Targeting of Tiam1 to the Plasma Membrane Requires the Cooperative Function of the N-terminal Pleckstrin Homology Domain and an Adjacent Protein Interaction Domain*

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The Rho-like GTPases Cdc42, Rac, and Rho play key roles in the regulation of the actin cytoskeleton and are implicated in transcriptional activation and cell transformation. We have previously identified the invasion-inducing Tiam1 gene, which encodes an activator of Rac. In fibroblasts, Tiam1 induces Rac-mediated membrane ruffling, which requires the N-terminal pleckstrin homology (PHn) domain. Here we show that this PHn domain is part of a protein interaction domain, which mediates membrane localization of Tiam1. After subcellular fractionation, up to 50% of Tiam1 is recovered in the Triton X-100-insoluble high speed pellet that contains small protein complexes. The regions in Tiam1 that are responsible for these protein interactions comprise the PHn domain, an adjacent putative coiled coil region (CC), and an additional flanking region (Ex). Deletions in each of these regions abolish membrane localization of Tiam1 and membrane ruffling, suggesting that they function cooperatively. Indeed, only polypeptides encompassing the PHn-CC-Ex region, and not the PHn-CC or the Ex region, localize at the membrane. These results indicate that the N-terminal PH domain is part of a larger functional Tiam1 domain that mediates protein complex formation and membrane localization of Tiam1.

Pleckstrin homology (PH) domains are protein motifs that have been identified in numerous signaling molecules (Refs. 1 and 2 and for reviews see Refs. 3 and 4). Proteins containing a PH domain include serine/threonine and tyrosine kinases, phospholipases, cytoskeletal proteins, and regulators of small GTPases of the Ras superfamily. PH domains have been implicated in the (transient) localization of proteins to the plasma membrane (3, 5–7). The precise binding properties of PH domains in vivo are largely unknown, but in vitro studies indicate that PH domains can bind to specific phosphoinositol lipids as well as to βγ subunits of heterotrimeric G proteins (3, 4), both providing a link with membrane localization. Many PH domains show in vitro a rather promiscuous interaction with phospholipids and βγ subunits, which raises the question how specificity is achieved in vivo.

A large group of proteins containing a PH domain is formed by regulators of Ras- and Rho-like small GTPases. Ras-like proteins primarily regulate growth and differentiation (8). Rho-like proteins, including RhoA, Rac1, and CDC42, regulate the organization of the actin cytoskeleton which plays an important role in adhesion and motility of cells (9, 10). In addition, Rho-like proteins have been implicated in cell cycle progression (11), transcriptional activation (12–14), and oncogenic transformation (15–17). Small GTPases are activated by the exchange of bound GDP for GTP, which is stimulated by guanine nucleotide exchange factors (GEFs). GEFs that activate Rho-like GTPases invariably contain a PH domain C-terminally adjacent to the catalytic Dbh homology (DH) domain (see Refs. 18–20), suggesting that the DH-PH combination forms a functional unit. Indeed, deletions in either the DH domain or the flanking PH domain of the proto-oncogenes Db1, Ost, Dbs and Lfc resulted in loss of transforming capacity (20).

Previously, we identified the Tiam1 gene by its capacity to induce invasion of T-lymphoma cells (21). Tiam1 encodes a GEF that activates the Rho-like GTPase Rac in vitro as well as in vivo (22). We recently showed that Tiam1 induces Rac-mediated membrane ruffling and stimulation of Jun N-terminal kinase (JNK) activity but only when Tiam1 is associated with the plasma membrane (23). The Tiam1 protein is myristoylated at the N terminus and contains various conserved domains implicated in membrane localization. In addition to the DH-PH combination, Tiam1 contains a second, N-terminally located PH domain and a discs large homology region (DHR), also named PDZ domain. DHRs have been implicated in the intracellular localization of proteins, especially by binding to membrane protein networks (24, 25). By deletion analysis we found that only the N-terminal PH domain (PHn) is required for the membrane localization of Tiam1 in fibroblasts. This domain could be functionally replaced by the membrane localization domain of c-Src (23).

In this study we show that, in addition to the PHn domain, sequences downstream of the PHn also are involved in proper function and membrane localization of Tiam1. We have identified a protein interaction domain in Tiam1 that encompasses the PHn domain, a C-terminally flanking putative coiled coil region (CC) and an additional adjacent region (Ex). This complete region (PHn-CC-Ex) is required for translocation of Tiam1 to the plasma membrane. Moreover, co-expression of the isolated PHn-CC-Ex domain with Tiam1 inhibits induction of membrane ruffling in COS-7 cells, whereas the PHn-CC do-

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† The abbreviations used are: PH, pleckstrin homology; PHn, N-terminal pleckstrin homology; DH, Dbh homology; DHR, discs large homology region; GEF, guanine nucleotide-exchange factor; MAPK, mitogen-activated protein kinase; FITC, fluorescein isothiocyanate; CSK, cytoskeleton; aa, amino acids; PAGE, polyacrylamide gel electrophoresis; TRITC, tetramethylrhodamine isothiocyanate.

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main alone does not. Our data indicate that the PHn-CC region and the adjacent Ex region both constitute protein interaction domains, which together determine the membrane localization and biological activity of Tiam1.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—Full-length Tiam1 (FL1591) and the truncated constructs N385 Tiam1, C1199 Tiam1, and C682 Tiam1 were cloned into the eukaryotic expression vector pcDNAe (17). C580 Tiam1 and C1199 Tiam1, carrying a hemagglutinin tag at the 3′ end, were cloned in the eukaryotic expression vector pUTSV1 (Eurogentec, Belgium) (23). Deletion constructs were derived from C1199 Tiam1. C1199 ΔDH-Tiam1 carries a deletion ranging from aa 853–905 which eliminates most of the DHR region (23). C1199ΔPHn1 Tiam1 carries an Xmn1-NlaIV deletion (aa 513–552), which ranges from the predicted sixth β sheet to the C-terminal end of the PH domain (23). In C1199ΔPHn3 Tiam1, the entire PH domain is deleted by an NsiI-NlaIV deletion (aa 420–552). PHn-CC-Ex-C570 and PHn-CC-C570 Tiam1 were constructed by deleting an SsrI-Bsa661 fragment (aa 731–1021) or a BstXI-Bsa661 fragment (aa 648–1021), respectively. C1199ΔCC Tiam1 carries a Ban1-PvuII deletion (aa 554–587; the putative coiled coil spans 458–580). In C1199Ex Tiam1 an ExoIII deletion resulted in deletion of aa 599–691. C1199Δ(772–853) Tiam1 carries an AclI-NcoI deletion resulting in deletion of aa 772–853. The competitive constructs, Tiam1 fragments were cloned behind a Myc-epitope tag in the eukaryotic expression vector pMT2SM. The PH-CC-Tiam1 fragment encodes aa 393–652, and the Ex(588–897) Tiam1 fragment encodes aa 858–897, and the Tiam1(794–1103) fragment encodes aa 794–1103, and the translucent pellet the “high speed cytoskeleton” (resuspended and centrifuged again) and taken as “low speed cytoskeleton.”

**Cells and Transfections—**NIH3T3 and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum or fetal calf serum, respectively. NIH3T3 cell lines expressing C1199 Tiam1 and C682 Tiam1 have been described (17). COS-7 cells were transiently transfected by using the DEAE-dextran method, as described (23). For competition experiments, plasmids encoding C1199 Tiam1 and C682 Tiam1 have been described (17). COS-7 cells were transiently transfected by using the DEAE-dextran method, as described (23). For competition experiments, plasmids encoding C1199 Tiam1 and C682 Tiam1 have been described (17). COS-7 cells were transiently transfected by using the DEAE-dextran method, as described (23). For competition experiments, plasmids encoding C1199 Tiam1 and C682 Tiam1 have been described (17)

**Triton X-100 Fractionation**—Cells were grown in 75-cm² flasks, washed twice with ice-cold phosphate-buffered saline, and lysed with 800 μl of cytoskeleton stabilizing buffer (CSK) containing 10 μM Heps, pH 6.8, 250 μM sucrose, 150 mM KCl, 1 mM EGTA, 3 mM MgCl₂, 0.5% Triton X-100 (26), and protease inhibitors aprotonin (2 μg/ml), leupeptin (2 μg/ml), soybean trypsin inhibitor (10 μg/ml), phenylmethylsulfonyl fluoride (0.2 mM), and Pefabloc (1 mM). All fractionation steps were performed at 4°C. Cells were scraped with a rubber policeman and left on ice for 30 min. A “total lysate” sample was taken and, after addition of cytoskeleton-stabilizing buffer (CSK) containing 10 μM Heps, pH 6.8, 250 μM sucrose, 150 mM KCl, 1 mM EGTA, 3 mM MgCl₂, 0.5% Triton X-100 (26), and protease inhibitors aprotonin (2 μg/ml), leupeptin (2 μg/ml), soybean trypsin inhibitor (10 μg/ml), phenylmethylsulfonyl fluoride (0.2 mM), and Pefabloc (1 mM), the cell suspension was centrifuged at 300 g for 2 min. The resulting supernatant was centrifuged again for 2 min at 4,000 g. After 2 min at 4,000 g, the supernatant was removed and the remaining Triton X-100 lysate was centrifuged for 10 min at 15,000 × g. The resulting pellet was washed once in lysis buffer (resuspended and centrifuged again) and taken as “low speed cytoskeleton fraction.”

**Immunoblot Analysis—**Lysates and fractions were solubilized in Laemmli sample buffer, containing 0.1% dithiothreitol and separated by SDS-PAGE. Proteins were blotted onto nitrocellulose and stained with Ponceau S, and filters were blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 3% non-fat dried milk for at least 2 h, as described (17, 23). The filters were probed with primary antibody in blocking buffer for at least 1 h, washed three times in TBST, incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Corp.) in blocking buffer for 30 min. Proteins were visualized using enhanced chemiluminescence (ECL, Amersham Corp.) secondary antibodies. Cells were stained simultaneously for F-actin with TRITC-labeled phalloidin (2 units/ml) (Molecular Probes). At least 100 positive cells were analyzed. Images were recorded separately with a Bio-Rad MRC-600 confocal laser scanning microscope. For immunoelectron microscopy, cells were fixed and immunostained with a rabbit polyclonal anti-MAPK antibody (9E10, Oncogene Science), and a rabbit polyclonal anti-MAPK antibody, provided by P. Horák.

**RESULTS**

**Tiam1 Is Associated with Protein Complexes**—Previously, we have shown that the C-terminal 1199 amino acids of Tiam1 (C1199 Tiam1), but not a smaller C-terminal region of Tiam1 lacking the PHn and DHR domains (C682 Tiam1), induce Rac-mediated formation of membrane ruffles in fibroblasts (23, see also Table I). Mutational analysis showed that the PHn domain in C1199 Tiam1 is required for membrane localization. Since Tiam1 regulates the membrane cytoskeleton and appears to co-localize with F-actin in membrane ruffles, we investigated whether association of Tiam1 with cytoskeletal components might be involved. For this, NIH3T3 cells, which stably expressed C1199 Tiam1 or C682 Tiam1, were lysed in cytoskeleton-stabilizing buffer (CSK) containing 10 μM Heps, pH 6.8, 250 μM sucrose, 150 mM KCl, 1 mM EGTA, 3 mM MgCl₂, 0.5% Triton X-100 (26), and protease inhibitors aprotonin (2 μg/ml), leupeptin (2 μg/ml), soybean trypsin inhibitor (10 μg/ml), phenylmethylsulfonyl fluoride (0.2 mM), and Pefabloc (1 mM). All fractionation steps were performed at 4°C. Cells were scraped with a rubber policeman and left on ice for 30 min. A ‘total lysate’ sample was taken and, after addition of cytoskeleton-stabilizing buffer (CSK) containing 10 μM Heps, pH 6.8, 250 μM sucrose, 150 mM KCl, 1 mM EGTA, 3 mM MgCl₂, 0.5% Triton X-100 (26), and protease inhibitors aprotonin (2 μg/ml), leupeptin (2 μg/ml), soybean trypsin inhibitor (10 μg/ml), phenylmethylsulfonyl fluoride (0.2 mM), and Pefabloc (1 mM), the cell suspension was centrifuged at 300 g for 2 min. The resulting supernatant was centrifuged again for 2 min at 4,000 g. After 2 min at 4,000 g, the supernatant was removed and the remaining Triton X-100 lysate was centrifuged for 10 min at 15,000 × g. The resulting pellet was washed once in lysis buffer (resuspended and centrifuged again) and taken as ‘low speed cytoskeleton fraction.’

**Sucrose Gradients—**NIH3T3 cells expressing C1199 Tiam1 or C682 Tiam1 (17) were lysed in 500 μl of CSK buffer containing protease inhibitors and 0.5% Triton X-100 as described above. The lysates were adjusted, and after clearance of the 15,000 × g pellet, half of the supernatant was layered on a linear 10–50% w/w sucrose gradient in TEN (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 100 mM NaCl). The other half of the supernatant was brought to 0.5 μM CaCl₂ and layered on a similar sucrose gradient containing 0.5 μM CaCl₂. Gradients were centrifuged for 14–18 h at 55,000 rpm at 4°C in a Beckman SW55.1 rotor. Fractions (0.5 ml each) were collected from the top and analyzed by Western blotting after separation on 6–20% gradient SDS-PAGE under reducing conditions.

**Immunoblot Analysis—**Lysates and fractions were solubilized in Laemmli sample buffer, containing 0.1% dithiothreitol and separated by SDS-PAGE. Proteins were blotted onto nitrocellulose and stained with Ponceau S, and filters were blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 3% non-fat dried milk for at least 2 h, as described (17, 23). The filters were probed with primary antibody in blocking buffer for at least 1 h, washed three times in TBST, incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Corp.) in blocking buffer for 30 min. Proteins were visualized using enhanced chemiluminescence (ECL, Amersham Corp.) secondary antibodies. Cells were stained simultaneously for F-actin with TRITC-labeled phalloidin (2 units/ml) (Molecular Probes). At least 100 positive cells were analyzed. Images were recorded separately with a Bio-Rad MRC-600 confocal laser scanning microscope. For immunoelectron microscopy, cells were fixed and immunostained with a rabbit polyclonal anti-MAPK antibody, provided by P. Horák.
TABLE I
Schematic representation of Tiam1 mutant proteins and their properties

A, properties of the Tiam1 proteins with respect to the amount of protein present in the high speed Triton X-100-insoluble fraction, the membrane localization, and the capacity to induce membrane ruffling in COS-7 and NIH3T3 cells. M, myristoylation site; P, PEST regions; PHn and PHc, N- and C-terminal pleckstrin homology domain; CC, putative coiled coil region; DHR, disc large homology region; DH, Dbl homology domain; Ex, Ex region; PHn-CC-Ex, a region encompassing the PHn, CC, and Ex region. FL 1591, full-length Tiam1 consisting of 1591 amino acid(aa). Nomenclature of the constructs C and N refer to the number of C-terminal or N-terminal aa of Tiam1. Δ domain constructs refer to the deletion made in this domain (see "Experimental Procedures"). If the position of a deletion is indicated this refers to the aa position of full-length Tiam1. For all constructs, transfection resulted in expression of proteins of the expected size, as confirmed by Western blot analyses (see Figs. 1 and 3). a, amount of protein in the high speed pellet: ++ represents 30–60%; +, 10–30%; −, less than 10% (examples in Figs. 1 and 3), n.t., not tested. b, membrane localization: the protein is predominantly present at the plasma membrane (+) or in the cytoplasm (−). At least 100 cells were inspected by confocal laser scanning microscopy. * indicate that results were confirmed by immunoelectron microscopy (see Ref. 23 and examples in Fig. 6). c, the protein induces membrane ruffling in more than 80% of the transfected cells (+) or is unable to induce membrane ruffling (−). *, C1199ΔEx Tiam1 induced some membrane ruffling in about 10% of the COS cells expressing this protein, but not in NIH3T3 cells. B, properties of Tiam1 polypeptides involved in protein interactions. Nomenclature refers to the position of aa in full-length Tiam1. d, inhibition of membrane ruffling is scored upon cotransfection of the indicated competition constructs with C1199 Tiam1: ++ and + indicate inhibition of C1199 Tiam1-induced ruffling in about 80% and 50% of the cells, respectively (see Fig. 7A). Inhibition in less than 10% of the cells expressing both constructs was considered negative (−). C, conserved domains between Tiam1 and SIF (32, 43). The aa identity between the different domains is indicated. The PHn-CC-Ex region in Tiam1 ranges from aa 431 to 670, and in SIF from 821 to 1057.

### A

| M | PP | PHn CC | DHR | DH | PHc |
|---|----|--------|-----|----|-----|
| FL 1591 | ++ | + | + | + |
| N385 | - | + | - |
| C682 | - | - | - |
| C580 | - | - | - |
| C1199 | + | + | + |
| C1199ΔHHR | + | + | - |
| C1199ΔPHn1 | + | + | - |
| C1199ΔPHn3 | + | + | - |
| PHn-CC-C570 | + | + | - |
| C1199ΔEx | + | + | - |
| C1199ΔCC | + | + | - |
| C1199Δ(772-853) | + | + | + |
| PHn-CC-Ex-C570 | n.t. | + | + |

![Diagram](image)

**Functional protein interaction domain**

### B

| PHn-CC-Ex | functional protein interaction domain |
|---|---|
| ++ | + |
| PHn-CC | + |
| Ex(588-897) | + |
| Tiam1(794-1103) | - |

### C

| PHn CC-Ex | DHR | DH | PHc |
|---|----|----|-----|
| Tiam1 | 56 | 55 | 20 |
| SIF | 38 | 41 | 20 | % identity
and analyzed as described in Experimental Procedures. Cell equivalent amounts of the fractions were separated on 6% SDS-PAGE gels, blotted, and incubated with αDH-Tiam1 antibodies (32). For detection of endogenous MAPK, 12% gels were used. The two bands represent the two isoforms p42 and p44 of MAPK. Total lysate (T), low speed cytoskeleton fraction (L), high speed Triton X-100-insoluble fraction (H), and soluble fraction (S). A, endogenous Tiam1 in NIH3T3 cells was detected with αDH-Tiam1 antibodies.

The C1199 Tiam1 protein could be progressively extracted from the high speed Triton X-100-insoluble pellet fraction by solubilization in CSK buffer containing increasing concentrations of NaCl or in buffer of pH 11.5 (not shown, see Fig. 2). This suggests that the presence of C1199 Tiam1 in the high speed pellet is due to hydrophilic protein-protein interactions that are easily disrupted.

To study further the properties of the Tiam1-containing complexes, NIH3T3 cells expressing C1199 or C682 Tiam1 (Table I) were lysed and analyzed on sucrose gradients. As shown in Fig. 2A, fraction 6 contains the most C1199 Tiam1 protein, but a significant amount is also present in the high density sucrose fractions. In contrast, the smaller C682 Tiam1 protein, as well as the soluble MAPK protein, fractionated as single peaks in fractions 5–6. The trailing of C1199 Tiam1 over all sucrose fractions (up to fraction 14 containing 50% sucrose) suggests that C1199 Tiam1 is associated with complexes of heterogeneous size. This indicates that the components in the complex are not bound in a fixed stoichiometry and/or that different proteins contribute to these complexes. When lysates were analyzed on sucrose gradients in the presence of 0.5 M NaCl, the C1199 Tiam1 protein migrated in a single broad peak, similar to C682 Tiam1 and MAPK, indicating that the complexes containing C1199 Tiam1 had been disrupted (Fig. 2B). Protein binding studies using differently tagged Tiam1 proteins did not reveal any self-association or dimerization of Tiam1. Whereas the exact nature of the complexes in the high speed pellet remains to be established, our data support a model in which Tiam1 is present in multi-protein complexes that may contain cytoskeletal as well as non-cytoskeletal proteins and that are required for the proper localization and function of the protein.

**Determination of the Protein Interaction Domain**—To determine the region of Tiam1 involved in complex formation, various truncation and deletion mutants of Tiam1 (see Table I) were transiently expressed in COS-7 cells. This yielded similar results as found in NIH3T3 fibroblasts, with respect to the induction of ruffling as well as to Triton X-100 fractionation (e.g. compare C1199 Tiam1 fractionation in Figs. 1A and 3). Analyses of cells expressing full-length Tiam1 (FL 1591), the N-terminal 385 amino acids (N385 Tiam1), and the C-terminal 1199 Tiam1 and C682 Tiam1 indicated that localization of Tiam1 in Triton-insoluble complexes is determined by the region N-terminal to C682 Tiam1, consisting of approximately 500 amino acids (see Table I). This region contains the N-terminal PH domain and the DHR domain, both of which have been implicated in protein-protein associations (3, 24, 25). Furthermore, the Coils program of Lupas (28) predicts a putative coiled coil sequence of about 42 amino acids immediately C-terminally adjacent to PHn (CC-region), which might also participate in protein interactions. To delineate further the sequences required for interaction, deletion mutants of C1199 Tiam1 were analyzed (see Table I and Fig. 3). Deletion of the entire DHR domain (C1199ΔDHR) or a small deletion in the PHn domain (C1199ΔPHn1) did not affect complex association. However, deletion of the entire PHn (C1199ΔPHn3) reduced association with complexes in the high speed Triton X-100-insoluble pellet about 2-fold. Similarly, a large deletion be-
between the PHn-CC and the catalytic DH domain (PHn-CC-C570), resulted in decreased association. This suggested that both PHn-CC and a region extending C-terminally of PHn-CC (termed Ex-region) contribute to protein complex association. This was corroborated by analysis of cells expressing polypeptides representing the respective regions. Polypeptides containing the PHn-CC region (aa 393–652) or the Ex region (aa 588–897) were both partly recovered in the high speed pellet, whereas a polypeptide containing aa 794–1103 was completely soluble (Table 1B). Thus, the putative protein interaction domain, termed PHn-CC-Ex, encompasses the PHn domain, the coiled coil region (CC), and a region (Ex) extending C-terminally adjacent to the coiled coil region (see Table 1).

**Biological Function of the PHn-CC-Ex Region**—The functional relevance of the PHn-CC-Ex region was studied by determining the capacity of mutants to induce membrane ruffles upon transient expression in COS-7 cells (summarized in Table I). All mutants were equally expressed as determined by Western blotting (examples in Figs. 1 and 3). Similar to deletions in PHn, small deletions in the putative coiled coil sequence (C1199ΔCC) as well as in the Ex region (C1199ΔEx) abrogated membrane localization of Tiam1 and induction of membrane ruffling (Fig. 4 and Table I). Apparently, the entire PHn-CC-Ex region is required for the proper localization of Tiam1. The C1199Δ(772–853) Tiam1 deletion mutant still induced membrane ruffling (Fig. 4), indicating that the localization domain does not extend beyond amino acid 772.

Further evidence for an autonomous membrane targeting function of the PHn-CC-Ex region was obtained by transient expression of Myc epitope-tagged polypeptides containing Tiam1 regions derived from this domain (see Table 1B). Both the PHn-CC and the Ex polypeptide were present in the cytoplasm and did not associate with the plasma membrane (Figs. 5). In contrast, the PHn-CC-Ex polypeptide, which contains the complete putative protein interaction domain, was predominantly present at the plasma membrane (Fig. 5). This finding was confirmed by immuno-electron microscopy. Only the PHn-CC-Ex polypeptide, but not PHn-CC, predominantly localized at the plasma membrane (Fig. 6), indicating that the PHn-CC region is not sufficient for membrane localization. Similar results were obtained when instead of a Myc tag, a glutathione S-transferase protein was fused C-terminally to PHn-CC or PHn-CC-Ex. Only the PHn-CC-Ex was capable of targeting a fused heterologous passenger protein to the membrane (not shown). Since the PHn-CC and the Ex polypeptides did not localize at the plasma membrane, but were present in the high speed fraction (Table 1B), the supposed protein associations do not just occur at the membrane but also in the cytoplasm. Since only the complete PHn-CC-Ex region mediates membrane localization, we concluded that the cooperative actions of PHn-CC and Ex are required for localization of Tiam1 at the plasma membrane.

**The PHn-CC-Ex Region Interferes with Tiam1 Function**—To corroborate the functional importance of the PHn-CC-Ex region, we performed competition experiments with different parts of the PHn-CC-Ex region. For this, COS-7 cells were co-transfected with C1199 Tiam1 cDNA and constructs encoding the different PHn-CC-Ex regions, and the effect on C1199 Tiam1-induced formation of membrane ruffling was determined in cells expressing both constructs. As shown in Table IB and Fig. 7A, PHn-CC, despite high expression levels, is unable to inhibit C1199 Tiam1-induced formation of membrane ruffles. As expected, also the Tiam1 polypeptide Tiam1(794–1103), located outside the PHn-CC-Ex region, was unable to inhibit C1199 Tiam1-induced formation of membrane ruffles. In contrast, the polypeptide containing the complete PHn-CC-Ex region inhibits the C1199 Tiam1-induced membrane ruffling in more than 80% of the cells co-expressing both constructs. The co-expression of the different polypeptides with C1199 Tiam1 did not affect C1199 Tiam1 expression (Fig. 7B). Interestingly, also the Ex(588–897) polypeptide was able to inhibit Tiam1-induced ruffling, although less efficiently than the complete PHn-CC-Ex region.

**Fig. 3. Triton X-100-insolubility of C1199 Tiam1 deletion mutants.** Constructs encoding various deletion mutants of Tiam1 (see Table I) were expressed in COS-7 cells for 48 h and subjected to fractionation as described under Experimental Procedures. Cell equivalent amounts of the fractions were separated on SDS-PAGE gels and analyzed for the distribution of the mutant Tiam1 proteins and endogenous MAPK by Western blotting. Shown are examples of the high speed Triton X-100-insoluble (H) and the soluble (S) fractions of the most relevant mutants analyzed, as listed in Table IA.

**Fig. 4. Deletions in the C-terminal portion of the protein interaction domain abolish membrane ruffling.** Confocal images of COS-7 cells transiently expressing C1199 Tiam1, C1199ΔPHn1, C1199ΔCC, C1199ΔEx, and C1199Δ(772–853). Cells were fixed and stained with αDH-Tiam1 and FITC-coupled secondary antibodies and with TRITC-labeled phalloidin for F-actin. Bar represents 25 μm.
Transfected COS cells were stained with polypeptides containing PHn-CC-Ex, PHn-CC, and Ex. Transiently transfected COS cells were stained with αMyc antibody and FITC-coupled secondary antibodies and with TRITC-labeled phalloidin for F-actin. Some nuclear localization of the truncated proteins is also observed. Note that PHn-CC-Ex is highly enriched at the plasma membrane. F-actin. Some nuclear localization of the truncated proteins is also observed. Note that PHn-CC-Ex is highly enriched at the plasma membrane.

This suggests that the Ex region constitutes a functional region by itself, which binds to and competes for proteins required for proper functioning of Tiam1.

The PHn-CC-Ex Region Is Highly Conserved—The relevance of PHn-CC-Ex is further supported by the structure of the Drosophila still life (SIF) protein, which has recently been identified (29). The SIF protein, a Drosophila homologue of Tiam1, affects locomotion and is involved in synapse formation. The most conserved regions (±55–60% identity) include the DH and the PHn domain (see Table IC). Strikingly, the C-terminal region flanking the PHn domain of the SIF protein (SIF, aa 935–1058) is also strongly conserved (55% identity), much more than the DHR/PDZ domain (24% identity) (29). Moreover, the Coils program (28) also predicts a coiled coil adjacent to the PHn of the SIF protein. Apparently, the SIF protein contains a similar functional PHn-CC-Ex domain. Furthermore, the sequence and structure similarities between Tiam1 and SIF suggest a limitation of the size of the functional Ex region in Tiam1 to about 75 amino acids, suggesting that the PHn-CC-Ex region of Tiam1 encompasses amino acids 431–670. Indeed, fusion of the thus determined PHn-CC-Ex domain to the catalytic domain of Tiam1 (C570 Tiam 1) caused membrane localization of PHn-CC-Ex-C570 Tiam1 and the formation of membrane ruffles (Table I and Fig. 8). In contrast, PHn-CC-C570 Tiam1, which lacks the Ex region, was unable to localize at the plasma membrane and to induce membrane ruffling (Table I and Fig. 8). This substantiates our conclusion that the Ex region functions cooperatively with the PHn-CC region in membrane localization of Tiam1.

DISCUSSION

Tiam1 Harbors a Functional Protein Interaction Domain That Includes the N-terminal PH Domain—Previously, we have shown that the N-terminal PH (PHn) domain of Tiam1 is essential for serum-induced translocation of Tiam1 to the plasma membrane (23). Here we show that the localization of Tiam1 at the plasma membrane is regulated by a larger region that encompasses the PHn domain, a C-termiically adjacent putative coiled coil (CC) region, and an additional flanking region (termed Ex). This PHn-CC-Ex region is also responsible for recovery of Tiam1 in the subcellular Triton X-100-insoluble (200,000 × g) high speed pellet fraction. Such fractions have been described to contain submembranous cytoskeleton complexes as well as associated signaling molecules (27, 30, 31). Tiam1 was not found in the low speed (15,000 × g) Triton X-100-insoluble pellet that contains large cytoskeletal structures as well as caveolae and nuclei (26, 32). Apparently, either Tiam1 does not associate with these structures or this association is disrupted after lysis of cells. Electron microscopic analysis of the high speed pellet did not reveal any vesicle structures, suggesting that this pellet fraction contains predominantly small protein complexes. Both the PHn-CC region and the Ex region participate in these protein interactions since polypeptides encompassing either region were found in the Triton X-100-insoluble high speed pellet. Analysis by sucrose gradient centrifugation supported that Tiam1 is present in protein complexes that are heterogeneous in size and disrupted by high salt or alkaline conditions, indicative of hydrophilic interactions. Recently, it was demonstrated that the N-terminal PH domain of Tiam1 is capable of binding phosphatidylinositol-3,4,5-triphosphate and, to a lesser extent, also phosphatidylinositol-4,5-biphosphate (47). However, it is unlikely that this would influence fractionation in our experimental conditions, i.e. detergent containing lysis buffer. It is tempting to speculate that protein-lipid and protein-protein interactions mediated by the PHn and PHn-CC-Ex region, respectively, act in concert to target Tiam1 to the membrane.

The Entire PHn-CC-Ex Region of Tiam1 Is Required for Membrane Localization—Deletions in the putative protein interaction region in either the PHn domain, the coiled coil region, or the Ex region abolished membrane localization of C1199 Tiam1 and induction of membrane ruffling in NIH3T3 fibroblasts or COS-7 cells. Furthermore, a polypeptide encompassing the complete PHn-CC-Ex region was markedly enriched at the plasma membrane, whereas polypeptides encompassing the PHn-CC or the Ex region localized in the cytoplasm. This indicates that the PHn-CC-and Ex regions act cooperatively in membrane localization of Tiam1 and suggests that the entire PHn-CC-Ex region constitutes a functional unit. Indeed, similar to the membrane localization domain of c-Src (23), PHn-CC-Ex region, but not PHn-CC region, is able to localize C570 Tiam1 at the plasma membrane. Furthermore, the entire PHn-CC-Ex region is highly conserved in the SIF protein (29), a Drosophila homologue of Tiam1 (see Table I). In competition experiments, the entire PHn-CC-Ex region was able to inhibit the Tiam1-induced membrane ruffling in COS-7 cells. Interestingly, a polypeptide encoding the Ex region, which localized in the cytoplasm, had similar capacities, suggesting that the Ex region binds competitively to cytoplasmic components that are required for Tiam1 function. The inhibitory function of PHn-CC-Ex most likely involves competition for binding partners. Interference with intramolecular interac-

![Fig. 5. The entire PHn-CC-Ex is required for membrane localization.](image)

Confocal images of COS-7 cells expressing myc epitope-tagged polypeptides containing PHn-CC-Ex, PHn-CC, and Ex. Transiently transfected COS cells were stained with αMyc antibody and FITC-coupled secondary antibodies and with TRITC-labeled phalloidin for F-actin. Some nuclear localization of the truncated proteins is also observed. Note that PHn-CC-Ex is highly enriched at the plasma membrane. Bar represents 25 μm.

![Fig. 6. Ultrastructural localization of PHn-CC-Ex and PHn-CC in COS-7 cells.](image)

Immunelectron microscopy analysis of COS-7 transfected expressing Myc epitope-tagged PHn-CC-Ex or PHn-CC. Frozen sections were stained with αMyc antibodies followed by gold-conjugated secondary antibodies. PHn-CC-Ex predominately localizes at the plasma membrane and PHn-CC in the cytoplasm. At least 40 sections were analyzed. Bar represents 400 nm.
The PH domain of Tiam1 induces membrane ruffling. Confocal images of COS-7 cells expressing PHn-CC-Ex-C570 and PHn-CC-C570. Transiently transfected COS-7 cells were stained with αDH-Tiam1 and FITC-coupled secondary antibodies and with TRITC-labeled phalloidin for F-actin. Bar represents 25 μm.

**Fig. 7.** The PHn-CC-Ex region is capable of inhibiting C1199 Tiam1-induced membrane ruffling. A, competition experiments in COS-7 cells co-expressing C1199 Tiam1 and PHn-CC, Ex or PHn-CC-Ex. C1199 Tiam1 expression was detected by staining with αDH-Tiam1 antibodies followed by FITC-coupled secondary antibodies. Expression of the competing protein was analyzed by staining with αMyc antibodies followed by Texas Red-coupled secondary antibodies. At least 100 cells that expressed both constructs were scored for the presence of membrane ruffles. B, Western blot analysis of cell lysates of co-transfected COS-7 cells (see A). Polypeptides were detected with αMyc tag antibodies and C1199 Tiam1 with αCT-Tiam1 antibodies. Note that the expression of the different polypeptides did not affect co-expression of C1199 Tiam1.

The fact that PH domains in proteins generally cannot be functionally exchanged (23, 37, 38) suggests that they provide distinct targeting signals or that they function in concert with adjacent sequences or other domains. The PH domain of β-adrenergic receptor kinase mediates protein binding through the C terminus and lipid binding through the N terminus, both of which are required for membrane localization and optimal kinase activation (6). For protein binding, an extension of about 25 amino acids is necessary. Similarly, in several other proteins, like Btk, Akt, and Tec, the PH domain is extended with approximately 30 amino acids (39–42). In Akt, this extension is required for the formation of protein complexes (35), whereas the extended PH domain of the Btk kinase binds protein kinase C at its N terminus and βγ subunits at the carboxyl end (40, 43). Apparently, short extensions of PH domains are often required for protein interactions, indicating that the structurally conserved minimal size of the PH domain does not exert all functions.

The N-terminal PH domain of Tiam1 was found to be part of a larger functional unit encompassing the PHn, the CC, and the Ex region. This organization of a PH domain in a functional unit is strikingly similar in the neuronal exchange factor Ras-GRF. In this RasGEF, the N-terminal PH domain is flanked by a coiled coil region followed by an IQ domain, which can bind calmodulin (37). Similar to that found for Tiam1, these domains function cooperatively in RasGRF activation and proper localization in the particulate fraction. Activation is mediated by calcium-induced calmodulin binding to the IQ domain, whereas the N-terminal PH domain is essential but not sufficient for particulate association (37). Virtually all other GEFs, like Dbl, Ost, Vav, Dbs and Lfc, contain one single PH domain, C-terminally adjacent to the DH domain (19, 20). These DH-flanking PH domains also have been shown to be essential for the intracellular localization and/or activity of these GEFs, but little is known whether additional sequences or domains play a role. Deletions in the PH domains of Dbl, Ost, Dbs, and Lfc abolished the transforming activity of these proteins (20). The transforming capacity of mutant Lfc, with a deletion in the PH domain, could be restored by the addition of a C-terminal Ras-CAAX motif, suggesting that this PH domain mediates localization of Lfc at the plasma membrane (44). In contrast, the Ras-CAAX motif could not replace the PH domain of Dbl for transforming activity (45). The PH domain of Dbl, however, was required for the presence of Dbl in the Triton X-100-insoluble portion of the particulate fraction of cells (45). Apparently, also Dbl requires specific protein interactions through its PH domain for proper functioning. In fibroblasts, Tiam1 localizes to the plasma membrane upon serum stimulation (23), suggesting that receptor stimulation regulates Tiam1 translocation. What kind of proteins associate with Tiam1 and play a role in membrane translocation remains to be established. It is tempting to speculate that adaptor proteins might be involved, in a way analogous to the activation of Ras by membrane-translocated Sos (46). The cooperative function of the PHn-CC and the Ex domain may allow integration of various signals, in response to receptor stimulation. Since the N-terminal PH domain of Tiam1 is capable of binding to phosphatidylinositol 3,4,5-triphosphate and, less strongly, to phosphatidylinositol...
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4,5-bisphosphate (47), this may involve specific lipids in combination with proteins that play a role in receptor signaling and cytoskeletal rearrangements.

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