Loss of Phosphatidylinositol 3-Phosphate Binding by the C-terminal Tiam-1 Pleckstrin Homology Domain Prevents in Vivo Rac1 Activation without Affecting Membrane Targeting

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Dbl family guanine nucleotide exchange factors (GEFs) for Rho family small GTPases invariably contain a pleckstrin homology (PH) domain that immediately follows their Dbl homology (DH) domain. Although the DH domain is responsible for GEF activity, the role of the PH domain is less clear. We previously reported that PH domains from several Dbl family members bind phosphoinositides with very low affinity (Kd values in the 10 μM range). This suggests that, unlike several other PH domains, those from Dbl proteins will not function as independent membrane-targeting modules. To determine the functional relevance of low affinity phosphoinositide binding, we mutated the corresponding PH domain from Tiam-1 to abolish its weak, specific binding to phosphatidylinositol 3-phosphate. We first confirmed that the C-terminal PH domain of Tiam-1 was required for maximum exchange activity of its adjacent DH domain, but that low affinity phosphatidylinositol 3-phosphate binding may be critical for in vivo regulation and activity of Tiam-1 but that the PH domain exerts its regulatory effects without altering membrane targeting. We suggest instead that ligand binding to the PH domain induces conformational and/or orientational changes at the membrane surface that are required for maximum exchange activity of its adjacent DH domain.

Tiam-1 (T-lymphoma invasion and metastasis 1) was first identified in a screen for genes that, when amplified, can increase invasiveness of normally noninvasive lymphoma cells (1). Its 1591-amino acid protein product, Tiam-1, is myristoylated at the N terminus (2) and contains two N-terminal PEST sequences (3), a PDZ (postsynaptic density-95/Discs large/Zona occludens-1) domain (4), a Ras-binding domain (5), two pleckstrin homology (PH)1 domains (6), plus a Dbl (diffuse B-cell lymphoma) homology (DH) domain (7) (Fig. 1). Tiam-1 is a member of the Dbl family of guanine nucleotide exchange factors (GEFs) for proteasome-like small GTPases (7) and is a specific activator of Rac1 in vivo (8, 9). By activating Rac1, Tiam-1 can influence a plethora of cellular processes including actin cytoskeleton dynamics, cell proliferation, gene transcription, cell survival, and the phagocytic NADPH oxidase complex, as reviewed by Bishop and Hall (10).

The 200-amino acid DH domain is both necessary and sufficient for the GEF activity of Dbl family proteins (7, 11–13). However, in all 46 or more Dbl family GEFs, a positively charged N-terminal region (9) of the PH domain immediately follows the DH domain (7). Dbl family GEFs account for some 18% of all known human PH domains. Deletion of the PH domain that follows the DH domain has been shown to either inhibit or stimulate the in vivo function of several Dbl family GEFs (7, 12, 14). Together with their invariable linkage, this has been taken to suggest a regulatory coupling of DH and PH domains, although the molecular basis for this remains unclear. In general, PH domains have been implicated in directing the subcellular localization of proteins that contain them (6), by binding to phosphoinositides and/or membrane-associated protein targets. Several reports have suggested that DH-associated PH domains function in targeting Dbl family members to membrane and/or cytoskeletal compartments in vivo (15–21). Studies of Tiam-1, however, indicate that the primary determinants of subcellular localization lie elsewhere in the molecule (21, 22), and most Dbl family members contain one or more additional potential targeting domains (7).

Several experimental observations indicate that PH domains of Dbl family proteins have specific roles in addition to (or instead of) the commonly assumed membrane targeting func-

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1 The abbreviations used are: PH, pleckstrin homology; C1199, C-terminal 1199 amino acids of Tiam-1; C-PH, C-terminal PH domain; GEF, guanine nucleotide exchange factor; GST, glutathione-S-transferase; LPA, lysophosphatidic acid; mant, N-methylanthraniloyl; PDGF, platelet-derived growth factor; PBD, p21-binding domain; PDE, platelet-derived growth factor; PH*L2, Tiam-1 mutated in the PH domain (variable loop 2); PH*L1/L2, Tiam-1 mutated in the PH domain (variable loops 1 and 2) of C-PH; FL, full-length.
tion. For example, the in vitro GEF activity of DH/PH fragments is far greater than that of isolated DH domains (11, 13, 23). Crystallographic studies of the Dbs (Dbl’s big sister) DH/PH fragment (23) showed that the PH domain makes direct contacts with Cdc42 that are important for exchange activity, providing one explanation for this observation. However, no contacts between the PH domain and the small GTPase were seen in the structure of the Tiam-1 DH/PH complex with Rac1 (24), suggesting that the PH domain may play a different role in this case. One proposal, stimulated by in vivo studies of Sos (son-of-sevenless) (25) and Vav (26, 27), is that the PH domain binds phosphoinositides, such as PI 3-kinase products, to sterically block access of the small GTPase to the relevant DH domain surface. Binding of the PH domain to phosphoinositides, such as PI 3-kinase products, has been suggested to reverse this inhibition (25–27).

We previously showed that the DH/PH fragments of inter- sectin, Dbs, and Tiam-1 bind phosphoinositides with only low affinity (29). The intersectin and Dbs DH/PH fragments were relatively promiscuous in their phosphoinositide binding, although PtdIns(4,5)P2 (because it is most abundant) is likely to be the most relevant ligand in vivo. The Tiam-1 DH/PH fragment, by contrast, showed clear selectivity (but still only low affinity) for PtdIns-3-P (29), a phosphoinositide that is enriched in endosomal compartments (30) and is generated in phagosomal membranes (31). The Tiam-1 DH/PH fragment, by contrast, showed clear selectivity (but still only low affinity) for PtdIns-3-P (29), a phosphoinositide that is enriched in endosomal compartments (30) and is generated in phagosomal membranes (31). The Tiam-1 DH/PH fragment, by contrast, showed clear selectivity (but still only low affinity) for PtdIns-3-P (29), a phosphoinositide that is enriched in endosomal compartments (30) and is generated in phagosomal membranes (31).

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs and Mutagenesis**—The coding region for the murine Tiam-1 DH/PH fragment (amino acids 1033–1406) was amplified by polymerase chain reaction and subcloned into the EcoRI site of pGEX-2TK (Amersham Biosciences) to express a GST fusion protein that could be labeled with 35S at the protein kinase A site between the GST moiety and the DH/PH fragment. A GST fusion protein, myristoylated full-length (human) Tiam-1 driven by a cytomegalovirus promoter (in pCaNmyc) was kindly provided by Anne Crompont (34). A construct expressing the C-terminal 1199 amino acids of murine Tiam-1 (C1199) was kindly provided by John Collard (8), and C1199 was resubcloned into a pcDNA3 derivative to incorporate an hemagglutinin (HA) tag at the N terminus of the fragment. The p21-binding domain (amino acids 70–117) from human p21-activated kinase 1, fused to GST (GST-PBD), was kindly provided by Dr. Jonathan Chernoff (Fox Chase Cancer Center).

PH domain mutations were made in the human FL Tiam-1 and murine C1199 (GST-DH/PH) backgrounds using the QuikChangeTM site-directed mutagenesis kit (Stratagene) as recommended by the manufacturers and making amino acid substitutions in the β1/β2 and β3/β4 loops of the PH domain as depicted in Fig. 2. Details are available upon request.

**Dot-blot Analysis of Phosphoinositide Binding**—GST-DH/PH fusion proteins were expressed in Escherichia coli, purified, and labeled with 32P (while bound to glutathione-agarose) exactly as described (38). 15 μg of mutated and wild type protein were labeled in parallel for each experiment to match specific activities as closely as possible, and 12 μg of eluted 32P-labeled protein was used to probe a nitrocellulose filter with 32P-labeled GST-DH/PH. This was performed essentially as described (37). COS1 cells were seeded at 2 × 105/35-mm well and were transfected the next day with wild type or PH domain-mutated versions of FL Tiam-1 or C1199 Tiam-1, using LipofectAMINE (Invitrogen) as directed by the manufacturer. After recovery in growth medium followed transfection, the cells were starved for 24 h in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum. The cells were then lysed in 300 μl of G protein lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 5 mM β-glycerophosphate, 0.5% Nonidet P-40, 1 mM dithiothreitol, plus 0.7 μg/ml pepstatin, 0.5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), containing 25 μg of a GST fusion protein of the PBD from human p21-activated kinase 1 (amino acids 70–117). The cell lysates were immediately clarified by centrifugation at 13,000 × g for 10 min at 4°C. An aliquot (30 μl) of the clarified lysate was removed for Western blot analysis; the remainder was precipitated using glutathione-Sepharose beads (Amersham Biosciences; 30-μl bead volume) for 30 min at 4°C. The beads were washed three times in G protein lysis buffer, boiled in sample buffer, resolved on 12% SDS-PAGE gels, and transferred to nitrocellulose. The top portion of the blot was probed with murine Tiam-1 (Santa Cruz Biotechnology, Inc. number sc-872; 1:1000); the bottom portion was probed with anti-Rac (Upstate Biotechnologies, Inc. number 05-389; 1:5000) to detect endogenous Rac and detected using Enhanced Chemiluminescence (Amersham Biosciences).
10^6 cells/35-mm dish. The following day, the cells were co-transfected (using LipofectAMINE) with the FL Tiam-1 mutant (or wild type) constructs and a luciferase reporter whose expression is driven by a mutated SRE that no longer binds ternary complex factor (40). Following transfection, the cells were allowed to recover overnight in 5% calf serum with Dulbecco’s modified Eagle’s medium and were then starved in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum. After 24 h of serum deprivation, the cells were harvested for luciferase assays using the Luciferase Assay System (Promega Corporation), following the manufacturer’s instructions. An aliquot of each cell lysate was also taken for SDS-PAGE and immunoblotting with anti-Tiam-1 antibody, to allow normalization of luciferase activity to Tiam-1 expression levels.

Nucleotide Exchange Assay—Nucleotide exchange assays were performed in vitro as described (25), monitoring incorporation of 32P-labeled GTP into Rac1 expressed and purified from E. coli. Exchange reaction assay mixtures containing 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, and 100 μM mant-GTP (Biomol), plus 2 μM Rac1 were prepared and allowed to equilibrate (with continuous stirring) in a Perkin-Elmer LS 50B spectrophotometer at 25 °C. After equilibration, the Tiam-1 GST-DH/PH protein (mutant or wild type) was added to 400 nM, and the rate of nucleotide loading was determined by monitoring the decrease in Rac1 tryptophan fluorescence λ₉₀ = 295 nm, λ₃₃₅ = 335 nm) upon binding of mant-GTP (41, 42). The rates of nucleotide exchange were determined by fitting the data as single exponential decays. The data were normalized to wild type curves to yield the percentage of GDP released.

Immunofluorescence Microscopy—NIH 3T3 cells (3.0 × 10⁵) were seeded on UV-irradiated glass coverslips in 35-mm dishes. The following day, the cells were transfected with 2 μg of plasmid encoding (Mye-tagged) FL wild type or PH*L1/L2 Tiam-1, using LipofectAMINE. The cells were then starved in 0.5% fetal bovine serum with Dulbecco’s modified Eagle’s medium for 24 h, stimulated for 10 min with platelet-derived growth factor (PDGF) at a final concentration of 10 ng/ml (Invitrogen), and fixed in 3.7% formaldehyde in phosphate-buffered saline. To localize Tiam-1, the cells were stained with anti-Myc antibody (9E10; 0.1 μg/ml) and visualized using Cy3-labeled donkey anti-mouse IgG (Jackson ImmunoResearch) diluted at 1:1,000. Fluorescence localization was simultaneously visualized using fluorescein isothiocyanate-labeled phallidin (Molecular Probes, Eugene, OR) diluted at 1:25 from a methanolic solution at 300 units/ml. The coverslips were mounted with SlowFade (Molecular Probes) and viewed on a Zeiss confocal microscope with LSM510 software, using excitation wavelengths of 488 nm (for fluorescein isothiocyanate) or 546 nm (for Cy3).

RESULTS

Mutation of Tiam-1 C-PH to Impair PtdIns-3-P Binding—To investigate the in vivo importance of low affinity PtdIns-3-P binding by the C-PH of Tiam-1, our first aim was to make mutations that would abolish this interaction. By contrast with PH domains that have specific, high affinity, phosphoinositide ligands, PH domains from Dbl family members do not contain clear sequence motifs at which mutations can be guaranteed to abolish phosphoinositide binding. For example, the critical arginine that can be mutated to prevent phosphoinositide binding by the phospholipase C-δ₁, Bruton’s tyrosine kinase, or protein kinase B PH domains (6) is not conserved in Tiam-1 C-PH. Therefore, assuming that phosphoinositides bind to the same face of Tiam-1 C-PH as they do to other PH domains, we substituted lysines in the β₁/β₂ (variable loop 1) and β₃/β₄ (variable loop 2) of Tiam-1 C-PH with glutamine (Fig. 2A) and evaluated the effects of these mutations on PtdIns-3-P binding in vitro. Because PtdIns-3-P binding by Tiam-1 C-PH is so weak (Kᵰ ≥ 10 μM), we used a semi-quantitative dot-blot approach to compare phosphoinositide binding by the mutated and wild type proteins. Identical quantities of 32P-labeled GST-DH/PH fusion proteins were used in parallel to probe nitrocellulose filters onto which PtdIns-3-P had been spotted (with PtdIns(4,5)P₂ as a control) as described under “Experimental Procedures.” As shown in Fig. 2B, a GST fusion protein containing the wild type DH/PH fragment gives a significant binding signal with PtdIns-3-P that only falls to background levels after three or four serial 2-fold dilutions of lipid. A weak signal is also seen with PtdIns(4,5)P₂ but not with phosphatidyl-serine, confirming the phosphoinositide binding specificity of this DH/PH fragment that we reported previously (29).

Mutation of four lysines in variable loop 2 to glutamine (PH*L1/L2; Fig. 2A) substantially reduced PtdIns-3-P binding by the DH/PH fragment (dropping to background after a single 2-fold lipid dilution) and gave no detectable signal with PtdIns(4,5)P₂. Additional mutation of three lysines in variable loop 1, to give the PH*L1/L2 mutant, appeared to reduce PtdIns-3-P binding further, so that it could barely be detected. The residues mutated are all solvent-accessible in the crystal structure of the Tiam-1 DH/PH fragment (24), and Fig. 2C shows that the mutations described here have no influence on the abundant soluble expression of the GST fusion proteins in E. coli at 37 °C or upon their integrity. Each of the lysines mutated in the Tiam-1 DH/PH fragment contributes to the characteristic positively charged face of the C-terminal Tiam-1 PH domain. Therefore, in addition to any possible effect on a specific PtdIns-3-P-binding site, mutating these lysines to glutamine will reduce the strength of delocalized (and nonspecific) electrostatic interactions between the positively charged face of the PH domain and negatively charged phosphoinositide-containing membranes.

Loss of Phosphoinositide Binding by C-PH Impairs the Ability of Tiam-1 to Activate Rac1 in Vivo—Tiam-1 serves as a Rac-specific GEF in vivo. To determine whether the loss of PtdIns-3-P binding affects Tiam-1 function, Rac activity was assessed in cells expressing either wild type (full-length) Tiam-1 or a form containing the C-PH mutations described above. Rac1 activation can be monitored by selectively precipitating the active, GTP-bound form from cell lysates with the PBD of p21-activated kinase, a physiological target of activated Rac (43). As shown in Fig. 3A, overexpression of FL wild type Tiam-1 in COS cells caused a substantial increase in the amount of activated Rac that can be precipitated by GST-PBD from lysates of serum-starved cells. By contrast, very little activated Rac could be seen in cells overexpressing the FL PH*L1/L2 or PH*L2 mutants or in the vector control. Western blots of whole cell lysates confirmed that the total levels of Rac and of the Tiam-1 proteins were comparable in all samples.

Identical results were obtained with a widely used form of Tiam-1 that removes the two PEST sequences at the N terminus, leaving only C1199 (Fig. 1), which is believed to lead to stabilization of the protein. As seen in Fig. 3B, C1199 potently activated Rac in COS cells, and mutation of the C-PH domain completely abrogated these effects (C1199 PH*L1/L2). These results therefore argue that loss of PtdIns-3-P binding by the C-terminal PH domain significantly impairs the ability of Tiam-1 to activate Rac in living cells.

Mutations in the C-terminal PH Domain of Tiam-1 Also Impair Its Ability to Activate Serum Response Factor (SRF)—One consequence of Rac activation in vivo is stimulation of SRF, which induces transcription of target genes by binding to SREs in their promoters (44). SRF activation can be monitored using an SRE-luciferase reporter gene. As shown in Fig. 4, transient overexpression of wild type FL Tiam-1 in NIH 3T3 cells resulted in robust SRE-dependent luciferase production to levels ~22-fold above background. By contrast, transfection with vectors encoding FL Tiam-1 bearing either the PH*L2 or PH*L1/L2 mutations resulted in only background levels of luciferase activity, no higher than induced with reporter plasmid alone. Although the C-PH mutants were expressed at slightly lower levels than the wild type protein (Fig. 4), it is highly unlikely that this modest difference can account for the complete loss of luciferase activation. Thus, this experiment supports the data described above and strengthens the argu-
ment that loss of PtdIns-3-P binding by C-PH of Tiam-1 abro-
gates its activation of Rac signaling pathways in vivo.

Inherent in Vitro Exchange Activity of DH/PH Fragments Is
Unaffected by PH Domain Mutations—The inability of Tiam-1
with C-PH mutations to activate Rac and SRF could have at
least two origins. The loss of function could reflect a misfolding
or stability artifact, or it could reflect a real requirement for
phosphoinositide binding by the C-PH domain for Tiam-1 to
exert its in vivo effects. The lack of any effect of the PH*L2 or
PH*L1/L2 mutations on expression of the DH/PH fragment in
E. coli (Fig. 2C) argues against a misfolding or stability arti-
fact. Indeed, the wild type and both mutated proteins were
readily expressed and purified. Similarly, the C-PH mutations
did not significantly impair expression levels of FL or C1199
Tiam-1 (Figs. 3 and 4). We also tested the possibility that C-PH
mutations might directly impair the GEF activity of the DH
domain for Tiam-1 to exert its in vivo effects. The lack of any effect of the PH*L2 or
PH*L1/L2 mutations on expression of the DH/PH fragment in
E. coli (Fig. 2C) argues against a misfolding or stability arti-
fact. Indeed, the wild type and both mutated proteins were
readily expressed and purified. Similarly, the C-PH mutations
did not significantly impair expression levels of FL or C1199
Tiam-1 (Figs. 3 and 4). We also tested the possibility that C-PH
mutations might directly impair the GEF activity of the DH
domain, which could explain the in vivo results. We employed
an in vitro nucleotide exchange assay using N-mant-GTP.
When mant-GTP binds to Rac, the intrinsic tryptophan fluo-
rescence of the small GTPase is quenched by energy transfer to
the mant moiety, and this effect can be monitored in a spec-
trofluorimeter to follow GTP loading in real time (23). As
shown in Fig. 5, the rate of guanine nucleotide exchange on
unprenylated Rac1 is indistinguishable for the wild type and
PH domain-mutated DH/PH fragments and was unaffected by
the addition of PtdIns-3-P (29). Each curve fit well to a single
exponential, with observed rates that differed by less 10%, as
listed in the legend to Fig. 5. It is therefore clear that the
PH*L2 and PH*L1/L2 mutations studied here do not affect the
intrinsic GEF activity of Tiam-1, so this cannot explain their
failure to activate Rac1 and SRF in vivo.

C-PH Mutations Do Not Influence Membrane Targeting of
Tiam-1—Previous studies have indicated that recruitment of
Tiam-1 to the plasma membrane is essential for its ability to
activate Rac in vivo. Because mutations that prevent PtdIns-
3-P binding by Tiam-1 C-PH impair cellular function without
affecting intrinsic GEF activity, we next tested the hypothe-
sis that these mutations might alter the membrane targeting
of Tiam-1. We first generated a green fluorescent protein
fusion protein of the Tiam-1 DH/PH fragment and examined
its subcellular location. Only diffuse green fluorescence was
observed, regardless of growth factor stimulation of the cells,
arguing that the DH/PH fragment alone is not a primary
determinant of Tiam-1 localization to the plasma membrane
(data not shown). This finding is consistent with multiple
other studies demonstrating that the N-terminal but not the

![Fig. 2. Mutation of Tiam-1 C-PH domain. A, the sequence from strand β1 to β4 of Tiam-1 C-PH is listed, with strands β1, β2, and β3 marked. Lysine to glutamine mutations in PH*L2 and PH*L1/L2 are in bold type. B, 12 μg of 32P-labeled GST-DH/PH was used to probe nitrocellulose onto which had been spotted serial 2-fold dilutions of PtdIns-3-P, PtdIns(4,5)P2, and phosphatidylserine (beginning at 2 mg/ml) as described under “Experimental Procedures.” The experiment was repeated for wild type DH/PH and mutated forms as labeled (see text). Dot-blots are representative of more than three independent experiments. A fixed amount (approximately 6 ng) of 32P-labeled protein was also spotted at the bottom left of each blot to serve as an exposure control. C, Coomassie Blue-stained SDS-PAGE gel of wild type and mutated forms of GST-DH/PH proteins loaded at 1 mg/ml. WT, wild type.](image-url)
C-terminal PH domain of Tiam-1 is necessary and sufficient for efficient membrane association (2, 22, 35).

We next examined the consequences of mutating Tiam-1 C-PH on membrane localization in the context of the FL Tiam-1 protein. Previous work has shown that PDGF can induce recruitment of Tiam-1 to the plasma membrane (45). Therefore, FL wild type or PH*L1/L2 Tiam-1 were transfected into NIH 3T3 cells, and their localization was monitored by confocal microscopy following stimulation of cells with PDGF. Both proteins were found predominantly in the cytoplasm and in a perinuclear region (Fig. 6). Strong plasma membrane localization of wild type Tiam-1 was additionally evident in ~5% of transfected cells. This low percentage may be due to the transient nature of Tiam-1 membrane recruitment in response to ligand, as suggested by previous studies (2), and/or the difficulty of clearly discerning membrane targeting at higher expression levels (when cytoplasmic staining is more intense). Neither the percentage of cells displaying strong plasma membrane localization of Tiam-1 nor the extent of membrane localization was affected by the PH domain mutations. A representative image demonstrating membrane localization of wild type versus PH*L1/L2 is shown in Fig. 6. When either form of Tiam-1 was plasma membrane associated, it appeared to colocalize with F-actin at sites of membrane ruffling. This result suggests that the inability of PH*L1/L2 to activate Rac in vitro does not derive from its aberrant subcellular localization and further confirms that the C-PH domain of Tiam-1 is dispensable for membrane recruitment.

DISCUSSION

In the relatively few cases where their function is clear, PH domains are known to target their host proteins to cellular membranes by binding phosphoinositides, sometimes in cooperation with other binding targets (6, 46). However, the function of most PH domains is not well understood, and the majority binds phosphoinositides with low affinity and with little if any specificity (6). The PH domains that invariably follow DH domains exhibit low affinity and usually promiscuous phosphoinositide binding in all cases studied (27, 29,
construct, starved, and then stimulated with PDGF (10 ng/ml) for 10 min. The cells were fixed and co-stained for Tiam-1 (using anti-Myc antibody) and F-actin (using fluorescein isothiocyanate-labeled phalloidin) as described under "Experimental Procedures." Significant membrane localization of Tiam-1 was observed in only ~5% of cells but was seen with an equivalent frequency for both wild type and PH*L1/L2 Tiam-1. Micrographs shown are representative of Tiam-1 staining when membrane localization was observed. As depicted in the merged images, Tiam-1 co-localized with F-actin at membrane ruffles.

There is no clear consensus on the role of PI 3-kinase in Tiam-1 activation. Tiam-1 translocation to the plasma membrane, mediated by its N-terminal region, does not appear to require PI 3-kinase activity (35, 45). The ability of Tiam-1 to activate Rac-dependent processes has been reported to be stimulated by PI 3-kinase products (35, 48), although other studies disagree (5, 45). If PI 3-kinase were able to regulate Tiam-1 through its C-terminal PH domain without affecting Tiam-1 localization, this would resemble the situation reported for P-Rex1, a phosphatidylinositol 3,4,5-trisphosphate-regulated Rac-GEF (36). P-Rex1 is regulated by PI 3-kinase and in vitro. However, this activation does not involve its translocation to the plasma membrane. Instead, a fraction of P-Rex1 that is membrane-targeted through other means is activated while present at that location by binding to phosphatidylinositol 3,4,5-trisphosphate (36), probably through its PH domain.

The fact that PtdIns-3-P is the preferred ligand for Tiam-1 C-PH merits discussion. This phosphoinositide is primarily considered as a marker for endosomal compartments in the cell (30). Interestingly, Michiels et al. (2) have observed localization of myristoylated Tiam-1 to intracellular vesicle-like structures. PtdIns-3-P is not generally considered to be a signaling phosphoinositide in the normal sense, because its levels remain quite constant with different stimuli (49). However, recent studies have implicated PtdIns-3-P in certain acute responses. There is clear evidence for acute de novo synthesis of PtdIns-3-P on phagosomes and in phagolysosomes during particle ingestion (31, 32) and for PtdIns-3-P accumulation at plasma membrane ruffles induced by invading Salmonella (33). It is therefore possible that PtdIns-3-P generation (or redistribution) could play a role in C-PH-mediated activation of Tiam-1. Indeed, a similar proposal has been made for the cytokine-independent survival kinase, which has an N-terminal (PtdIns-3-P-specific) phox homology domain that must bind PtdIns-3-P for cytokine-independent survival kinase activity (50). One output for which the presence of PtdIns-3-P and activated Rac must coincide is in the assembly and activation of the neutrophil NADPH oxidase complex. PtdIns-3-P promotes assembly of this complex through the phox homology domain of p40phox (51), and activated Rac is known to be required for oxidase activity (52). However, Tiam-1 has not been directly implicated in this process to our knowledge.

An important implication of our findings is that binding of phosphoinositides (or other ligands) to the C-PH domain of...
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Tiam-1 must occur once the protein is already membrane-targeted. The effective local concentration of phosphoinositides in the vicinity of membrane-targeted Tiam-1 will be very high. Therefore, an interaction that is weak (K_d > 10 μM) when the two components are free in solution (such as between Tiam-1 DH/PH fragment and PtdIns-3-P in vitro) would be rather strong in the context of membrane-targeted Tiam-1. A local concentration effect of this sort presents complications for interpreting lipid binding specificity from in vitro studies. Whereas we found that C-PH of Tiam-1 is PtdIns-3-P-specific in vitro, we also demonstrated that the PH domain of Tiam-1 associates strongly with the Dbl DH/PH domain actually enhanced association of the protein. Loss of phosphoinositide binding by the Dbl PH domain therefore mirrors our findings with Tiam-1. Der and Booden et al. (2002) have provided explanations for the variably observed PI 3-kinase dependence of a Dbl DH/PH fragment (20) and FL proto-Dbl (53). However, recent studies by Eva and co-workers (20, 53) have provided a slightly different picture for the role of the DH domain-specific regions of the membrane that are enriched in a particular lipid (such as PtdIns-3-P). No evidence exists for such “lateral targeting,” although it may simply be beyond the resolution of approaches used to study localization. The second, more favored possibility is that binding of membrane phosphoinositide or other ligand to the PH domain optimizes the relationship between the DH and PH domains of the GEF and the lipid-anchored small GTPase to maximize exchange activity. This could simply involve promotion of an ideal orientation of the DH domain with respect to the GTPase. Alternatively, as suggested for Sos (28), ligand binding by the PH domain may disfavor an auto-inhibitory relationship between the DH and PH domains. Another mode, suggested by crystallographic studies of the Dbs DH/PH fragment bound to Cdc42 (23), involves promotion of critical contacts between the PH domain and the small GTPase at the membrane surface. The different relationships between DH domain, PH domain, and small GTPase in the structures determined to date (23, 24, 28, 55) suggest that the precise mode of regulation by PH domain ligand binding may differ between subclasses of Dbl family GEF.

We have suggested several hypotheses for how low affinity phosphoinositide binding by C-PH could play a critical role in Tiam-1 regulation without being required for membrane targeting. However, we cannot exclude the possibility that our C-PH mutations abolish binding to Tiam-1 of some other regulatory component, such as another lipid or a protein that is required for Tiam-1 to be able to activate Rac in vivo. Indeed, an accumulating body of data (6, 46, 56) suggests that PH domains that bind phosphoinositides with low affinity may often have additional (or alternative) binding targets. Regardless of whether such an additional target exists for Tiam-1 C-PH, the data presented here support the idea that this PH domain plays a regulatory role that is quite different from that seen in Bruton’s tyrosine kinase, protein kinase B, and other examples. Understanding the diverse ways in which such PH domains can regulate their host proteins will be an interesting future challenge.

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