Lysophosphatidic Acid Induces Threonine Phosphorylation of Tiam1 in Swiss 3T3 Fibroblasts via Activation of Protein Kinase C*

(Received for publication, September 9, 1997, and in revised form, October 20, 1997)

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The Rho family of GTPases plays an important role in the control of cell shape, adhesion, movement, and growth. Several guanine nucleotide exchange factors have been identified that activate Rho family GTPases by promoting the binding of GTP to these proteins. However, little is known concerning the regulation of these GDP/GTP exchange factors. In this study, we demonstrate that lysophosphatidic acid (LPA) induces a rapid, sustainable phosphorylation of the Rac1-specific nucleotide exchange factor Tiam1 in Swiss 3T3 fibroblasts. LPA stimulated Tiam1 phosphorylation in a dose-dependent manner, and the protein was phosphorylated on threonine, but not tyrosine or serine. Tiam1 phosphorylation was also induced by platelet-derived growth factor, endothelin-1, bombesin, and bradykinin but not by epidermal growth factor. Significantly, pretreatment of Swiss 3T3 fibroblasts with 1 μM phorbol 12-myristate 13-acetate for 24 h, or with the selective protein kinase C inhibitor Ro-31–8220, reduced LPA-stimulated phosphorylation of Tiam1 by approximately 75%. Moreover, acute stimulation with 100 nM phorbol 12-myristate 13-acetate was sufficient to induce Tiam1 phosphorylation in vivo, and protein kinase C could phosphorylate purified Tiam1 on threonine residues in vitro. These data indicate that agonist-induced phosphorylation of Tiam1 is a general mechanism and suggest that it is likely to be important in its regulation. Protein kinase C appears to play a key role in phosphorylation of Tiam1.

The proteins of the Rho family cycle between GTP-bound (active) and GDP-bound (inactive) states, aided by a number of regulatory proteins. A number of guanine nucleotide exchange factors, which promote binding of GTP to Rho family members by facilitating the release of GDP, have been identified (16). Nucleotide exchange factors that act on Rho proteins contain two conserved domains: a Dbl homology domain which is believed to be responsible for catalyzing GDP/GTP exchange, and a pleckstrin homology domain that seems to be important for cellular localization through interaction with lipids and/or proteins (16). Several GTPase-activating proteins (GAPs), which enhance the intrinsic GTPase activity of Rho proteins, have also been characterized (17). Rho family members also bind to a cytosolic regulatory protein, Rho-GDI,1 which inhibits GDP dissociation (18) and GTP hydrolysis (19) and is believed to be important in localizing the GTPases predominantly in the cytosolic compartment (20).

It is now well established that nucleotide exchange on Ras is stimulated by tyrosine phosphorylation of growth factor receptors and recruitment of the Sos exchange factor to the plasma membrane with the aid of the Grb2 adapter protein (21, 22) and that many receptors coupled to heterotrimeric G-proteins also activate Ras through a similar mechanism involving Gp2 subunits (reviewed in Ref. 23). However, little is known concerning the regulation of Rho family nucleotide exchange factors. Crespo et al. (24) recently demonstrated that tyrosine phosphorylation of the oncogene Vav results in increased GDP/GTP nucleotide exchange on Rac1 in Cos cells co-transfected with Vav and Lck. On the other hand, Michiels et al. (25) have shown that the Rac1-specific exchange factor Tiam1 becomes associated with the membrane fraction upon addition of serum to NIH3T3 cells transiently transfected with N-terminally truncated Tiam1. However, it is not yet clear whether phosphorylation or relocalization of Rho family exchange factors plays an important role in their regulation in nontransfected cells. Therefore, to further elucidate the mechanisms of regulation of the Rho family nucleotide exchange factors, we investigated the effect of agonist treatment on the subcellular distribution and phosphorylation state of Tiam1 in Swiss 3T3 fibroblasts.

EXPERIMENTAL PROCEDURES

Materials—Swiss 3T3 fibroblasts were obtained from the American Type Culture Collection. Fetal bovine serum, Dulbecco’s modified Eagle’s medium (DMEM), penicillin, and streptomycin were from Life Technologies, Inc. LPA (1-oleoyl) was from Avanti Polar Lipids. Platelet-derived growth factor (PDGF-βb) was from Upstate Biotechnology.

1 The abbreviations used are: Rho-GDI, Rho GDP dissociation inhibitor; DMEM, Dulbecco’s modified Eagle’s medium; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RIPA buffer, radiimmune precipitation buffer; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid.
Inc. Bradykinin was from Novabiochem USA. Bombesin, endothelin-1, phorbol 12-myristate 13-acetate (PMA), sodium orthovanadate, leupeptin, antipain, phenylmethylsulfonyl fluoride, sodium fluoride, sodium pyrophosphate, Tween 20, Triton X-100, and fatty acid-free bovine serum albumin were obtained from Sigma. Ro-31–8220, bisindolylmaleimide I, and staurosporine were from Calbiochem. Tiam1 and PY20 antibodies and A-agarose beads were from Santa Cruz Biotechnology. Phosphothreonine and phosphoserine-specific antibodies were obtained from Zymed Laboratories, Inc. [γ-32P]ATP was from NEN Life Science Products.

Cell Culture Conditions—Swiss 3T3 fibroblasts were maintained in HEPES-buffered DMEM with 4 mM L-glutamine supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. For all experiments, cells were grown on 100-mm dishes for 1–2 days to subconfluency (60–70%). The medium was then replaced with a low serum medium (DMEM containing 1% fetal bovine serum, 0.5% (v/v) bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin) for 24 h to allow the cells to become quiescent. The cells were then treated with serum-free medium (DMEM containing 0.5% bovine serum albumin and antibiotics) for 1 h prior to agonist stimulation.

Agonist Treatment and Preparation of Membrane Fraction—Serum-starved cultures on 100-mm dishes were treated with various concentrations of LPA at 37 °C for different times as noted in the experiments. The medium was removed, and the cells were washed three times with 5 ml of ice-cold PBS containing 500 μM sodium orthovanadate and scraped in 400 μl/dish lysis buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 2 mM EDTA, 10 μg/ml antiapain and leupeptin, 1 μg phenylmethylsulfonyl fluoride, 500 μM sodium orthovanadate, 10 mM pyrophosphate, 10 mM sodium fluoride, and 1 mM dithiothreitol). The cells were lysed by five passes through a 27-gauge needle (26) on ice. Lysates were centrifuged at 120,000 × g for 45 min to prepare cytosolic and total particulate fractions. The membrane pellet was washed twice with lysis buffer to remove cytosolic proteins. Protein determination was done by the method of Bradford (27).

Immunoprecipitation of Tiam1—Serum-starved cultures on 100-mm dishes were treated with various concentrations of LPA, PDGF, endothelin-1, bombesin, or Bradykinin at 37 °C for different times as noted in the experiments. The medium was removed, and the cells were washed twice with 5 ml of ice-cold PBS containing 500 μM sodium orthovanadate and scraped in 0.4 μl/dish RIPA buffer (PBS containing 0.1% SDS, 1% Nonidet P-40, 0.25% deoxycholate, 10 μg/ml antiapain and leupeptin, 1 μg phenylmethylsulfonyl fluoride, 500 μM sodium orthovanadate, 10 mM pyrophosphate, 10 mM sodium fluoride, and 1 mM dithiothreitol). The cells were lysed by five passes through a 27-gauge needle (26) on ice. Lysates were centrifuged at 120,000 × g for 45 min to prepare cytosolic and total particulate fractions. The membrane pellet was washed twice with lysis buffer to remove cytosolic proteins. Protein determination was done by the method of Bradford (27).

Purification and Phosphorylation of Tiam1 by Protein Kinase C—An N-terminally truncated form of Tiam1, GST-C1199-Tiam1 (25), was transfected into COS-7 cells and purified using glutathione-Sepharose beads essentially as described (28), in the presence of 0.1% (v/v) Triton X-100. Silver staining analysis indicated that the purified GST-Tiam1 was almost homogeneous.

Purified GST-Tiam1 (5 μl) was incubated for 1 h at 30 °C in the presence and absence of 0.3 units of purified rat brain protein kinase C (Sigma) in 20 mM MOPS buffer, pH 7.2, containing 25 mM glycine-3-phosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM CaC12, 0.1 mg/ml phosphatidylserine, 0.01 mg/ml diacylglycerol, 15 mM MgCl2, and 100 μM ATP. Assays were carried out either using non-radiolabeled ATP and phosphorylation analysis by Western blotting or with [γ-32P]ATP (specific activity 5 × 106 dpm/nmol) and phosphorylation analysis by autoradiography.

RESULTS

As a first step toward studying the regulation of Tiam1, cytosol and membranes from Swiss 3T3 fibroblasts were analyzed for the presence of Tiam1 by Western blotting (Fig. 1). A protein band with a molecular mass of approximately 190 kDa was identified in membranes from rat brain, a tissue with high Tiam1 expression (29), and Swiss 3T3 cells using the Santa Cruz Tiam1 antibody (Fig. 1). Antibody recognition of this protein band was specifically blocked by preincubation of the antibody with the peptide against which it was raised (Fig. 1). The same protein band was also strongly recognized by a different polyclonal antibody (not shown), raising against the C-terminal part of Tiam1 (30), confirming that it represents Tiam1. Interestingly, in growing Swiss 3T3 fibroblasts, Tiam1 was detected in both cytosol and membrane fractions (Fig. 1). Moreover, Western blotting indicated that the enzyme was predominantly localized in the membrane fraction (78 ± 4%), providing further evidence that Tiam1 is present in the membrane fraction of growing cells (25).

In NIH3T3 cells transiently transfected with N-terminally truncated Tiam1, addition of serum induces membrane localization of Tiam1 and subsequent membrane ruffling (25). Since LPA is an important constituent of serum (31), activates several signaling pathways in fibroblasts which involve Rho family GTPases (2, 9, 32, 33), and also translocates these GTPases to membranes (26), we decided to study the effect of this mitogen on the regulation of Tiam1 in Swiss 3T3 fibroblasts.

In Swiss 3T3 fibroblasts, LPA treatment did not cause a significant change in the subcellular distribution of Tiam1 (data not shown). However, it is possible that LPA may induce a redistribution of Tiam1 to a specific membrane fraction, which we could not detect by our procedures. Alternatively, agonist-stimulated Tiam1 redistribution may be lost during cell lysis. On the other hand, stimulation of Swiss 3T3 cells with LPA caused an electrophoretic retardation of the membrane-associated Tiam1 (Fig. 2, A and B). Preincubation of the Tiam1 antibody with its immunopeptide specifically blocked antibody
membranes (8 M A) 
molar concentrations of 1 mM PDGF for 15 min (A) or for the indicated time (B) and then lysed and fractionated as described under "Experimental Procedures." Cytosol (20 µg) and membranes (7 µg) (A) and membranes (8 µg) (B) were analyzed for Tiam1 content by Western blotting. Data are representative of three (A) or four (B) independent experiments.

Fig. 2. Lysophosphatidic acid induces an electrophoretic retardation of Tiam1 in Swiss 3T3 cells. Swiss 3T3 cells were treated with (+) or without (−) 100 µM LPA for 15 min (A) or for the indicated time (B) and then lysed and fractionated as described under "Experimental Procedures." Cytosol (20 µg) and membranes (7 µg) (A) and membranes (8 µg) (B) were analyzed for Tiam1 content by Western blotting. Data are representative of three (A) or four (B) independent experiments.

To obtain further evidence that Tiam1 is phosphorylated by agonist treatment, Tiam1 was immunoprecipitated from Swiss 3T3 fibroblasts treated with and without 100 µM LPA for 15 min, and the immunoprecipitates were probed with phospho-tyrosine-, phosphothreonine-, and phosphoserine-specific antibodies. As expected, the 190-kDa protein designated as Tiam1 was immunoprecipitated by the antibody raised against the C-terminal part of Tiam1 and by the Santa Cruz Tiam1 antibody but could not be detected in those immunoprecipitates generated in the presence of the immunizing peptide or with a nonspecific rabbit IgG antibody (results not shown). Tiam1 was immunoprecipitated equally well from control and LPA-treated cells (Fig. 3A). Significantly, after LPA treatment, Tiam1 immunoprecipitates contained a 190-kDa protein band that strongly reacted with the phosphothreonine-specific antibody. However, the PY20 antibody did not recognize the Tiam1 band, either before or after LPA treatment, but specifically recognized a highly phosphorylated 190-kDa protein in PDGFβ receptor immunoprecipitates from PDGF-treated Swiss 3T3 cells (Fig. 3A), suggesting that LPA does not stimulate tyrosine phosphorylation of Tiam1 to any significant extent. Similarly, the phosphoserine antibody did not recognize the Tiam1 band in control or LPA-treated immunoprecipitates but did recognize several protein bands in phosphoserine immunoprecipitates from PMA-stimulated Swiss 3T3 cells (Fig. 3A), indicating that LPA does not significantly stimulate serine phosphorylation of Tiam1. Therefore, these data indicate that the phosphothreonine antibody interacts specifically with Tiam1 after LPA treatment, providing strong evidence that the agonist selectively stimulates phosphorylation of Tiam1 threonine residues.

Importantly, the phosphothreonine antibody could immunoprecipitate a 190-kDa protein that was recognized by the Tiam1 antibody, and the amount of the 190-kDa protein immunoprecipitated by this antibody was significantly increased by LPA treatment (Fig. 3B). These data confirm that the 190-kDa protein that is phosphorylated on threonine residues by LPA treatment is indeed Tiam1.

Several other agonists were also tested for their ability to phosphorylate Tiam1 on threonine to ascertain whether Tiam1 phosphorylation is an LPA-specific event. The heterotrimeric G-protein-mediated agonists endothelin-1, bombesin, and bradykinin (Fig. 4) and sphingosine-1-phosphate (not shown) all induced phosphorylation of Tiam1, showing that LPA is not the only agonist which stimulates Tiam1 phosphorylation. In addition, incubation of Swiss 3T3 cells with PDGF (50 ng/ml) also caused Tiam1 phosphorylation (Fig. 4), whereas epidermal growth factor treatment (50 ng/ml) had no effect on phosphorylation of this protein (not shown), indicating that some growth factors can stimulate phosphorylation of Tiam1. Importantly, the different agonists tested were able to phosphorylate Tiam1 to different extents; LPA, PDGF, and endothelin-1 induced strong phosphorylation of Tiam1, whereas bradykinin and bombesin only caused a weak phosphorylation of this protein.
Analysis of Tiam1 immunoprecipitates indicated that the LPA-induced phosphorylation of Tiam1 is very rapid. Phosphorylation became evident after 15 s of LPA stimulation, was maximal at 2.5 min LPA stimulation, and began to decrease after 10 min of LPA treatment (Fig. 5A). However, Tiam1 phosphorylation was still readily detectable after 60 min of LPA treatment (Fig. 5A). LPA stimulated phosphorylation of Tiam1 in a dose-dependent manner; phosphorylation was half-maximal at approximately 100 nM LPA and maximal at concentrations of 1 μM and higher (Fig. 5B). Importantly, several LPAs containing different fatty acid chains could induce phosphorylation of Tiam1 (not shown). 1-Oleyl-LPA was approximately 6-fold more potent than 1-palmitoyl-LPA and 40-fold more effective than 1-myristoyl-LPA at inducing phosphorylation of Tiam1. Indeed, the rank order of potency of the various LPAs correlates with their ability to bind to the LPA binding site in Swiss 3T3 membranes (36), indicating that the effects are transduced by a specific receptor.

LPA-induced Tiam1 phosphorylation was not affected by pretreating Swiss 3T3 cells with 100 ng/ml pertussis toxin for 24 h (not shown), indicating that the effect does not involve stimulation of a heterotrimeric G-protein of the G i/G o family. Therefore, since LPA activates protein kinase C (PKC) via a pertussis toxin-insensitive heterotrimeric G-protein, at concentrations of 100 nm and higher (37), and all of the agonists tested that stimulate phosphorylation of Tiam1 also activate PKC, we investigated the possibility that PKC is involved in Tiam1 phosphorylation. Indeed, pretreatment of Swiss 3T3 fibroblasts with 5 μM Ro-31–8220, a specific inhibitor of protein kinase C, inhibited the LPA-stimulated phosphorylation of Tiam1 by approximately 70% (Fig. 6A), suggesting that this kinase phosphorylates Tiam1.

Although some phosphorylation of Tiam1 was observed in the absence of PKC (Fig. 7), perhaps due to a protein kinase which co-purifies with the GST-Tiam1, addition of PKC significantly enhanced [32P]-labeled phosphorylation of the exchange factor (Fig. 7A), suggesting that this kinase phosphorylates Tiam1. Indeed, inclusion of PKC stimulated phosphorylation of Tiam1 on threonine (Fig. 7B), providing strong evidence that this kinase directly phosphorylates the exchange factor.

**DISCUSSION**

The results presented here provide strong evidence that the Rac1-specific nucleotide exchange factor Tiam1 is phosphorylated by a cellular threonine protein kinase in Swiss 3T3 cells stimulated with LPA. This is the first study providing evidence that nucleotide exchange factors which act on Rho family GTPases are phosphorylated in vivo by agonist treatment. Moreover, the rapid threonine-specific phosphorylation of Tiam1 after addition of LPA (Fig. 5A) suggests that this event is likely to be functionally important in the action of this mitogen. Indeed, PDGF, endothelin-1, and to a lesser extent bombesin and bradykinin (Fig. 4), also induce phosphorylation of Tiam1, indicating that agonist-induced phosphorylation of this protein is a general mechanism and is likely to play an important role in Tiam1 regulation.

LPA activates a number of well characterized signaling pathways and processes, via the heterotrimeric G-proteins G i and G o, namely, inhibition of adenylate cyclase, activation of Ras and the Raf/MAP kinase pathways, stimulation of PLC and PLD, and stress fiber formation (37). Significantly, LPA-stimulated phosphorylation of Tiam1 was not inhibited by pretreating cells with pertussis toxin, indicating that it does not involve inhibition of adenylate cyclase or activation of the Ras/Raf/MAP kinase pathway, which are regulated via G i. On the other hand, nanomolar concentrations of LPA activate Tiam1 phosphorylation (Fig. 5B) and a phosphoinositide-specific PLC (37)
factor, which produces barely detectable phosphoinositide hydrolysis in Swiss 3T3 cells (38), we tested the possibility that PKC is involved in the phosphorylation studied here.

Several lines of evidence indicate that PKC plays an important role in LPA-stimulated Tiam1 phosphorylation. First of all, preincubation of Swiss 3T3 cells with the specific PKC inhibitors Ro-31–8220 and bisindolylmaleimide I reduced LPA-induced Tiam1 phosphorylation by nearly 70% (Fig. 6A). Second, 24-h pretreatment of Swiss 3T3 cells with PMA, to down-regulate the cellular level of non-atypical PKC isozymes, reduced LPA-stimulated Tiam1 phosphorylation by approximately 75% (Fig. 6B). In addition, acute treatment with PMA was sufficient to induce phosphorylation of Tiam1 (Fig. 6C). Finally, purified rat brain PKC could phosphorylate purified GST-Tiam1 in vitro on threonine residues (Fig. 7). These data provide strong evidence that PKC plays a role in the phosphorylation of Tiam1 and that PKC directly phosphorylates this exchange factor, although it remains possible that PKC may also activate another protein kinase (or inactivate a phosphatase) that controls the phosphorylation state of Tiam1. Further support that PKC can phosphorylate Tiam1 comes from the fact that most serine/threonine protein kinases predominantly phosphorylate serine residues, whereas PKC can also phosphorylate threonine. In addition, Tiam1 is particularly rich in serine and threonine residues (29) and contains several potential PKC phosphorylation consensus sequences. It seems likely that PKC isoforms of the classical or novel family catalyzes the phosphorylation described here since long-term pretreatment of cells with PMA reduces the phosphorylation by approximately 75%. Moreover, a PKC isoform of the classical PKC family would be a good candidate for stimulating this phosphorylation since LPA activates a phosphoinositide-specific PLC (37) and Tiam1 phosphorylation via similar signaling pathways. Interestingly, although PKC inhibitors and long-term PMA treatment reduced the LPA-stimulated Tiam1 phosphorylation to a similar extent, neither manipulation completely abrogated the LPA effect. This suggests that a second protein kinase may be involved in LPA-stimulated Tiam1 phosphorylation. This hypothesis is supported by the fact that PMA does not stimulate Tiam1 phosphorylation to the same extent as LPA. Thus PKC may synergize with another protein kinase to phosphorylate Tiam1 in response to LPA stimulation. The second putative protein kinase could be an atypical PKC or an enzyme from a different kinase family that is capable of phosphorylating threonine residues.

The relationship between the electrophoretic retardation of Tiam1 on SDS-polyacrylamide gel electrophoresis and phosphorylation of that protein is not yet clear. Protein phosphorylation probably plays a part in LPA-induced gel retardation of Tiam1 since the magnitude of this bandshift is reduced by the protein kinase inhibitor staurosporine. Moreover, LPA-induced Tiam1 phosphorylation (Fig. 5B) and the Tiam1 bandshift are both induced by LPA concentrations of 100 nM and higher, suggesting that both effects may be activated via a common pathway. However, the time-courses of the two effects are considerably different. Tiam1 phosphorylation becomes detectable after 15 s of LPA treatment and is maximal after 2.5 min (Fig. 5A), whereas electrophoretic retardation of this protein becomes detectable after 2.5 min of LPA stimulation and is maximal after 5 min (Fig. 2B). One possibility is that LPA induces phosphorylation of Tiam1 on several amino acids and that this hyperphosphorylation causes the Tiam1 bandshift. Alternatively, the Tiam1 bandshift may be caused by the second protein kinase suggested above.

It has been proposed that regulated membrane localization of Tiam1 may be important in its activation and that an intact

via a pertussis toxin-insensitive G-protein. PLC stimulation results in the generation of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, which activate PKC and mobilize Ca\(^{2+}\), respectively. Therefore, since Tiam1 phosphorylation is also stimulated by treatment with PDGF, endothelin-1, bombesin, and bradykinin (Fig. 4), agonists which activate PLC and PKC (38, 39, 40), but not by epidermal growth
pleckstrin homology domain is critical for the membrane association of this protein (25). Moreover, protein phosphorylation plays an important role in activation of the Ras exchange factor Sos by facilitating recruitment of the Sos/Grb2 complex to the plasma membrane (21, 22), suggesting that a phosphorylation/dephosphorylation mechanism could also be involved in Tiam1 translocation. However, it seems unlikely that this is the case in Swiss 3T3 cells since LPA treatment induced a strong phosphorylation of Tiam1 (Fig. 3) but did not cause a significant change in its subcellular distribution.

Phosphorylation of the nucleotide exchange factors Ras-GRF (41) and Vav and Rac has been reported recently. However, Tiam1, Ras-GRF, and Vav appear to be phosphorylated by completely different signaling pathways. Tiam1 is phosphorylated on threonine residues by a PKC-dependent pathway (Fig. 6), Ras-GRF is phosphorylated on serine/threonine residues by a PKC-independent mechanism (41), and Rac is phosphorylated on tyrosine by a src family tyrosine kinase (24). Significantly, phosphorylation of both Ras-GRF and Vav increased the GDP/GTP exchange activity exerted on their target GTPases, Ras and Rac1, respectively (24, 41). Further work will be required to determine whether phosphorylation of Tiam1 alters its nucleotide exchange activity.

Tiam1 is believed to act as a Rac1-specific exchange factor in vitro (28). Indeed, in NIH3T3 cells, serum induces membrane localization of Tiam1, membrane ruffling and Jun kinase activation (25). However, neither PDGF or insulin could substitute for serum in the induction of membrane ruffling (25). Therefore since LPA is a major constituent of serum (31) and stimulates localization of Tiam1, membrane ruffling and Jun kinase activation (25). Moreover, protein phosphorylation of both Ras-GRF and Vav increased the GDP/GTP exchange activity exerted on their target GTPases, Ras and Rac1, respectively (24, 41). Further work will be required to determine whether phosphorylation of Tiam1 alters its nucleotide exchange activity.

We are currently investigating which protein kinases, besides PKC, phosphate purified Tiam1 in vitro and the effect of protein phosphorylation on the rate of Tiam1-catalyzed GDP/GTP exchange on Rac1.

Acknowledgments—We thank Dr. Frits Michiels for helpful advice on Tiam1 and Judy Childs for typing this manuscript.

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