) P_3 were synthesized as described in [30]. Plasmids for expression of GST-Rac1 and GST-p21 (Cdc42/Rac1)-binding domain (PBD) of p21-activated kinase (PAK) in *Escherichia coli* were kindly given by Professor A. Hall (MRC Laboratory for Molecular Cell Biology, University College London, London, U.K.). Plasmid for expression of CD2-tagged PI 3-kinase was from Dr D. Cantrell (Imperial Cancer Research Fund, London, U.K.), and the inactive Δ p85 subunit of PI 3-kinase was from Dr M.C. Frame (Beatson Institute, Bearsden, Glasgow, Scotland, U.K.).

Preparation of Tiam1 constructs

PCR primers were designed, as described previously [31], to amplify the N-PH (amino acids 403–572) and C-PH (amino acids 1255–1400) domains from a Tiam1 template, with flanking *Bam*HI and *Sal*I restriction sites, to allow directional cloning into appropriate expression vectors. Each construct was verified by nucleic acid sequencing, then subcloned into a pGEX-4T vector (Pharmacia) to allow expression of GST-tagged proteins in *E. coli*, and into a pEGFP vector (Clontech), to allow expression of green fluorescent protein (GFP)-tagged PH domains in mammalian cells for fluorescence studies.

Three Tiam1 PH domain deletion mutants were constructed from a Tiam1 template lacking the N-terminal 392 amino acids (C1199-Tiam1) [32] by PCR. An N-terminal PH deletion mutant (Δ N-PH-Tiam1) was prepared by deleting the DNA encoding amino acids 403–572, and a C-terminal PH deletion mutant (Δ C-PH-Tiam1) prepared by deleting the bases encoding amino acids 1255–1400. These constructs were verified by nucleic acid se-

quencing, and a PH-less Tiam1 mutant (Δ -PH-Tiam1) was then prepared by digesting the single PH domain mutants with *Pst*I and *Xba*I, and ligating the DNA encoding amino acids 392–1101 of Δ N-PH-Tiam1 to the nucleotides encoding amino acids 1101–1599 of Δ C-PH-Tiam1. These three Tiam1 deletion mutants, and C1199-Tiam1, were subcloned into the pEGFP vector for fluorescence studies, and into pMT2SM (Pharmacia) to allow expression of GST-tagged Tiam1 proteins in mammalian cells. Western-blotting analysis confirmed that all of the constructs were expressed in 293 cells, and were of the correct molecular mass (results not shown). The various GST-tagged proteins were purified using GSH-agarose beads.

Cell-culture conditions

HEK-293 and Swiss 3T3 cells were maintained in modified Eagle's medium supplemented with 4 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C in a humidified atmosphere of CO₂/air (1:19). For the GFP localization and membrane-ruffling studies, cells were plated out in 35 mm-diameter plates containing coverslips, and FuGene 6-mediated transfection carried out using 1 μ g of the indicated Tiam1 construct according to the manufacturers instructions (Roche Diagnostics Ltd.). Cells were washed twice with PBS, fixed for 30 min with PBS containing 4% (w/v) paraformaldehyde, rinsed once with PBS, then carefully mounted on to a drop of Mowiol 40-88 (Aldrich) on a slide. The slides were dried at room temperature for 2 h and analysed by confocal microscopy.

For the Rac1-activation assay, cells were seeded at approx. 25% confluency on 100 mm-diameter dishes. After 2 h, 10 μ g of each DNA construct was transfected into the appropriate plate using calcium phosphate transfection [33] and the cells allowed to grow for 48 h before harvesting.

Membrane-ruffling assay

At 12 h after transfection, Swiss 3T3 cells were serum-starved for 4 h in Dulbecco's minimal essential medium containing 0.5% BSA and subsequently transferred to modified Krebs buffer [25 mM Hepes (pH 7.4)/118.4 mM NaCl/4.7 mM KCl/1.18 mM MgSO₄/1.29 mM CaCl₂/1.18 mM K₂HPO₄/11.7 mM glucose] before stimulation with 10 ng/ml platelet-derived growth factor (PDGF) for 10 min at 37 °C. Cells were then fixed in 3% paraformaldehyde solution in PBS for 10 min, washed three times in PBS, permeabilized with 1% Triton X-100 in PBS for 5 min, then washed three times in PBS before staining polymerized actin with 0.1 μ g/ml rhodamine phalloidin (Sigma Ltd.) in PBS for 5 min. Cells were then washed three times in PBS and mounted for confocal microscopy. Confocal images of the cells were collected using a Bio-Rad confocal-microscope system.

Rac1 activation assay

The Rac1 activation assay was done essentially as described in [34,35]. The p21 PBD of PAK was expressed in *E. coli* as a GST fusion protein, purified using GSH beads and used to precipitate GTP-bound Rac1 from cell lysates.

Transfected HEK-293 cells were washed with 5 ml of PBS and scraped into 0.5 ml of lysis buffer [1% Nonidet P40/150 mM NaCl/50 mM Tris (pH 7.4)/10% (v/v) glycerol/2.5 mM MgCl₂/10 μ g/ml antipain/leupeptin/1 mM PMSF/500 μ M sodium orthovanadate/10 mM pyrophosphate/10 mM NaF/1 mM dithiothreitol]. Cells were lysed using a Jencons sonicator at 40% power setting for two 5 s bursts, then clarified by centrifugation.

Figure 1 Interaction of various Tiam1 mutants and the PLC δ 1-PH domain with inositol phospholipids

Fat Western blotting was carried out as described in the Experimental section, using various amounts (3–100 pmol) of the indicated phosphoinositides. Proteins analysed were C1199-Tiam1 (**A**), PLC δ 1-PH domain (**B**), Tiam1 N-terminal PH domain (**C**), Tiam1 C-PH domain (**D**) or Δ C-PH-Tiam1 (**E**). Lipids used in these experiments contained dipalmitoyl fatty acids and were: (1) PtdIns; (2) PtdIns3P; (3) PtdIns4P; (4) PtdIns5P; (5) PtdIns(3,4)P₂; (6) PtdIns(3,5)P₂; (7) PtdIns(4,5)P₂; (8) PtdIns(3,4,5)P₃. Results are representative for at least three independent experiments.

gation at 12000 *g* for 10 min. Each supernatant was incubated with 10 μ g of immobilized GST-PBD for 1 h at 4 °C. Beads were collected by centrifugation (2000 *g*, 5 min), washed four times in lysis buffer, resuspended in 30 μ l of Laemmli sample buffer and the amount of precipitated Rac1 analysed by Western blotting.

SDS/PAGE and Western-blot analysis

SDS/PAGE was performed on 6% or 12% (w/v) polyacrylamide gels and proteins transferred on to PVDF membranes (Millipore) for 1 h at 14 V using a semi-dry transfer unit. The membranes were blocked overnight with 5% (w/v) non-fat dried milk. For the Tiam1 blots, antibody was used at a dilution of 1:2000 in TTBS [50 mM Tris (pH 8.0)/150 mM NaCl/0.1% Tween 20], containing 1% BSA, for 1 h. Rac1 antibody was used at a dilution of 1:500 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 (Scottish Antibody Production Unit, Law Hospital, Carlisle, Lanarkshire, Scotland, U.K.) prior to development using an enhanced chemiluminescence kit (ECL®; Amersham Corp.).

Fat Western blotting

The lipid-binding properties of proteins were assessed using the relatively new fat Western method of Stevenson et al. [36], with some modifications. Lipids were solubilized in chloroform/methanol/water (5:11:4, by vol.) and 1 μ l aliquots, containing various amounts of the indicated lipids, were spotted on to Hybond-C extra nitrocellulose membrane and allowed to dry at room temperature for 1 h. The membrane was blocked in 2% fatty acid-free BSA in TTBS for 1 h, then incubated overnight in the same buffer, containing 0.1 μ g/ml of the indicated protein of interest, at 4 °C. Membranes were either incubated for 1 h with Tiam1 (diluted 1:2000) or GST (diluted 1:1000) antibody in TTBS containing 1% BSA, then for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody, and bound proteins were detected using ECL®.

Tiam1 exchange assay

C1199-Tiam1, with an N-terminal hexahistidine tag, was expressed in Sf9 (*Spodoptera frugiperda*) cells and purified using Talon metal-affinity resin (Clontech) in a 25 mM Tris buffer, pH 8.0, containing 0.5 μ M β -mercaptoethanol and 100 mM NaCl. Tiam1 was eluted from the beads using 100 mM imidazole and dialysed prior to freezing. GST-Rac1 was expressed in *E. coli*, and purified using GSH-agarose beads in a 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 exchange assay was carried out as described previously [27]. Purified GST-Rac1 (120 pmol) was preloaded with [³H]GDP (30 μ M; 25 Ci/mmol) in 60 μ l of binding buffer. An 8 μ l portion of the preloaded GTPase was added to 32 μ l of exchange mixture, which contained 5 pmol of Tiam1 or BSA, and 1 mM GTP in exchange buffer. At the indicated times, 8 μ l aliquots were pipetted into 1 ml of stopping buffer [50 mM Tris (pH 7.4)/5 mM MgCl₂/50 mM NaCl], and the [³H]GDP bound to Rac1 was analysed by filtering the mixture through nitrocellulose. Tiam1 assays were carried out in the presence of the indicated lipid vesicles to determine their effect on exchange

activity. The amount of [^3H]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 [^3H]GDP which is released from Rac1 during the assay.

RESULTS

The various Tiam1 deletion mutants were expressed in HEK-293 cells, and the isolated Tiam1 PH domains expressed in *E. coli* as GST-fusion proteins. The proteins were purified by affinity chromatography on GST beads, and migrated at the expected molecular mass on SDS/PAGE (results not shown). The lipid-binding specificity of the Tiam1 mutants was investigated by Fat Western blotting [36], using a series of phospholipids, over a range of different concentrations. C1199-Tiam1 did not bind to phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine or phosphatidic acid (results not shown). On the other hand, inositol phospholipids did interact with C1199-Tiam1: the enzyme bound preferentially to $\text{PtdIns}(3,4,5)\text{P}_3$, but also interacted with $\text{PtdIns}(3,4)\text{P}_2$ and, to a lesser extent, $\text{PtdIns}(4,5)\text{P}_2$ (Figure 1A). In contrast, the PH domain of the $\text{PtdIns}(4,5)\text{P}_2$ -hydrolysing enzyme, phospholipase $\delta 1$ (PLC $\delta 1$), bound preferentially to $\text{PtdIns}(4,5)\text{P}_2$ (Figure 1B), demonstrating the distinctive lipid-binding properties of Tiam1.

The N-terminal PH domain of Tiam1 also bound to inositol phospholipids (Figure 1C), with the same binding affinity and specificity as C1199-Tiam1. Intriguingly, the C-PH domain of Tiam1 also bound to these lipids (Figure 1D), with the same

binding specificity as Tiam1, but this interaction was only detected at relatively high lipid concentrations, indicating that the C-PH domain interacts more weakly than the N-PH domain or C1199-Tiam1. Significantly, the $\Delta\text{C-PH-Tiam1}$ mutant (Figure 1E) bound to the different inositol phospholipids with the same pattern as Tiam1 and the N-PH domain of Tiam1, whereas the $\Delta\text{N-PH-Tiam1}$ and $\Delta\text{PH-Tiam1}$ proteins showed no detectable lipid binding (results not shown). Hence these data indicate that the high-affinity inositol-lipid-binding capability of Tiam1 is accounted for by the N-PH domain.

Several different signalling proteins can be recruited to the membrane fraction of cells by inositol phospholipids, and PH domains often play a role in this. We therefore used the Tiam1 mutants to investigate the function of each PH domain in the subcellular localization of Tiam1. Subcellular fractionation of transfected 293 cells (results not shown) and confocal microscopy using a GFP-tagged protein (Figure 2A), confirm that C1199-Tiam1 is predominantly localized in the membrane fraction of growing cells [25]. On the other hand, the isolated N-PH (Figure 2G) and C-PH (Figure 2H) domains of Tiam1 were found in the nucleus and the cytosol and did not translocate to the membrane upon stimulation of HEK-293 cells with 1 μM insulin for 10 min or serum for 30 min (results not shown). Moreover, the $\Delta\text{N-PH-Tiam1}$ (Figure 2E) and $\Delta\text{PH-Tiam1}$ (Figure 2F) deletion mutants were also largely cytosolic, whereas the $\Delta\text{C-PH-Tiam1}$ protein (Figure 2C) was mainly membrane-associated. Therefore, whereas the individual PH domains alone are not sufficient for membrane localization, the N-PH domain, but not the C-PH

Figure 2 Membrane localization of Tiam1 requires the N-terminal pleckstrin homology domain, but not PI 3-kinase products

Transfected HEK-293 cells were fixed with paraformaldehyde, mounted with Mowiol and analysed by confocal microscopy as described in the Experimental section. Some cells (B and D) were pretreated with wortmannin (100 nM for 30 min) prior to being fixed. Cells were transfected with GFP-C1199-Tiam1 (A and B), GFP- $\Delta\text{C-PH-Tiam1}$ (C and D), GFP- $\Delta\text{N-PH-Tiam1}$ (E), GFP- $\Delta\text{PH-Tiam1}$ (F), GFP-N-PH of Tiam1 (G) or GFP-C-PH of Tiam1 (H). Results are representative for at least three different experiments.

Figure 3 Effect of inositol phospholipids on the GDP $\leftrightarrow$ GTP exchange activity of Tiam1

Nucleotide exchange reactions were carried out as described in the Experimental section, using [^{3} H]GDP loaded GST-Rac1 as substrate, for 25 min. Exchange assays were carried out with and without Tiam1 in the presence of phosphatidylcholine (PC)/phosphatidylserine (PS)/polyphosphoinositide vesicles (70:27:3 molar ratio). Assays were carried out in the presence of vesicles containing 10 μ M PtdIns(3,4) P_2 (PI34P2), PtdIns(4,5) P_2 (PI45P2) or PtdIns(3,4,5) P_3 (PI345P3) or no inositol phospholipid (**A**). Experiments were also done using vesicles containing 10 μ M PtdIns(4,5) P_2 (PI45P2) and PtdIns(3,4,5) P_3 (PI345P3) (**B**). 'GDP released (%)' is the percentage of [^{3} H]GDP which is released from [^{3} H]GDP-Rac1 during the assay. Results are means $\pm$ S.E.M. for seven (**A**) or three (**B**) independent experiments.

domain, contains an important element for localizing Tiam1 to the membrane. Pretreatment with wortmannin (100 nM for 30 min), however, had no effect on the subcellular distribution of these Tiam1 proteins (Figure 2), suggesting that 3-phosphorylated inositol phospholipids either do not have a major function in recruiting Tiam1 to the membrane, or that there is an alternative mechanism which can also carry out this function.

Guanine nucleotide exchange activity was assessed *in vitro* by monitoring the dissociation of [^{3} H]GDP from GST-Rac1 in the presence and absence of purified hexahistidine-tagged Tiam1

Figure 4 Various PtdIns(3,4,5) P_3 molecules with different fatty acid chains interact with and activate Tiam1

Fat Western blotting was carried out with C1199-Tiam1 as described in the Experimental section, using various amounts (3–30 pmoles) of the indicated phosphoinositides (**A**). Lipids used in the experiments were: (1) *sn*-1,2-dipalmitoyl PtdIns(3,4,5) P_3 (16:0); (2) SAL-PtdIns(3,4,5) P_3 ; (3) SAD-PtdIns(3,4,5) P_3 ; (4) *sn*-1,2-dioleoyl PtdIns(3,4,5) P_3 (18:2). (**B**) Nucleotide exchange assays were carried out, as described in the Experimental section, in the presence of phosphatidylcholine (PC)/phosphatidylserine (PS)/polyphosphoinositide vesicles (70:27:3 molar ratio) containing 10 μ M of the indicated PtdIns(3,4,5) P_3 , or no inositol phospholipid. 'GDP released (%)' is the percentage of [^{3} H]GDP which is released from [^{3} H]GDP-Rac1 during the assay. Results are representative for three independent experiments (**A**) or the means $\pm$ S.E.M. for three independent experiments (**B**).

[27]. Inositol phospholipids were introduced to exchange assays as phosphatidylcholine/phosphatidylserine/polyphosphoinositide vesicles (70:27:3 molar ratio). Assays were carried out in the presence of vesicles to give a final concentration of 10 μ M PtdIns(3,4) P_2 , PtdIns(4,5) P_2 or PtdIns(3,4,5) P_3 , or no inositol phospholipid. In the absence of Tiam1, inositol phospholipids had no effect on the release of [^{3} H]GDP from Rac1 (Figure 3A). This basal rate of [^{3} H]GDP release from GST-Rac1 was enhanced by addition of Tiam1 (Figure 3A), as shown previously [27]. Moreover, inclusion of PtdIns(3,4) P_2 or PtdIns(3,4,5) P_3 in the lipid vesicles enhanced the GDP $\leftrightarrow$ GTP exchange activity of Tiam1 approx. 2-fold compared with control vesicles (Figure 3A). In contrast, PtdIns(4,5) P_2 had no effect on Tiam1 exchange activity *in vitro* (Figure 3A), and inclusion of PtdIns(4,5) P_2 in PtdIns(3,4,5) P_3 -containing vesicles had no effect on the PtdIns(3,4,5) P_3 -dependent activation of Tiam1 (Figure 3B).

Figure 5 PI 3-kinase products enhance activation of Rac1 by Tiam1 *in vivo*

(A) HEK-293 cell lysates were incubated with 1 mM GDP or 100 μ M GTP[S] ('GTP γ S'). (B) HEK-293 cells were transiently transfected with: (1) empty vector; (2) CD2-PI 3-kinase; (3) C1199-Tiam1 + inactive p85; (4) C1199-Tiam1 + CD2-PI 3-kinase + wortmannin; (5) C1199-Tiam1 + CD2-PI 3-kinase; or (6) C1199-Tiam1. GTP-bound Rac1 was affinity-precipitated with GST-PBD, as described in the Experimental section, and the amount of precipitated Rac1 assessed by Western blotting. Results are representative for three independent experiments. The 47 kDa band in the above blots is probably due to interaction of the secondary antibody with an unidentified protein which co-precipitates with the GST-CRIB domain.

These data therefore show that the 3-phosphorylated inositol lipids PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 directly and selectively enhance the GDP $\leftrightarrow$ GTP exchange activity of Tiam1 *in vitro*.

Several PtdIns(3,4,5) P_3 phospholipids with different fatty-acid chains were used to investigate further the nature of the PtdIns(3,4,5) P_3 effect (Figure 4). Tiam1 bound to all of the PtdIns(3,4,5)- P_3 molecules tested (Figure 4A), with the enzyme binding best to PtdIns(3,4,5) P_3 species containing C_{18:2} and C_{16:0} fatty acid chains, and less well to *sn*-1-stearoyl-2-arachidonoyl D-PtdIns(3,4,5) P_3 [SAD-PtdIns(3,4,5) P_3] and *sn*-1-stearoyl-2-arachidonoyl L-PtdIns(3,4,5) P_3 [SAL-PtdIns(3,4,5) P_3]. Interestingly, unlike protein kinase B [37], Tiam1 could not differentiate between the SAD-PtdIns(3,4,5) P_3 and SAL-PtdIns(3,4,5) P_3 enantiomers (Figure 4A). All of these different PtdIns(3,4,5) P_3 molecules were able to enhance the exchange activity of Tiam1 to a similar extent (Figure 4B), indicating that the enantiomeric configuration of the inositol headgroup, or the length and saturation of the fatty acid chains, has a limited influence on this PtdIns(3,4,5) P_3 effect.

The GST-tagged PBD from PAK was used to estimate Rac1 activation *in vivo*. This domain binds specifically to the GTP-liganded form of Rac1 and Cdc42, allowing the activated form of these GTPases to be selectively precipitated from cell lysates [34,35]. HEK-293 cell lysates were incubated with 1 mM GDP or 100 μ M guanosine 5'-[γ -thio]triphosphate (GTP[S]) to preload Rho-family GTPases with these nucleotides, then affinity pre-

Figure 6 PI 3-kinase products stimulate Tiam1 GDP $\leftrightarrow$ GTP exchange activity *in vivo* via the N-PH domain

HEK-293 cells were transiently transfected with (1) C1199-Tiam1, (2) Δ N-PH-Tiam1, (3) Δ C-PH-Tiam1 or (4) Δ PH-Tiam1 in the presence and absence of CD2-PI 3-kinase. (A) Expression of the various mutants was assessed by probing lysates (15 μ g) with antibody to Tiam1. (B) GTP-bound Rac1 was affinity-precipitated with GST-PBD, as described in the Experimental section, and the amount of precipitated Rac1 determined by Western blotting. Results are representative for two independent experiments.

cipitated with GST-PBD, and the amount of Rac1 present in the pellet assessed by Western blotting. The results (Figure 5A) demonstrate that the PBD associates with Rac1 preloaded with GTP[S], but not GDP, confirming that the protein only interacts with GTP-bound Rac1.

HEK-293 cells were transiently transfected with Tiam1 in the presence and absence of a constitutively active PI 3-kinase (CD2-tagged PI 3-kinase), or a dominant negative Δ p85 subunit of PI 3-kinase, to ascertain the effect of 3-phosphorylated inositol lipids on Tiam1 activity *in vivo*. Western blotting with anti-CD2, anti-Tiam1 and anti-p85 antibodies confirmed that each of the constructs was efficiently expressed in the appropriate lysate (results not shown). Expression of the constitutively active PI 3-kinase alone had no detectable effect on Rac1 activation (Figure 5B). Expression of C1199-Tiam1 caused a small, but variable, increase in GTP-bound Rac1, which was consistently enhanced by co-expression with the active PI 3-kinase (Figure 5B). This effect was abolished by preincubating the cells with wortmannin (100 nM for 30 min) and was not detected when Tiam1 was co-expressed with the inactive Δ p85 PI 3-kinase subunit (Figure 5B). These data demonstrate that PI 3-kinase products are important regulators of the Rac1 exchange factor, Tiam1, *in vivo*.

To elucidate further the molecular mechanisms by which PI 3-kinase products activate Rac1, the various Tiam1 deletion mutants were transfected into HEK-293 cells, in the presence and absence of the CD2-PI 3-kinase. Western blotting confirmed that all of the Tiam1 mutants were expressed at similar levels in the lysates, and were of the expected molecular mass (Figure 6A). Expression of the Tiam1 mutants alone caused little or no activation of Rac1, whereas co-expression of the PI 3-kinase with C1199-Tiam1 or Δ C-PH-Tiam1 caused a significant activation of

Figure 7 Effect of wortmannin on Tiam1-induced membrane ruffling

Transfected Swiss 3T3 cells were fixed and analysed by confocal microscopy as described in the Experimental section. Some cells (**B** and **D**) were pretreated with wortmannin (100 nM for 30 min) prior to being fixed. Cells were transfected with GFP-C1199-Tiam1 (**A** and **B**), GFP-ΔC-PH-Tiam1 (**C** and **D**), GFP-ΔN-PH-Tiam1 (**E**) or GFP-ΔPH-Tiam1 (**F**). Each slide was analysed in the green channel for GFP-expressing cells (left-hand panel), and in the red channel for rhodamine phalloidin staining of polymerized actin (right-hand panel). In (**A**)–(**D**) asterisks have been used to highlight transfected cells, and triangles to indicate membrane ruffles. Overexpression of Tiam1 causes a characteristic pattern of membrane ruffling around the cell periphery (**A**–**C**). The phalloidin staining pattern observed in (**D**) does not highlight membrane ruffling. Results are representative for at least two independent experiments.

Table 1 Effect of Tiam1 mutants and PI 3-kinase inhibitors on membrane ruffling

Swiss 3T3 cells were transfected with the indicated Tiam1 mutant, fixed, and analysed for GFP-expressing cells and membrane ruffling by confocal microscopy, as described in the Experimental section. Some cells were pretreated with wortmannin (100 nM for 30 min) or LY294002 (10 μ M for 30 min) before being fixed. For each Tiam1 mutant, the percentage of GFP-expressing cells which contain discernible membrane ruffles was scored.

Construct	Percentage of transfected cells ruffling (%)		
	Control	+ Wortmannin	+ LY294002
C1199-Tiam1	87	84	88
ΔN-PH-Tiam1	28		
ΔC-PH-Tiam1	85	33	28
ΔPH-Tiam1	17		
GFP control	20		

Rac1 in the GST-PBD precipitate (Figure 6B). No activation of Rac1 was detected, however, when the PI 3-kinase was co-expressed with ΔN-PH-Tiam1 or ΔPH-Tiam1 (Figure 6B). Thus the N-PH domain is required for this effect, consistent with the idea that PI 3-kinase products stimulate Tiam1 exchange activity by interacting with the N-PH domain.

PtdIns(3,4,5) P_3 plays an important role in Rac1-dependent membrane ruffling [29]. Since transfection of Tiam1 into NIH3T3

fibroblasts induces Rac1-mediated membrane ruffling [38], and PtdIns(3,4,5) P_3 binds to the N-PH domain of Tiam1 (Figure 1), stimulating exchange activity *in vitro* (Figures 3 and 4) and *in vivo* (Figures 5 and 6), this lipid could regulate ruffling through its binding to, and activation of, Tiam1. To address this possibility, GFP-tagged Tiam1 deletion mutants were transiently transfected into Swiss 3T3 fibroblasts and their effects on membrane ruffling were analysed. As expected [38], transfection of C1199-Tiam1 into Swiss 3T3 cells stimulated membrane ruffling (Figure 7A). ΔC-PH-Tiam1 also induced ruffling (Figure 7C), but ΔN-PH-Tiam1 (Figure 7E) and ΔPH-Tiam1 (Figure 7F) did not, confirming that the N-PH domain is required for this response [32]. Significantly, preincubation with the PI 3-kinase inhibitor wortmannin had no effect on C1199-Tiam1-induced membrane ruffling (Figure 7B), but clearly abolished ruffling stimulated by the ΔC-PH-Tiam1 mutant (Figure 7D).

These data were further analysed by scoring the percentage of transfected cells (those expressing GFP) which show discernible membrane ruffles (Table 1). C1199-Tiam1 and ΔC-PH-Tiam1 induced membrane ruffling in approx. 85% of Swiss 3T3 cells expressing GFP-tagged protein, whereas ruffling was only observed in about 20% of cells transfected with a control GFP vector (Table 1). Moreover, ΔN-PH-Tiam1 and ΔPH-Tiam1 only stimulated ruffling in about 20–30% of GFP-labelled cells (Table 1), i.e. equivalent to GFP-labelled cells. Furthermore, whereas wortmannin and LY294002 had no effect on the proportion of ruffling cells expressing C1199-Tiam1, these inhibitors decreased the percentage of ruffling cells from 85% to

approx. 30 % in Δ C-PH-Tiam1-expressing cells (Table 1). These data indicate that the Δ C-PH-Tiam1 mutant requires PI 3-kinase products to stimulate membrane ruffling, but that C1199-Tiam1 does not. One possible explanation is that PtdIns(3,4,5) P_3 enhances Tiam1 GDP/GTP exchange activity by associating with the N-PH domain, and the C-PH domain also regulates Tiam1, but through a mechanism which does not involve PtdIns(3,4,5) P_3 . Therefore Tiam1 GDP $\leftrightarrow$ GTP exchange activity may be regulated by more than one signalling pathway.

DISCUSSION

The data presented here demonstrate that inositol phospholipids bind to Tiam1 primarily via the N-PH domain, and that, of the lipids tested, PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 bind with the highest affinity (Figure 1). Furthermore, this interaction is functionally significant, since PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 enhance Tiam1 GDP $\leftrightarrow$ GTP exchange activity *in vitro* (Figures 3 and 4), and PI 3-kinase products activate Tiam1 *in vivo* (Figures 5 and 6). Moreover, wortmannin inhibits Rac1-dependent membrane ruffling stimulated by a Tiam1 mutant lacking the C-terminal PH domain (Figure 7). Since PtdIns(3,4,5) P_3 activates the Rac1 exchange factor Vav [21], this inositol phospholipid may be a general regulator for Rac1 exchange proteins.

PH motifs, which are poorly conserved modules of approx. 120 amino acids, bind a variety of ligands, including proteins and polyphosphoinositides. We show here that the N-PH of Tiam1 interacts with inositol phospholipids in the rank order, PtdIns(3,4,5) P_3 > PtdIns(3,4) P_2 $\gg$ PtdIns(4,5) P_2 (Figure 1). These data are in general agreement with the findings of Rameh et al. [20], which show that radiolabelled PtdIns(3,4,5) P_3 binds to the Tiam1 N-PH domain and can be displaced by unlabelled PtdIns(4,5) P_2 and PtdIns(3,4) P_2 , indicating that these lipids also bind. Interestingly, the isolated C-terminal PH domain of Tiam1 interacts with the same inositol phospholipids as the N-PH domain, but with a weaker affinity (Figure 1), consistent with observations made using protein-depleted bovine brain membranes as a source of inositol lipids [31]. Significantly, the N-PH domain accounts for the lipid binding capability of full-length Tiam1: the Δ C-PH-Tiam1 has the same lipid-binding properties as C1199-Tiam1 (Figure 1), whereas the Δ N-PH-Tiam1 and Δ PH-Tiam1 proteins show no detectable lipid binding. Binding of polyphosphoinositides to the C-PH domain may be an artifact of expressing this domain in isolation, or this motif could be masked in the intact Tiam1 protein, perhaps interacting with lipids only after secondary modification of the protein (e.g. protein phosphorylation).

PtdIns(3,4,5) P_3 is involved in the recruitment of a number of signalling proteins to the plasma membrane. However, the PI 3-kinase inhibitor wortmannin has no effect on the subcellular localization of C1199-Tiam1 or Δ C-PH-Tiam1 (Figure 2), indicating that 3-phosphorylated inositol lipids are not required to target Tiam1 to membranes. Indeed, whereas the N-PH and C-PH motifs clearly bind to polyphosphoinositides *in vitro* (Figure 1), neither insulin nor serum [which both increase PtdIns(3,4,5) P_3 levels in 293 cells] affect the localization of GFP-tagged N-PH or C-PH domains (results not shown). This agrees with the data of Stam et al. [39] which indicate that the isolated N-PH motif is not localized at the membrane. Therefore, whereas increased PtdIns(3,4,5) P_3 levels are sufficient to recruit the PH domain of ADP-ribosylation factor nucleotide-binding site opener (ARNO) to the membrane [40], this lipid alone does not appear to relocalize either PH domain of Tiam1.

Significantly, whereas the isolated Tiam1 N-PH domain is not itself membrane-localized, this motif is required for membrane

association of full-length Tiam1, since the Δ N-PH-Tiam1 mutant is not found in the membrane (Figure 2E). This agrees with the data of Collard and associates [32,39], which demonstrate that the N-PH domain and an adjacent protein-interaction domain are both required for membrane localization of the exchange factor. Interestingly, Wang et al. [31] suggest that the C-PH of Tiam1 can bind weakly to Cos-7 membranes. However, in HEK-293 cells, the C-PH domain is not membrane-associated, and the Δ C-PH-Tiam1 has the same cellular localization as C1199-Tiam1 (Figure 2), indicating that the C-PH motif has little or no role in Tiam1 membrane association.

Although PI 3-kinase products do not play a significant role in the recruitment of Tiam1 to the membrane (Figure 2), PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 clearly enhance the exchange activity of purified Tiam1 *in vitro*, by approximately twofold (Figures 3 and 4). The GDP/GTP exchange activity of Vav is also activated by PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 *in vitro*, by a similar magnitude [21]. However, whereas PtdIns(4,5) P_2 inhibits Vav exchange activity [21], it has no detectable effect on Tiam1 activity (Figure 3A) and does not influence the regulation of Tiam1 by PtdIns(3,4,5) P_3 (Figure 3B), perhaps because it interacts with Tiam1 much more weakly than does PtdIns(3,4,5) P_3 . Furthermore, co-transfection of an active PI 3-kinase with Tiam1 significantly enhanced the amount of GTP-bound Rac1 in HEK-293 cells, whereas overexpression of Tiam1 or PI 3-kinase alone had little or no effect on Rac1 activation (Figure 5), confirming that PI 3-kinase products also activate the enzyme *in vivo*. Indeed, Tiam1-induced migration and E-cadherin-mediated cell-cell adhesion are dependent on PI 3-kinase [41].

Removal of the Tiam1 N-PH domain, but not the C-PH domain (Figure 6B), eliminates the PtdIns(3,4,5) P_3 -dependent activation of its exchange activity. These observations, coupled with the fact that the N-PH domain accounts for the inositol-phospholipid-binding capability of Tiam1 (Figure 1), suggest that the PtdIns(3,4,5) P_3 -dependent stimulation of Tiam1 is mediated by the N-PH domain. It is not yet apparent how PtdIns(3,4,5) P_3 causes these effects, but it seems likely that it induces a key change in the conformation of these proteins. This could involve reorientation of the DH domain and PH domains [42], perhaps allowing the GTPase better access to its binding site on Tiam1. Indeed, removal of the PH domain from Vav [43], or Sos [44], produced constitutively active proteins, whereas removal of the PH motif from Trio reduced exchange activity by 100-fold [45], indicating that PH domains have an important influence on the basal GDP $\leftrightarrow$ GTP exchange activity of these enzymes. However, removal of the N-PH or C-PH domains had no detectable effect on basal Tiam1 activity *in vivo* (Figure 6B). This may partly be because the N-PH domain is necessary for membrane association (Figure 2) [32,39], and this localization is required for Tiam1 activity [32]. Alternatively, removal of the PH domains may only cause a small change in Tiam1 basal activity, which could not be detected by this assay.

In fibroblasts, membrane ruffling is stimulated by PDGF [1,3], inhibited by the PI 3-kinase inhibitor wortmannin [29] and induced by expression of an active form of PI 3-kinase [46]. G-protein-coupled receptors can also induce cytoskeletal reorganization through activation of PI 3-kinase, a guanine nucleotide exchange protein and Rac1 [43], and PDGF increases GTP loading of Rac1 in Swiss 3T3 cells through activation of a PI 3-kinase [47]. Indeed, we show here that wortmannin inhibits Δ C-PH-Tiam1-induced membrane ruffling (Figure 7 and Table 1), demonstrating that PI 3-kinase products are involved in Tiam1 activation. However, C1199-Tiam1-stimulated membrane ruffling is not affected by wortmannin (Figure 7 and Table 1), indicating that Tiam1 can also be activated by a mechanism

which does not involve PI 3-kinase products. These results could be due to subtle differences in the affinity of PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 for binding to C1199-Tiam1 and Δ C-PH-Tiam1. However a more likely hypothesis is that PtdIns(3,4,5) P_3 enhances Tiam1 GDP $\leftrightarrow$ GTP exchange activity, by associating with the N-PH domain, but that the C-PH domain also participates in regulating Tiam1 activity, via a PI 3-kinase-independent pathway. The nature of such a pathway is not clear at present, but could involve serine/threonine phosphorylation [25–27] or the $\beta\gamma$ subunits of heterotrimeric G-proteins [31].

The results presented above suggest that the N-PH domain of Tiam1 has at least two separate functions: to activate GDP $\leftrightarrow$ GTP exchange by binding to PtdIns(3,4,5) P_3 (Figure 6B) and to participate in targeting the enzyme to the membrane (Figure 2) [32,39]. While PI 3-kinase products regulate the GDP $\leftrightarrow$ GTP exchange rate of Tiam1 (Figures 3–6), they are not required for membrane association (Figure 2). One possible explanation is that the weak interaction of the N-PH domain with PtdIns(4,5) P_2 (Figure 1) co-operates with the adjacent protein-interaction domain to recruit Tiam1 to the membrane. Membrane association may allow inositol phospholipids and proteins to influence Tiam1 GDP $\leftrightarrow$ GTP exchange activity, or co-localization of Tiam1 and Rac1 at the membrane surface may aid nucleotide exchange.

It is now apparent that several signalling pathways regulate the Rac1 exchange factor Tiam1. The enzyme is directly activated by PtdIns(3,4,5) P_3 (Figure 3–6) and via threonine phosphorylation by CamKII [27]. Tiam1 is also phosphorylated by protein kinase C [25–27], and may associate with $\beta\gamma$ subunits of heterotrimeric G-proteins [31], although the function of these signals is not yet clear. Significantly, the N-PH domain and an adjacent protein interaction domain are both required for membrane localization of Tiam1 [39] and serum starvation reduces the amount of membrane-bound Tiam1 [32]. Therefore, one interesting possibility is that phosphorylation of the N-PH–protein interaction domain region of Tiam1 by protein kinase C or CamKII influences the subcellular localization of Tiam1, similar to the regulation of ARNO [48]. Indeed, CamKII facilitates relocation of Tiam1 to the membrane fraction [49], but it is not yet known which phosphorylation site(s) are involved in this process. Interestingly, the N-PH domain of Tiam1 can be functionally replaced by the myristoylated localization domain of c-Src [32]. This observation suggests that deletion of the N-PH domain either produces a constitutively active Tiam1 protein (as discussed above), or that a membrane-associated Tiam1 can be activated via a PtdIns(3,4,5) P_3 /N-PH-independent mechanism. This could involve phosphorylation by CamKII [27], or a signal which acts through the C-PH domain (as discussed above).

We suggest that Tiam1, and perhaps other large exchange proteins, integrate a variety of signals that ultimately control Rho-family-dependent signalling pathways. The N-PH domain primarily functions in membrane association and regulation by PI 3-kinase lipid products. The C-PH domain may confer PI 3-kinase-independent regulation by an as-yet-unknown mechanism. Serine/threonine phosphorylation events appear to have additional modulatory roles. The interplay between these various inputs is the subject of ongoing work.

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. We are also very grateful to Dr G. Kular for providing the GST-PLC δ 1-PH domain, and Dr N. Leslie for his invaluable help in designing primers and with molecular-biological techniques. This work was supported by Medical Research Council grant no. G940316 to C.P.D.

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Received 25 May 2000/11 July 2000; accepted 24 July 2000