Translocation of the Rac1 Guanine Nucleotide Exchange Factor Tiam1 Induced by Platelet-derived Growth Factor and Lysophosphatidic Acid*

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Several guanine nucleotide exchange factors for the Rho family of GTPases that induce activation by exchanging GDP for GTP have been identified. One of these is the tumor invasion gene product Tiam1, which acts on Rac1. In this study, we demonstrate that platelet-derived growth factor (PDGF) and lysophosphatidic acid induce the translocation of Tiam1 to the membrane fraction of NIH 3T3 fibroblasts in a time-dependent manner. Previously, we have shown that Tiam1 is phosphorylated by protein kinase C (PKC) and calcium/calmodulin kinase II (CaMK II) after stimulation with agonists. Here we show, by pretreatment of cells with kinase inhibitors, that CaMK II, but not PKC, is involved in the membrane translocation of Tiam1. Addition of the calcium ionophore ionomycin alone induced the translocation of Tiam1. However, the cell-permeable diacylglycerol oleoylacylglycerol was without effect and did not enhance the effect of ionomycin. These data further indicated a role for CaMK II and not PKC. Inhibition of phosphoinositide 3-kinase by wortmannin had little effect on the translocation of Tiam1. The role of phosphorylation was further studied by comparing the phosphorylation pattern of Tiam1 in the membranes versus whole cell Tiam1. PDGF-induced phosphorylation of membrane-associating Tiam1 occurred more rapidly than that of the total Tiam1 pool, and CaMK II, but not PKC, played a significant role in this process. Furthermore, by using the p21-binding domain of PAK-3, we show that PDGF, but not lysophosphatidic acid, activates Rac1 in vivo and that this activation involves CaMK II and PKC, but not 3-phosphoinositides. Our results indicate that Tiam1 is translocated to and phosphorylated at membranes after agonist stimulation and that CaMK II, but not PKC, is involved in this process. Also, these kinases are involved in the activation of Rac in vivo.

Rho family GTPases are a subfamily of the Ras superfamily of small G proteins. Better characterized members are RhoA, RhoB, RhoC, Rac1, Rac2, and Cdc42. These proteins mediate the actions of various extracellular agonists to elicit different forms of cytoskeletal reorganization and other cellular responses. Agonists such as lysophosphatidic acid (LPA),1 which stimulate heterotrimeric G protein-coupled receptors, induce stress fiber and focal contact formation through the activation of Rho-mediated pathways. Receptors with tyrosine kinase activity that are activated by growth factors such as platelet-derived growth factor (PDGF) or insulin stimulate the generation of lamellipodia (membrane ruffles) through the activation of Rac-mediated pathways. Other agonists like bradykinin induce the Cdc42-mediated formation of filopodia (1–3). Rho family GTPases are regulated by the binding of guanine nucleotides. They are inactive when bound to GDP and active when bound to GTP. Guanine nucleotide exchange factors (GEFs) exchange GTP for GDP, thus rendering the G proteins active (4). GTPase-activating proteins increase the intrinsic GTPase activity of the G proteins, which switches them to an inactive state following the hydrolysis of GTP to GDP (5). The Rho family GEFs are characterized by Dbl homology and pleckstrin homology (PH) domains in their sequences. The Dbl homology domain is proposed to be responsible for the GEF activity, whereas the PH domain is a binding site of lipids such as phosphatidylinositol 4,5-bisphosphate and/or phosphatidylinositol 3,4,5-triphosphate (PIP3) (6).

Tiam1 is a guanine nucleotide exchange factor for Rac1 that encodes two PH domains and one Dbl homology domain. It was first characterized by its ability to induce T lymphoma cells to invade monolayers of fibroblasts (7). Previously, we showed that Tiam1 is phosphorylated by protein kinase C (PKC) and calcium/calmodulin kinase II (CaMK II) in vitro and upon treatment with PDGF or LPA in vivo (8, 9). Phosphorylation of the N-terminally truncated form of Tiam1 by CaMK II increases the GEF activity toward Rac in vitro, indicating an important role for CaMK II activity in vivo (10). In contrast, in vitro phosphorylation of truncated Tiam1 by PKCα has no effect on GEF activity toward Rac1. Recent studies have shown that the generation of PIP3 by the activation of phosphoinositide 3-kinase (PI3K) can increase the amount of GTP bound to Rac after PDGF stimulation (5). The PI3K inhibitor wortmannin has also been shown to inhibit the formation of Rac-GTP and also membrane ruffling in Swiss 3T3 fibroblasts while not affecting stress fiber formation mediated by Rho (11). Wortmannin also blocks Rac2 activation induced by N-formylmethioninyl-leucyl-phenylalanine in neutrophils (12).

The co-localization of proteins and/or lipids is an important factor in the regulation of signaling cascades. Different agonists induce distinctive reorganization of the actin cytoskeleton* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; GEF, guanine nucleotide exchange factor; PKC, protein kinase C; CaMK II, calcium/calmodulin protein kinase II; PI3K, phosphoinositide 3-kinase; OAG, 1-oleoyl-2-acetyl-sn-glycerol; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid/acetoxymethyl ester; PBS, phosphate-buffered saline; RIPA, radioimmune precipitation assay; GTPγS, guanosine 5’-O-(3-thiotriphosphate).
and induce the translocation of Rho family proteins. For example, the treatment of Swiss 3T3 fibroblasts with LPA induces the translocation of Rho, but not Rac, to a membrane fraction including caveolae. Conversely, the treatment of fibroblasts with PDGF induces the translocation of Rac, but not Rho, to the membranes and caveolae (13, 14).

There have been few reports of the translocation of GEFs in response to agonists. The Ras family GEF SOS has been shown to translocate to membranes upon stimulation with insulin, epidermal growth factor, or macrophage-stimulated protein (15, 16). Moreover, the translocation of SOS alone is sufficient to observe activation of Ras (17). There is little information on agonist-induced translocation of Ras family GEFs. Tiam1 has been shown to associate with the membrane upon serum stimulation, and membrane association of Tiam1 is required for membrane ruffling (18). Furthermore, the membrane association of Tiam1 is dependent upon the N-terminal PH domain and an as yet undetermined adjacent protein interaction domain (19). To further define the role of translocation in the activation of small G proteins, we examined if agonists could induce membrane association of Tiam1 and explored what role phosphorylation served in this process.

Until recently, the study of the in vivo activation of small G proteins has involved the observation of downstream morphological changes. As stated above, Rac activation leads to membrane ruffling, and Rho activation leads to stress fiber formation. Current research has provided a new assay for the quantitation of the in vivo activity of the small G proteins. Downstream protein kinases such as the PAK and PRK isoforms have been shown to bind to Rho family GTPases (20, 21). The association occurs when the small G proteins are in an active GTP-bound form, and this association activates the kinase (22, 23). The binding domains of these kinases have been identified and are currently being used to determine the in vivo activity of the G proteins. We utilized the p21-binding domain of PAK-3 (24) to determine what effects phosphorylation and PI3K activity have on agonist-induced activation of Rac1.

**Experimental Procedures**

**Materials**—NIH 3T3 fibroblasts were obtained from the American Type Culture Collection. Calf serum, Dulbecco’s modified Eagle’s medium, penicillin, and streptomycin were from Life Technologies, Inc. Lysophosphatidic acid and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were from Avanti Polar Lipids. Ro-31-8220, KN-93, ionomycin, and BAPTA/AM were from Calbiochem. Wortmannin, leupeptin, antipain, sodium orthovanadate, Tween 20, Triton X-100, sodium fluoride, sodium pyrophosphate, and phenylmethylsulfonyl fluoride were from Sigma. BCA protein determination reagents were from Pierce. Rac1 and β1 integrin antibodies were from Transduction Laboratories. The p21-binding domain of PAK-3 was a kind gift from Dr. Richard Cerione (Cornell University, Ithaca, NY). The phosphothreonine antibody was from Zymed Laboratories Inc., and the Tiam1 antibody was from Santa Cruz Biotechnology. Platelet-derived growth factor was from Roche Molecular Biochemicals. Horseradish peroxidase-conjugated secondary antibodies were from Vector Laboratories, Inc. Polyvinylidene difluoride membranes were from Millipore Corp.

**Cell Culture Conditions**—NIH 3T3 fibroblasts were maintained in HEPES-buffered Dulbecco’s modified Eagle’s medium with 4 mm L-glutamine supplemented with 10% (v/v) calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2. Cells were grown in 100-mm dishes for 1–2 days to a confluency of 60–70%. The medium was replaced with a reduced serum medium (Dulbecco’s modified Eagle’s medium containing 0.5% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) for 24 h to induce quiescence of the cells. The cells were then washed twice with PBS and resuspended in 1 ml of lysis buffer. The cells were disrupted by brief sonication and centrifuged at 100,000 × g for 60 min. The pellets were resuspended in RIPA buffer containing 1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1% dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml antipain, 1 mM phenylmethylsulfonyl fluoride, 500 μM sodium orthovanadate, 10 mM sodium pyrophosphate, and 10 mM sodium fluoride and incubated for 1 h at 4 °C with Tiam1 antibody. Protein A-agarose beads were added, and the lysate was rocked for an additional 1 h at 4 °C. The beads were collected by centrifugation and washed three times with RIPA buffer. The beads were then resuspended in 1× Laemmli sample buffer and boiled for 5 min.

**Immunoprecipitation of Tiam1 from Membrane Fractions—Quiescent cells were treated with inhibitors and/or agonists at 37 °C for various times as indicated in the figure legends. The cells were rinsed twice in ice-cold PBS and scraped in 500 μl of lysis buffer. The cells were then disrupted by brief sonication and centrifuged at 100,000 × g for 60 min. The pellets were resuspended in RIPA buffer containing 0.1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1% dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml antipain, 1 mM phenylmethylsulfonyl fluoride, 500 μM sodium orthovanadate, 10 mM sodium pyrophosphate, and 10 mM sodium fluoride and incubated for 1 h at 4 °C with Tiam1 antibody. Protein A-agarose beads were added, and the lysate was rocked for an additional 1 h at 4 °C. The beads were collected by centrifugation and washed three times with RIPA buffer. The beads were then resuspended in 1× Laemmli sample buffer and boiled for 5 min.

**Immunoprecipitation of Tiam1 from Whole Cells—Quiescent cells were treated with inhibitors and/or agonists at 37 °C for various times as indicated in the figure legends. The cells were rinsed twice in ice-cold PBS and scraped in 500 μl of lysis buffer. The cells were then disrupted by brief sonication and centrifuged at 10,000 × g for 10 min. The supernatant was incubated with PAK-3, 20 μl of buffer containing 20 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 100 mM NaCl, 10 mM MgCl2, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml antipain, 1 mM phenylmethylsulfonyl fluoride, 500 μM sodium orthovanadate, 10 mM sodium pyrophosphate, and 10 mM sodium fluoride. The cells were disrupted by five passes through a 27-gauge needle and incubated at 4 °C for 30 min. The lysate was centrifuged at 10,000 × g for 10 min. The supernatant was incubated with antibody conjugated to glutathione S-transferase on Sepharose beads (or glutathione S-transferase-Sepharose alone) at 4 °C. (Prior to incubation with PAK-3, 20 μl of supernatant was removed to determine equal amounts of Rac.) The beads were collected by centrifugation and washed three times with the above buffer. The beads were then resuspended in 1× Laemmli sample buffer and boiled for 5 min.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—SDS gel electrophoresis was performed on 6% Tris/glycine-polyacrylamide gels at 100 V for 2.5 h, and the proteins were transferred onto polyvinylidene difluoride membranes using a Novex wet transfer apparatus at 20 V for 90 min. The membranes were blocked overnight in 5% (w/v) nonfat dry milk (Bio-Rad). The blots were incubated for 1 h in the presence of primary antibody, rinsed three times in Tris-buffered saline containing Tween 20, and incubated with the corresponding secondary antibody conjugated to horseradish peroxidase. Bands were visualized with the enhanced chemiluminescence kit from Amersham Pharmacia Biotech.

**Results**

**Translocation of Tiam1 Induced by PDGF and LPA—**Tiam1 has been shown to associate with the membrane fraction, and mutational studies have indicated that this association is mediated by the N-terminal PH domain, an adjacent coiled-coil region, and a surrounding sequence (18, 19). To determine what factors induce this association, we examined the translocation of Tiam1. Tiam1 was induced by two different agonists, PDGF and LPA, in NIH 3T3 cells. Both agonists caused translocation of Tiam1 to the membrane fraction in a time-dependent manner (Fig. 1). The increase in Tiam1 at the membrane induced by PDGF was apparent in 1 min and was increasingly sustained through 30 min. Membrane fractions were also blotted with β1 integrin to show equal loading. Tiam1 translocation was de-
Western-blotted with Western blotting with Tiam1 antibody. Membrane fractions were also prepared as described under “Experimental Procedures.” The amount of Tiam1 in the cytosol or membrane was determined by Western blotting with Tiam1 antibody. Membrane fractions were also Western-blotted with β1 integrin to show equal loading. Results are representative of three different experiments.

tectable with 5 ng/ml PDGF and maximal with 50 ng/ml (data not shown). The amount of Tiam1 in the cytosol steadily declined and was absent at the 30-min time point, thus mirroring the increase in the membrane. LPA also induced a similar time course of translocation, except that membrane association of Tiam1 returned to basal levels by 30 min (Fig. 1). In general, the effect was smaller and less sustained than that of PDGF and was induced by LPA concentrations as low as 25 μM. As seen with the PDGF-induced translocation, the level of Tiam1 in the cytosol upon LPA stimulation correlated with the level of Tiam1 in the membrane. The cytosolic level of Tiam1 decreased as the membrane level increased through 10 min of stimulation. Furthermore, at 30 min, the loss of Tiam1 in the membrane fraction was mirrored by an increase in the cytosol. These two distinct time courses illustrate the different effects of PDGF and LPA on the translocation of Tiam1.

Effect of Ro-31-8220 and KN-93 on the PDGF- and LPA-induced Translocation of Tiam1—Previously, we showed that two protein kinases, PKC and CaMK II, phosphorylate Tiam1 in vivo and in vitro (8, 9). To determine if these kinases play a role in the translocation of Tiam1, we used inhibitors to block their activity and then observed the translocation induced by PDGF or LPA. The staurosporine analog Ro-31-8220 has been shown to inhibit the kinase activity of PKC (25), and KN-93 has been shown to inhibit CaMK II activity (26). As shown in Figs. 2 and 3, Ro-31-8220 had no consistent effect on the translocation of Tiam1 to the membrane induced by either PDGF or LPA. However, in the presence of the inhibitor KN-93, the PDGF- or LPA-induced translocation of Tiam1 was greatly reduced (Figs. 2 and 3).2 These data indicate that the kinase activity of CaMK II, but not that of PKC, is involved in the translocation of Tiam1. Previously, we observed that the inhibition of PKC or CaMK II alone reduced the phosphorylation of Tiam1 on threonine and that inhibition of both kinases virtually abolished all threonine phosphorylation of Tiam1 (10). Therefore, we tested if inhibition of both kinases could completely inhibit membrane translocation of Tiam1. However, the combination of inhibitors had no greater effect than that of KN-93 alone (Figs. 2 and 3), indicating again that CaMK II activity, but not PKC activity, is involved in the translocation of Tiam1.

The pH domain has been shown to bind phosphatidylinositol phosphates such as PIP_3 (27), which is the product of PI3K action (28). Since PDGF treatment stimulates PI3K activity (29), we sought to determine if inhibition of PIP_3 generation would affect the translocation of Tiam1. Cells were pretreated with the PI3K inhibitor wortmannin and then stimulated with PDGF or LPA. In the presence of wortmannin, we observed a small, but reproducible decrease in the translocation of Tiam1 induced by PDGF (Fig. 2). However, no consistent effect of the inhibitor was observed on the smaller translocation induced by LPA (Fig. 3). These data indicate that the generation of PIP_3 plays a lesser role in the translocation of Tiam1 induced by PDGF than does CaMK II activity. Membrane samples from Figs. 2 and 3 were blotted with β1 integrin to show equal loading, and cytosolic blots of Tiam1 complemented those of the membrane fractions, as shown in Fig. 1 (data not shown).

Effect of Calcium and Diacylglycerol on the Translocation of Tiam1—To further investigate the role of phosphorylation in the translocation of Tiam1 to the membrane, we sought to determine if a rise in Ca^{2+} could stimulate translocation. The calcium ionophore ionomycin has been shown to increase intracellular Ca^{2+} levels (30). This increase in Ca^{2+} can serve to activate CaMK II and can also participate with diacylglycerol in the activation of classical isozymes of PKC. Therefore, NIH 3T3 fibroblasts were treated with ionomycin and/or OAG, a water-soluble form of diacylglycerol, to determine if they affected membrane translocation of Tiam1. As shown in Fig. 4, treatment with ionomycin resulted in a substantial increase in Tiam1 in the membrane fraction, suggesting that activation of CaMK II alone can translocate Tiam1. In contrast, treatment with OAG did not induce translocation of Tiam1 and did not alter the effect of ionomycin. As noted in Figs. 2 and 3, membrane fractions were blotted with β1 integrin to verify equal protein loading, and changes in cytosolic Tiam1 reflected those in the membrane fractions (data not shown). These data support the conclusion that activation of CaMK II, but not PKC, is able to translocate Tiam1 to the membrane fraction.

Phosphorylation of Tiam1 at the Membrane—To further explore the role of phosphorylation in the translocation of Tiam1, we compared the phosphorylation patterns of Tiam1 in whole cells versus that in the membrane fraction in NIH 3T3 fibro-

\[ \text{PDGF} \]

\[
\begin{array}{cccccc}
0 & 1 & 5 & 10 & 30 & \\
\text{membrane} & \text{cytosol} & \text{Tiam1} & \beta_1 \text{Integrin} & \\
\end{array}
\]

\[ \text{LPA} \]

\[
\begin{array}{cccccc}
0 & 1 & 5 & 10 & 30 & \\
\text{membrane} & \text{cytosol} & \text{Tiam1} & \beta_1 \text{Integrin} & \\
\end{array}
\]

\[ \text{FIG. 1.} \quad \text{Platelet-derived growth factor and lysophosphatidic acid induce the translocation of Tiam1 to the membrane. NIH 3T3 fibroblasts were treated with either 10 ng/ml PDGF or 100 μM LPA for the indicated times. Cells were lysed, and cytosol and membrane fractions were prepared as described under “Experimental Procedures.” The amount of Tiam1 in the cytosol or membrane was determined by Western blotting with Tiam1 antibody. Membrane fractions were also Western-blotted with β1 integrin to show equal loading. Results are representative of three different experiments.} \]

\[ \text{FIG. 2.} \quad \text{Effect of PKC and CaMK II inhibitors on PDGF-induced translocation of Tiam1. NIH 3T3 fibroblasts were preincubated with vehicle (Me}_2\text{SO;} -\text{, 5 μM Ro-31-8220 (Ro; +)} \text{ for 60 min, 20 μM KN-93 (+) for 24 h, or 1 μM wortmannin (Wort; +)} \text{ for 15 min. Cells were then treated with 10 ng/ml PDGF for the indicated times. Cells were lysed, and cytosol and membrane fractions were prepared as described under “Experimental Procedures.” The amount of Tiam1 at the membrane was determined by Western blotting with Tiam1 antibody. Results are representative of three different experiments.} \]

\[ \text{The cells were treated with KN-93 for 24 h to be consistent with our earlier study (10). However, an inhibitory effect of KN-93 could be observed after 2 h of treatment.} \]
Translocation of the Rac1 GEF Tiam1 Induced by PDGF and LPA

**FIG. 3.** Effect of PKC and CaMK II inhibitors on LPA-induced translocation of Tiam1. NIH 3T3 fibroblasts were preincubated with vehicle (Me2SO; −), 5 μM Ro-31-8220 (Ro; +) for 60 min, 20 μM KN-93 (+) for 24 h, or 1 μM wortmannin (Wort; +) for 15 min. Cells were then treated with 100 μM LPA for the indicated times. Cells were lysed, and the cytosol and membrane fractions were prepared as described under "Experimental Procedures." The amount of Tiam1 at the membrane was determined by Western blotting with Tiam1 antibody. Results are representative of three different experiments.

**FIG. 4.** Tiam1 is translocated to the membrane fraction by an increase in intracellular calcium. NIH 3T3 fibroblasts were treated with 10 ng/ml PDGF, 5 μM ionomycin (Iono), and/or 40 μM OAG for 5 min. Cells were lysed, and cytosol and membrane fractions were prepared as described under "Experimental Procedures." The amount of Tiam1 at the membrane was determined by Western blotting with Tiam1 antibody. Results are representative of three different experiments.

**FIG. 5.** Agonist-induced phosphorylation of Tiam1 in the membrane fraction versus total cellular Tiam1. NIH 3T3 fibroblasts were treated with either 10 ng/ml PDGF (upper) or 100 μM LPA (lower) for the indicated times. Cells were lysed, and Tiam1 was immunoprecipitated from whole cells; or the cells were lysed, membrane fractions were prepared, and Tiam1 was immunoprecipitated as described under "Experimental Procedures." The amount of Tiam1 phosphorylation was determined by Western blotting with phosphothreonine antibody. Results are representative of three different experiments.

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blasts treated with agonists. The cells were either lysed in RIPA buffer or fractionated in a non-detergent buffer. Tiam1 was then immunoprecipitated from whole cells or the membrane fraction, and Western blotting was performed with phosphothreonine antibodies. In whole cells, PDGF induced phosphorylation of Tiam1, which reached a maximum at 10 min (Fig. 5, upper). This is consistent with the previously reported time course in Swiss 3T3 fibroblasts (9). However, a very different time course of Tiam1 phosphorylation was seen in the membrane fraction. The time course of phosphorylation of membrane-associated Tiam1 was much faster than that of total (cytosol + membrane) Tiam1 (Fig. 5, upper). The phosphorylation of Tiam1 in the membranes was maximal at 1–5 min and nearly disappeared at 30 min, whereas total phosphorylation was maximal at 10 min and still evident at 30 min. These data indicate that Tiam1 is very rapidly phosphorylated in membranes and then becomes dephosphorylated since Tiam1 protein is still present in the membranes at 10 and 30 min (see Fig. 1). On the other hand, total phosphorylation, which mainly represents that in the cytosol, occurs more slowly and correlates with membrane association of Tiam1 (Fig. 1).³

Interestingly, the LPA-induced phosphorylation of total and membrane pools of Tiam1 showed a more similar time course. As Fig. 5 (lower) shows, the phosphorylation of Tiam1 increased by 1 min and was sustained through 30 min in both fractions. There was no delay in total Tiam1 phosphorylation as seen with PDGF. There was a decrease in phosphorylated Tiam1 at 30 min in both total and membrane fractions. The latter corresponded with the loss of Tiam1 from the membranes (Fig. 1).

Effect of Kinase Inhibitors on the Agonist-induced Phosphorylation of Membrane-associated Tiam1—To further define what role phosphorylation plays in the translocation of Tiam1, we pretreated NIH 3T3 fibroblasts with Ro-31-8220, KN-93, or wortmannin and then induced the translocation and phosphorylation of Tiam1 with agonists. Addition of the PKC inhibitor Ro-31-8220 had no effect on the phosphorylation of membrane-associated Tiam1 (Fig. 6), just as it had no effect on the translocation of Tiam1 (Fig. 2). Although inhibition of PKC has been shown to reduce total cellular Tiam1 phosphorylation (9), the data of Fig. 6 indicate that this primarily involves cytosolic Tiam1. PDGF-induced phosphorylation of membrane Tiam1 was greatly reduced by addition of the CaMK II inhibitor KN-93. However, this is probably mostly due to the loss of Tiam1 at the membrane (Fig. 6; cf. Fig. 2). Although the inhibition of PI3K by addition of wortmannin caused a slight decrease in the PDGF-induced translocation of Tiam1 (Fig. 2), wortmannin had no detectable effect on the phosphorylation of Tiam1 at the membrane (Fig. 6).

Similar results were observed when cells were treated with LPA. As shown in Fig. 7, the PKC inhibitor Ro-31-8220 had no effect on the phosphorylation of membrane-bound Tiam1. The CaMK II inhibitor KN-93 produced a decrease in Tiam1 phosphorylation, which, again, is probably due mostly to the loss of Tiam1 from the membrane (Fig. 3). As in the case of PDGF, inhibition of PI3K by wortmannin had no effect on the phosphorylation of membrane-associated Tiam1 by LPA.

Effect of PDGF and LPA on the in Vivo Activation of Rac—To determine the role of translocation and phosphorylation in the in vivo activation of Tiam1, we observed the activation of Rac1 under similar conditions. As stated above, small G proteins such as Rac are active when bound to GTP and inactive when bound to GDP. Also, the guanine nucleotide exchange factor Tiam1 serves to exchange the GDP for GTP, thus rendering Rac active. A novel set of binding domains have been used to determine the amount of small G protein that is active (GTP-associated). These binding domains include PRK-1 (31) and rhoetokin (32), which bind only to Rho-GTP, and the p21 (Cdc42 and Rac)-binding domain of PAK-3, which binds to Rac-GTP and Cdc42-GTP (24). Therefore, by using these domains, the active forms of small G proteins can be affinity-purified. As shown in Fig. 8, treatment of NIH 3T3 fibroblasts with PDGF induced a time-dependent increase in the amount of affinity-purified Rac1-GTP, which was maximal at 4 min.

³ We were unable to consistently immunoprecipitate Tiam1 from the cytosol to determine the phosphorylation of Tiam1 in this fraction alone.
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FIG. 6. Effect of Ro-31-8220, KN-93, and wortmannin on the PDGF-induced phosphorylation of membrane-associated Tiam1. NIH 3T3 fibroblasts were preincubated with vehicle (Me2SO; −), 5 μM Ro-31-8220 (Ro; +) for 60 min, 20 μM KN-93 (+) for 24 h, or 1 μM wortmannin (Wort; +) for 15 min. Cells were then treated with 10 ng/ml PDGF for the indicated times. Cells were lysed, and membrane fractions were prepared. Tiam1 was immunoprecipitated from the membrane fraction as described under “Experimental Procedures.” The amount of Tiam1 phosphorylation was determined by Western blotting with phosphothreonine antibody. Results are representative of three different experiments.

FIG. 7. Effect of Ro-31-8220, KN-93, and wortmannin on the LPA-induced phosphorylation of membrane-associated Tiam1. NIH 3T3 fibroblasts were preincubated with vehicle (Me2SO; −), 5 μM Ro-31-8220 (Ro; +) for 60 min, 20 μM KN-93 (+) for 24 h, or 1 μM wortmannin (Wort; +) for 15 min. Cells were then treated with 100 μM LPA for the indicated times. Cells were lysed, and membrane fractions were prepared. Tiam1 was immunoprecipitated from the membrane fraction as described under “Experimental Procedures.” The amount of Tiam1 phosphorylation was determined by Western blotting with phosphothreonine antibody. Results are representative of three different experiments.

The treatment of cells for longer periods of time (up to 60 min) did not show any increase in Rac1-GTP above basal levels (data not shown). Interestingly, this time course of Rac1 activation more closely resembles the time course of Tiam1 phosphorylation at the membrane than that of total Tiam1 phosphorylation (cf. Fig. 5). Unlike PDGF, the treatment of cells with LPA did not produce any consistent increase in the levels of Rac1-GTP over basal levels (Fig. 8). These data demonstrate that although LPA can induce the translocation and phosphorylation of Tiam1, LPA does not induce a significant activation of Rac.

Effect of PKC and CaMK II Inhibitors on the PDGF-induced Activation of Rac—Since CaMK II is involved in the translocation (Fig. 2) and membrane phosphorylation (Fig. 6) of Tiam1, we sought to determine if CaMK II activity is required for the PDGF-mediated activation of Rac. In the presence of the CaMK II inhibitor KN-93, the amount of GTP-associated Rac1 seen in the presence of PDGF was reduced by >50% (Fig. 9). However, unlike the translocation and membrane phosphorylation data, addition of the PKC inhibitor Ro-31-8220 blocked the activation of Rac1 in a similar manner. Surprisingly, Ro-31-8220 increased the basal concentration of Rac-GTP. However, this compound also increased the basal translocation of Tiam1 as well (Fig. 2). We have also found that phosphorylation in general is important in Rac1 activation since the removal of phosphatase inhibitors from the affinity purification buffer resulted in the loss of most all Rac1-GTP complexes. Since guanine nucleotide exchange factors such as Tiam1 have been shown to contain a PH domain, we were also interested in determining the role of PIP3 in the activation of Tiam1. As shown in Fig. 9, addition of wortmannin, which blocks the formation of PIP3, had no effect on the activation of Rac1. These 4 These effects of Ro-31-8220 may be related to its stimulating effect on inositol phosphate production in fibroblasts (41).

5 F. G. Buchanan, C. M. Elliot, M. Gibbs, and J. H. Exton, unpublished observations.

FIG. 8. PDGF- and LPA-induced activation of Rac. NIH 3T3 fibroblasts were treated with 10 ng/ml PDGF or 100 μM LPA for the indicated times. Rac associated with GTP was immunoprecipitated from the cell lysates as described under “Experimental Procedures” with the p21-binding domain of PAK-3 (PBD-PAK-3) immobilized on agarose beads. The amount of Rac-GTP was visualized by Western blotting with antibody specific for Rac1 (top blots). The bottom blots show 2% of the total cell lysate used for the affinity purification. Results are representative of three different experiments.

FIG. 9. Effect of kinase inhibitors and wortmannin on the PDGF-induced activation of Rac. NIH 3T3 fibroblasts were preincubated with vehicle (Me2SO (DMSO)), 5 μM Ro-31-8220 for 60 min, or 20 μM KN-93 for 24 h (A) or with vehicle (Me2SO; −) or 1 μM wortmannin (+) for 15 min (B). Cells were then treated with 10 ng/ml PDGF for 4 min. Rac associated with GTP was immunoprecipitated from the cell lysates as described under “Experimental Procedures” with the p21-binding domain of PAK-3 (PBD-PAK-3) immobilized on agarose beads. The amount of Rac-GTP was visualized by Western blotting with antibody specific for Rac1 (top blots). The bottom blots show 2% of the total cell lysate used for the affinity purification. Results are representative of three different experiments.
results, along with those in Fig. 2 and 6, indicate that a
decrease in PIP3 has little effect on the PDGF-induced activation
of Rac1 via Tiam1.

**DISCUSSION**

Membrane translocation and/or co-localization of proteins to
distinct subcellular compartments is an important component
of cell signaling. Upon stimulation with agonists, cells are
induced to reorganize the location of signaling proteins. For
example, the translocation of PKC isoforms from the cytosol to
membranes in association with the activation of these enzymes
has been observed in most cell types (33). Likewise, activation
of growth factor receptors results in the recruitment of a mul-
titude of signaling proteins to the receptors, including phospholipase Cγ, Src, Grb2, and PI3K (34, 35). Furthermore, agonist
stimulation of Swiss 3T3 fibroblasts induces specific types of
Rho family GTPases to translocate to the membrane fraction
(14, 36). SOS-1, a GEF for Ras, has been shown to translocate
upon stimulation of Rat1 cells with insulin, epidermal growth
factor, or the macrophage-stimulating protein (15, 16). Not
only do agonists induce the re-localization of SOS, but the presence
of SOS at the membrane alone has been shown to induce Ras
activation (17). Similarly, Tiam1, a GEF for Rac, has been
shown to associate with the membrane fraction, and this asso-
ciation is required for membrane ruffling and c-Jun N-terminal
kinase activation (18). This interaction of Tiam1 with the mem-
brane is dependent upon its N-terminal PH domain and an
adjacent protein interaction domain (19).

The data presented here demonstrate that Tiam1 is translo-
cated to the membrane by treatment of NIH 3T3 cells with
PDGF and LPA in a time- and dose-dependent manner (Fig. 1
and data not shown). PDGF also induces the translocation of
Rac to the membrane fraction (14, 36), and the observation that
GTPγS can mimic the effect of PDGF (13) suggests that the
translocation is associated with activation of Rac. The obser-
vation that PDGF stimulates the translocation of both Tiam1
and Rac to the membrane fraction suggests that the re-local-
ization of Tiam1 is involved in Rac activation. The idea that
PDGF-induced membrane translocation of Rac reflects activa-
tion of Rac is consistent with the observation that PDGF treat-
ment of fibroblasts induces Rac-mediated membrane ruffling
(37). However, the present study demonstrates directly that
PDGF increases the level of active Rac1 (Fig. 8). The membrane
translocation (Fig. 1) and phosphorylation (Fig. 5) of Tiam1
occur sufficiently and rapidly enough to be involved in this
activation.

Previously, we have shown that LPA or PDGF induces the
phosphorylation of Tiam1 in Swiss 3T3 fibroblasts (8–10).
Inhibitor studies and in vitro kinase assays demonstrated that
PKC and CaMK II are two protein kinases involved in this
process (8–10). Importantly, we observed that the phosphoryl-
ation of N-terminally truncated Tiam1 by CaMK II increased
the in vitro GEF activity of Tiam1 toward Rac1. However,
phosphorylation by PKCα had no effect (10). Similarly, through
the use of kinase inhibitors, we have now found that the trans-
location of Tiam1 induced by PDGF (Fig. 2) or LPA (Fig. 3)
is mediated by CaMK II, but not by PKC. Furthermore, an in-
crease in intracellular Ca2+ induced by ionomycin was capable
of inducing the translocation of Tiam1 to the membrane (Fig.
4). In contrast, addition of OAG, a cell-permeable activator of
PKC, did not induce translocation of Tiam1. Furthermore,
OAG did not increase the translocation of Tiam1 over that
observed with the Ca2+ ionophore alone (Fig. 4). In contrast
to the marked effect of inhibition of CaMK II activity, inhibition
of PI3K activity by wortmannin had little effect on the translo-
cation of Tiam1 (Figs. 2 and 3).

To further explore the role of phosphorylation in the trans-
location of Tiam1, we compared the time course of phosphoryl-
ation of Tiam1 in whole cells versus that in the membrane
fraction. PDGF induced a different time course of phosphoryl-
ation in these two fractions. Membrane-associated Tiam1 was
phosphorylated in a much faster time course compared with
total Tiam1 (Fig. 5). Also, the phosphorylation of Tiam1 in the
membrane fraction was greatly reduced by 30 min, whereas
the total pool of Tiam1 was still phosphorylated at this time.
The results shown in Fig. 1 show that Tiam1 is still strongly pres-
ent in the membrane at this time. This indicates that phosphi-
rylation and dephosphorylation of Tiam1 in the membrane are
more rapidly controlled than in the cytosol. Unfortunately, we
were unable to prove this directly since we could not consist-
tently immunoprecipitate Tiam1 from the cytosol. Interest-
ingly, LPA generated a similar time course of phosphorylation
of Tiam1 in the membrane fraction as in the total pool of Tiam1
(Fig. 5). This suggests that PDGF and LPA may translocate
Tiam1 to different membrane fractions, in which it is differen-
tially phosphorylated and dephosphorylated. This is intrigu-
ing because although LPA can induce the translocation and phos-
phorylation of Tiam1, it cannot induce Rac translocation (14),
Rac activation (Fig. 8), or Rac-mediated signaling events (2).
This could be explained if the membrane fraction to which LPA
directs Tiam1 is not involved in Rac activation or if LPA-
induced translocation and phosphorylation of Tiam1 have no
effect on the activation of Rac. This would also suggest that
additional changes besides the phosphorylation and translo-
cation of Tiam1 are required for Rac-mediated signaling, such as
the translocation of Rac and its effectors.

Although LPA and PDGF induce different time courses of
phosphorylation of Tiam1 (Fig. 5), the phosphorylation of
Tiam1 at the membrane induced by either agonist is mediated
principally by CaMK II (Figs. 6 and 7). Surprisingly, inhibition
of PKC by Ro-31-8220 had little effect on the phosphorylation
of Tiam1 at the membrane, although this compound and the
CaMK II inhibitor KN-93 markedly inhibit the phosphoryla-
tion of Tiam1 in whole cells (9). These findings suggest that
PKC and CaMK II are both involved in agonist-induced phos-
phorylation of Tiam1 in the cytosol, whereas CaMK II plays a
significant role in its phosphorylation at the membrane.

The minimal role of PKC in the membrane phosphorylation
of Tiam1 corresponded to its much smaller effect on the mem-
brane association of Tiam1 compared with that of CaMK II
(Figs. 2 and 3), suggesting that the two events are related.
Wortmannin had no detectable effect on PDGF- or LPA-
induced phosphorylation of Tiam1 at the membrane (Figs. 6 and
7), implying a small role (if any) for PI3K.

Although PDGF and LPA can induce the translocation and
phosphorylation of Tiam1, only PDGF induces the in vitro ac-
ivation of Rac1. PDGF treatment of NIH 3T3 fibroblasts in-
creased the amount of active (GTP-ligated) Rac1 in a time
course that was as rapid as that seen in the translocation and
membrane phosphorylation of Tiam1 (Fig. 8; cf. Figs. 1 and 5).
The increase in Rac1-GTP was dependent upon both CaMK II
and PKC activities, based on the effects of KN-93 and Ro-31-
8220 (Fig. 9). However, the data of Figs. 2, 4, and 7 indicate a
major involvement of CaMK II in the membrane translocation
and phosphorylation of Tiam1, with a small role for PKC. This
suggests that the activation of Rac1 by PDGF may involve
another PKC-dependent mechanism and possibly another GEF
for Rac1 (24, 38). This idea is reinforced by the finding that
the phosphorylation of Tiam1 by CaMK II in vitro activates its
GEF activity, whereas the phosphorylation by PKCα is without
effect (10). The inhibition of PI3K had no effect on the PDGF-
duced increase in Rac1-GTP (Fig. 9). This is consistent with
our finding that this inhibition had little or no effect on the
translocation and phosphorylation of Tiam1 (Figs. 2 and 6).

These data indicate that Tiam1 translocates to the membrane fraction of NIH 3T3 cells upon stimulation with either LPA or PDGF and that the translocation is dependent upon CaMK II, but not PKC. The time course of translocation was similar, except that membrane association of Tiam1 was lost at 30 min with LPA. Both agonists induced phosphorylation of Tiam1 at the membrane, but PDGF acted more rapidly than did LPA. Inhibitor studies indicated the involvement of CaMK II, but not PKC, in this phosphorylation, whereas total Tiam1 phosphorylation involved both kinases. Since these kinases are predominately cytosolic, these findings suggest that Tiam1 becomes phosphorylated by CaMK II, but not PKC, after it becomes associated with a membrane fraction and that some factor inhibits its phosphorylation by PKC. Surprisingly, despite the fact that both PDGF and LPA cause the translocation and phosphorylation of Tiam1, only PDGF activates Rac, in agreement with indirect conclusions from previous studies (36, 37, 39, 40). This indicates that PDGF provides another factor(s) involved in Rac activation. Although we suspected this might be PIP₃, the negative findings with wortmannin (Fig. 9) did not support this.

Another issue in this study is the role of PKC. Although this kinase causes significant phosphorylation of Tiam1 in vitro and of total cellular Tiam1 in vivo (8–10), it plays a small role, if any, in the membrane translocation and phosphorylation of Tiam1 (Figs. 2, 3, 6, and 7) and does not alter the GEF activity of Tiam1 in vitro (10). The observation that a PKC inhibitor consistently inhibited PDGF-induced Rac activation was therefore somewhat of a surprise and indicated an additional PKC-dependent mechanism of Rac activation, which could be due to an indirect action of PKC on Tiam1 or could involve another GEF for Rac. Exploring the mechanisms and sites of Tiam1 translocation and also the phosphorylation sites on Tiam1 and their relation to its activity will be the subjects of future work.

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