Phospholipase C-γ, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II are involved in platelet-derived growth factor-induced phosphorylation of Tiam1

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Abstract In Swiss 3T3 fibroblasts, the Rac1-specific guanine nucleotide exchange factor Tiam1 is phosphorylated by several different agonists. We show here that PDGF induces threonine phosphorylation of Tiam1 in a time- and dose-dependent manner. Tiam1 phosphorylation was significantly reduced by the selective protein kinase C inhibitor Ro-31-8220 and by KN93, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. The Ca^{2+} chelator BAPTA/AM totally abrogated Tiam1 phosphorylation, indicating that Ca^{2+} is essential for this phosphorylation. Moreover, PDGF-stimulated Tiam1 phosphorylation was markedly reduced by 72 ± 10% in PLC-γ1 deficient mouse fibroblasts, compared to wild-type cells, indicating that phosphoinositide phospholipase C is involved.

Key words: Platelet-derived growth factor; Tiam1; Phosphorylation; Protein kinase C; Protein kinase II; Phospholipase C-γ

1. Introduction

Rho family small GTPases play an important role in the regulation of several cellular functions including cell morphology [1–4], cell cycle progression [5], gene transcription through activation of the serum response factor [6], activation of the Jun kinase and p38 mitogen-activated protein kinase signalling cascades [7–10], and Ras-stimulated transformation of NIH3T3 fibroblasts [11,12].

While Rho GTPases are activated by guanine nucleotide exchange factors, which promote binding of GTP to these proteins, the mechanism(s) of activation of these factors is largely unknown. All Rho family nucleotide exchange factors identified thus far contain a Dbl homology domain which is believed to catalyse GDP/GTP exchange, and a pleckstrin homology domain which may be important in controlling the subcellular localization of these proteins, by binding to lipids and/or proteins [13]. Membrane localization of Tiam1 stimulates Rac-dependent membrane ruffling and Jun kinase activation in NIH3T3 cells [14], and the N-terminal pleckstrin homology domain and an adjacent protein interaction domain are required for membrane association of this exchange factor [14,15]. Significantly, the N-terminal pleckstrin homology domain of Tiam1 binds to PIP_2 and PIP_3 [16], raising the possibility that these lipids have a role in determining the cellular localization of this exchange factor. In addition, there is growing evidence that protein phosphorylation is involved in the regulation of Rho family exchange factors. Dbl [17] and Ost [18] are both phosphoproteins in vivo. Moreover, tyrosine phosphorylation of Vav by Lck stimulates Vav-catalysed GDP/GTP nucleotide exchange on Rac1 in Cos cells [19], and PIP_3 enhances phosphorylation and activation of this exchange factor [20]. We recently demonstrated that lysophosphatidic acid (LPA) induces threonine phosphorylation of Tiam1 in Swiss 3T3 cells via activation of protein kinase C (PKC) [21]. We now show that platelet-derived growth factor (PDGF) also stimulates phosphorylation of Tiam1 on threonine, in a time- and dose-dependent manner, and that a classical isoform of PKC acts in concert with Ca^{2+}/calmodulin-dependent protein kinase II to catalyse this phosphorylation. In addition, we present evidence that PLC-γ1 plays an important role in this signalling pathway.

2. Materials and methods

2.1. 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-β) was from Upstate Biotechnology Incorporated. Phorbol 12-myristate 13-acetate (PMA), sodium orthovanadate, leupeptin, antipain, phenylmethylsulphonyl 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, KN93 and BAPTA/AM were from Calbiochem. Tiam1 and PY20 antibodies and A-agarose beads were from Santa Cruz. Phosphothreonine- and phosphoserine-specific antibodies were obtained from Zymed.

2.2. Cell culture conditions

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% CO_2 and 95% air. For all experiments, cells were grown on 100-mm dishes for 1–2 days to subconfluency (60–70%). Prior to experiments, Swiss 3T3 cells were incubated for 24 h in low serum medium (DMEM containing 1% fetal bovine serum, 0.5% (w/v) bovine serum albumin, 100 Units/ml penicillin and 100 μg/ml streptomycin) for 24 h, then in serum-free medium (DMEM containing 0.5% bovine serum albumin and antibiotics) for 1 h. Mouse embryonic fibroblasts were starved in serum-free medium for 24 h before agonist stimulation.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; PI 3-kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; RIPA buffer, radioimmune precipitation buffer

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2.3. Immunoprecipitation of Tiam1

Serum-starved Swiss 3T3 cells were incubated with the indicated concentrations of PDGF for various times at 37°C. The medium was then removed, the cells washed twice with 5 ml of ice-cold PBS containing 500 μM sodium orthovanadate, and scraped in 0.5 ml/dish of RIPA buffer (PBS containing 0.1% SDS, 1% NP40, 0.25% deoxycholate, 10 μg/ml antipain and leupeptin, 1 mM 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 [22], on ice. Lysates were clarified by centrifugation at 3000×*g* for 10 min and precleared by incubation with 1 μg rabbit IgG and 20 μl A-agarose beads for 1 h at 4°C. After removal of A-agarose beads by centrifugation (1000×*g* for 10 min), the supernatants were transferred to new tubes for immunoprecipitation. Supernatants were incubated with 4 μl Tiam1 antibody for 1 h, 20 μl A-agarose beads were then added and the samples rocked at 4°C overnight. Beads were collected by centrifugation (1000×*g* for 10 min), washed four times in RIPA buffer and further analyzed by Western blotting.

Immunoprecipitation experiments with mouse embryonic fibroblasts were done essentially as described above, except that the cells were scraped and broken in PBS containing phosphatase and protease inhibitors (0.5 ml/plate). Protein concentrations were then determined using the method of Bradford [23] and the protein concentrations equalized between the different samples. 2×RIPA buffer was added to make the samples to a final concentration of 1×RIPA buffer, and the immunoprecipitations completed as described above.

2.4. SDS-polyacrylamide gel electrophoresis and Western analysis

SDS-polyacrylamide gel electrophoresis was performed on 6% acrylamide gels (Novel Experimental Corp.) and proteins transferred onto PVDF membranes (Millipore) for 1.5 h at 20 V using a Novex wet transfer unit. The membranes were blocked overnight with 5% (w/v) non-fat dried milk. Blots were incubated for 1 h with Tiam1 antibody (diluted 1:2000) in 1% bovine serum albumin, then 1 h with a horseradish peroxidase-conjugated secondary antibody (Vector laboratories), prior to development using an enhanced chemiluminescence kit (Amersham Corp.).

The other Western blots were carried out essentially as described above, except that the PVDF membranes were blocked overnight in 1% (w/v) BSA. The PY20, phosphothreonine and phosphoserine antibodies were all used at a dilution of 1:1000.

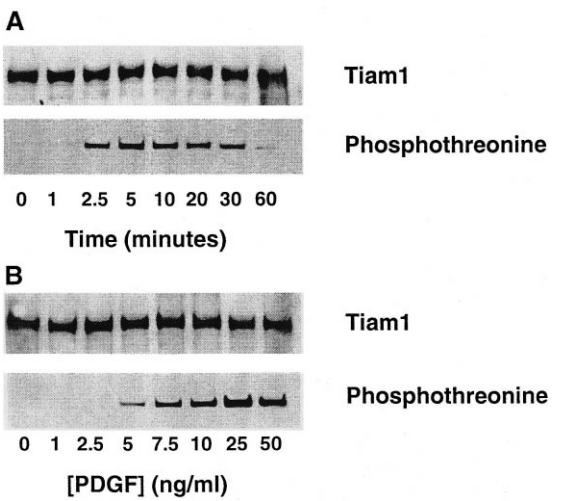

Fig. 1. Platelet-derived growth factor induces phosphorylation of Tiam1 in a time- and concentration-dependent manner. A: Swiss 3T3 fibroblasts were stimulated with 10 ng/ml PDGF for different times. B: Cells were treated with the indicated concentrations of PDGF for 5 min. The cells were lysed in RIPA buffer and the Tiam1 immunoprecipitated as described in Section 2. After immunoprecipitation, the A-agarose beads bound to Tiam1 were resuspended in 80 μl Laemmli buffer, and 30 μl of each sample analyzed for phosphothreonine and Tiam1 content by Western blotting. Data are representatives of five (A) or four (B) independent experiments.

3. Results and discussion

In Swiss 3T3 fibroblasts, LPA, PDGF, endothelin-1, bombesin, bradykinin and sphingosine-1-phosphate stimulate phosphorylation of Tiam1 [21]. While LPA stimulates Tiam1 phosphorylation through activation of PKC [21] it is not yet known how other agonists induce phosphorylation of this exchange factor. Therefore, to elucidate the mechanism of PDGF-induced Tiam1 phosphorylation, Tiam1 was immunoprecipitated, as described previously [21], from Swiss 3T3 fibroblasts stimulated with or without 50 ng/ml PDGF for 10 min, and the immunoprecipitates probed with phosphotyrosine-, phosphothreonine- and phosphoserine-specific antibodies. Tiam1 was immunoprecipitated from both control and PDGF-treated cells. In agreement with earlier findings [21], Tiam1 immunoprecipitates contained a 190 kDa protein band which strongly reacted with the phosphothreonine-specific antibody after PDGF treatment (data not shown). In contrast, the phosphotyrosine antibody did not recognize the Tiam1 band, either before or after PDGF treatment, but recognized a highly phosphorylated 190 kDa protein in PDGF-β receptor immunoprecipitates from PDGF-treated Swiss 3T3 cells (data not shown), indicating that this agonist does not stimulate tyrosine phosphorylation of Tiam1. Similarly, the phosphoserine antibody did not recognize the Tiam1 band in control or PDGF-treated immunoprecipitates (data not shown), indicating that the agonist does not significantly stimulate serine phosphorylation of Tiam1. Therefore, since Tiam1 only interacts with the phosphothreonine antibody after PDGF treatment, these data show that PDGF selectively stimulates phosphorylation of Tiam1 on threonine.

PDGF stimulates Tiam1 phosphorylation in a time-dependent manner (Fig. 1A). Tiam1 phosphorylation became apparent at 2.5 min of PDGF stimulation, was maximal after 5 min PDGF stimulation and decreased thereafter until phosphorylation was no longer detectable after 60 min PDGF treatment (Fig. 1A). In Swiss 3T3 cells, LPA-induced Tiam1 phosphorylation [21] is more rapid and is sustained for longer than PDGF-activated phosphorylation. These different time-courses of phosphorylation probably reflect the different downstream coupling and downregulation mechanisms of the two agonists and may be important in their distinct cellular responses.

Tiam1 phosphorylation was also dose-dependent. Phosphorylation became detectable at 5 ng/ml PDGF and was maximal at concentrations of 10 ng/ml and higher (Fig. 1B). Several signal transduction molecules bind to the activated PDGF receptor, namely phosphatidylinositol 3-kinase, src family tyrosine kinases, phospholipase C-γ, Grb2, Ras GTPase activating protein, Nck and protein tyrosine phosphatase 1D (reviewed in [24]). Src family kinases are not involved, since Tiam1 phosphorylation is not on tyrosine. Similarly, Ras GTPase activating protein is only tyrosine phosphorylated by PDGF concentrations higher than 7.5 ng/ml in Swiss 3T3 cells [25], indicating that Tiam1 phosphorylation is probably not activated via this signalling pathway. Pretreatment of Swiss 3T3 fibroblasts with the phosphatidylinositol 3-kinase inhibitor wortmannin (100 nM for 30 min) had no effect on Tiam1 phosphorylation (not shown) indicating that phosphatidylinositol 3-kinase is probably not involved. This is in contrast to Vav phosphorylation and activation by Lck, which is enhanced by PIP_3 [20]. On the other

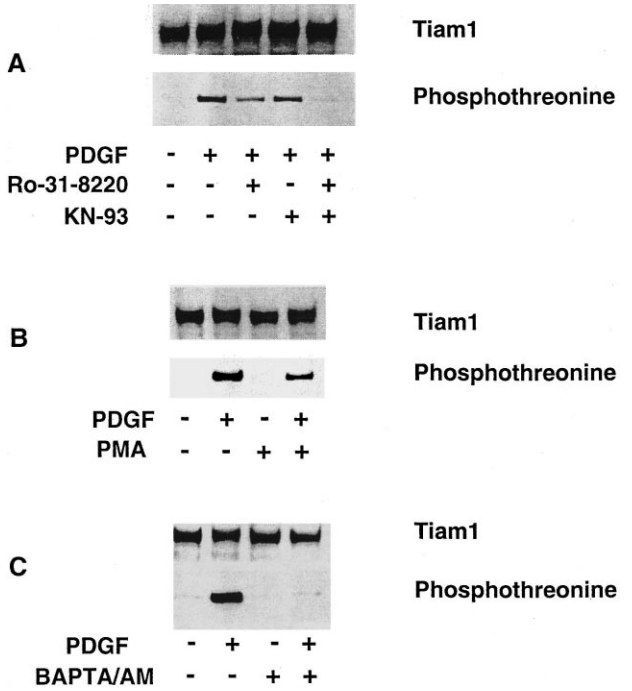

Fig. 2. Protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II are involved in Tiam1 phosphorylation. A: Swiss 3T3 fibroblasts were preincubated with (+) 5 μM Ro-31-8220 for 30 min, 20 μM KN93 for 24 h or DMSO (−) as indicated, prior to stimulation with (+) or without (−) 10 ng/ml PDGF for 5 min. B: Cells were preincubated with DMSO (−) or 1 μM PMA (+) for 24 h prior to stimulation with (+) or without (−) 10 ng/ml PDGF for 5 min. C: Cells were preincubated with 20 μM BAPTA/AM (+) or DMSO (−) for 30 min, prior to stimulation with (+) or without (−) 10 ng/ml PDGF for 5 min. Cells were lysed in RIPA buffer and the Tiam1 immunoprecipitated as described in Section 2. After immunoprecipitation, the A-agarose beads bound to Tiam1 were resuspended in 80 μl Laemmli buffer, and 30 μl of each sample analyzed for phosphothreonine and Tiam1 content by Western blotting. Data are representatives of at least three independent experiments.

hand, half maximal tyrosine phosphorylation of phospholipase C-γ is stimulated by 6.5 ng/ml PDGF in Swiss 3T3 cells [25] indicating that this enzyme is a good candidate for participating in the pathway studied here (see below).

To investigate the possible role of PKC in PDGF-stimulated Tiam1 phosphorylation, Swiss 3T3 cells were pretreated with the selective PKC inhibitor Ro-31-8220 for 1 h, or with 1 μM PMA for 24 h to downregulate the classical and novel PKC isoforms. The results (Fig. 2A) indicate that Ro-31-8220 inhibited PDGF-stimulated Tiam1 phosphorylation by approximately 70 ± 10%. PMA pretreatment also reduced the magnitude of PDGF-induced Tiam1 phosphorylation (Fig. 2B), by approximately 60 ± 11%. These results provide strong evidence that PKC is involved in PDGF-stimulated Tiam1 phosphorylation and suggest that a classical or novel PKC isozyme catalyses this reaction. In addition, KN93, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II, reduced PDGF-stimulated Tiam1 phosphorylation by 34 ± 18% and eliminated the Ro-31-8220-independent Tiam1 phosphorylation (Fig. 2A), indicating that this kinase may also contribute to Tiam1 phosphorylation. Indeed, purified Tiam1 is efficiently phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II in vitro (Hess, J.A., Ji, Q-S., Carpenter, G. and Exton, J.H., unpublished observations), confirming that the exchange factor is a good substrate for this kinase. In addition, the Ca^{2+} chelator BAPTA/AM totally eliminated PDGF-stimulated Tiam1 phosphorylation (Fig. 2C) indicating that Ca^{2+}-activated protein kinases are involved. The chelator also abolished the phosphorylation of Tiam1 induced by LPA (Fig. 3A) indicating the involvement of Ca^{2+}-activated kinases in the action of this agonist also. A role for Ca^{2+} was further supported by the finding that the Ca^{2+} ionophore ionomycin induced threonine phosphorylation of Tiam1 (lower band in Fig. 3B). Since Swiss 3T3 cells only contain PKC-α, -δ, -ε and -ζ [26], these and earlier data [21] show that PDGF and LPA stimulate Tiam1 phosphorylation through activation of the classical Ca^{2+}-dependent PKC isoform, PKC-α, and a Ca^{2+}-activated protein kinase, probably Ca^{2+}/calmodulin-dependent protein kinase II.

Phospholipase C 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, the observation that Tiam1 phosphorylation (Fig. 1B) and PLC activation [25] are stimulated by similar PDGF concentrations, and the indications that PKC, Ca^{2+}/calmodulin-dependent protein kinase II and Ca^{2+} (Fig. 2) are involved in PDGF-induced Tiam1 phosphorylation suggest that PLC may participate in the phosphorylation studied here. This hypothesis is supported by the fact that Tiam1 phosphorylation is stimulated by LPA, PDGF, endothelin-1, bombesin and bradykinin [21], agonists which activate PLC [27–29], but not by EGF, which does not significantly activate phosphoinositide hydrolysis in Swiss 3T3 cells [27].

To test the possibility that PDGF stimulates Tiam1 phosphorylation through activation of PLC-γ, wild-type and PLC-γ1 deficient mouse embryonic fibroblasts [30] were stimulated with LPA or PDGF for various times. Tiam1 was efficiently immunoprecipitated from both the wild-type and null fibroblasts (Fig. 4). LPA and PDGF stimulated threonine phosphorylation of Tiam1 in a time-dependent manner, with maximum phosphorylation detected after 5 min of LPA or PDGF treatment in wild-type and PLC-γ1 null cells (not shown). However, in PLC-γ1 deficient cells the magnitude of PDGF-induced Tiam1 phosphorylation was only 28 ± 10% of that of the wild-type cells (Fig. 4). In contrast, LPA, which stimulates

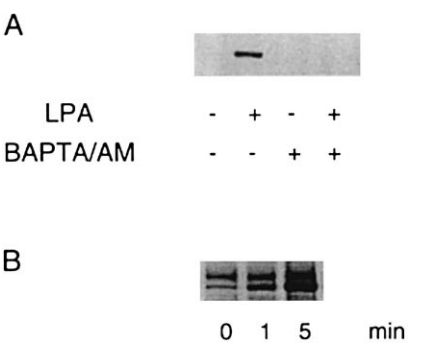

Fig. 3. Role of Ca^{2+} in LPA-induced Tiam1 phosphorylation. A: Swiss 3T3 fibroblasts were preincubated with 20 μM BAPTA/AM (+) or DMSO (−) for 30 min and then stimulated with (+) or without (−) 100 μM LPA for 10 min. B: Cells were stimulated with 1 μM ionomycin for the indicated times. Cells were lysed and analyzed for phosphothreonine and Tiam1 content by Western blotting as described in the legend to Fig. 2. The Tiam1 content of each lane was similar in A and in B (not shown). Data are representatives of at least three independent experiments.

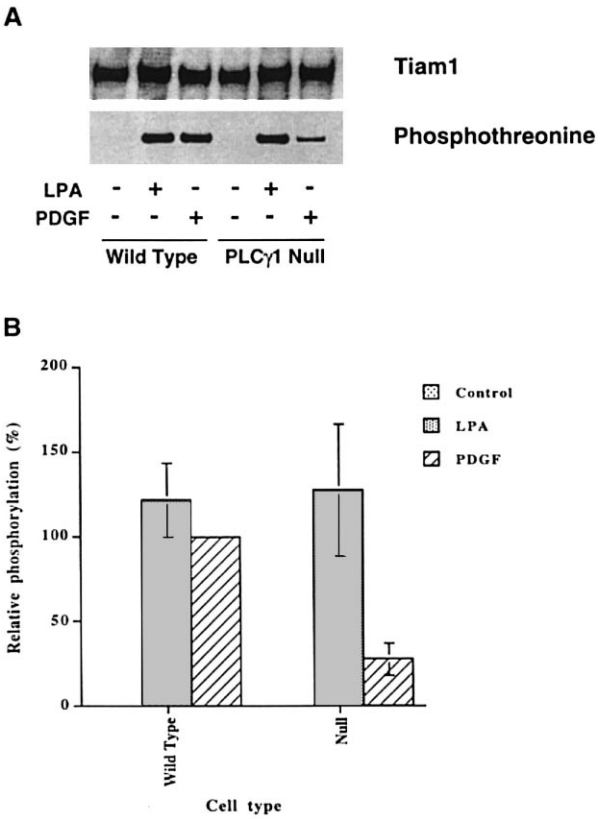

Fig. 4. PDGF-induced Tiam1 phosphorylation is reduced in PLC-γ1 deficient fibroblasts. Wild-type and PLC-γ1 null mouse embryonic fibroblasts were incubated with buffer, 10 ng/ml PDGF or 100 μM LPA for 5 min, as indicated. The cells were lysed and the Tiam1 immunoprecipitated as described in Section 2. After immunoprecipitation, the A-agarose beads bound to Tiam1 were resuspended in 80 μl Laemmli buffer, and 30 μl of each sample analyzed for phosphothreonine and Tiam1 content by Western blotting. A: Data are representatives of five independent experiments. B: Data are expressed as a percentage of the PDGF-stimulated Tiam1 phosphorylation in wild-type cells, and are the averages ± standard deviation from five independent experiments.

Ca^{2+} release and PKC through PLC-β, showed unimpaired inositol phosphate production (Hess, J.A., Ji, Q-S., Carpenter, G. and Exton, J.H., unpublished observations) and comparable Tiam1 phosphorylation in both wild-type and PLC-γ1 null cells (Fig. 4). The residual phosphorylation in the PLC-γ1 null cells treated with PDGF can be attributed to the presence of some PLC activity due to upregulation of PLC-γ2 (Hess, J.A., Ji, Q-S., Carpenter, G. and Exton, J.H., unpublished observations). These data provide evidence that PLC-γ1 plays a major role in PDGF-induced Tiam1 phosphorylation. This is also consistent with the fact that PMA and a Ca^{2+} ionophore stimulate Tiam1 phosphorylation in an additive manner (Fleming, I.N., Elliott, C.M. and Exton, J.H., unpublished observations).

Tiam1 acts as a Rac1-specific exchange factor in vivo [31] and transfection of this exchange factor into NIH3T3 cells stimulates Rac-dependent membrane ruffling and Jun kinase activation [14]. Rac1 can activate a number of processes, including membrane ruffling [1,4,32], serum response factor gene transcription [6], the Jun kinase pathway [7–9], Ras-stimulated transformation of NIH3T3 fibroblasts [11,12] and phospholipase D [33]. Membrane ruffling in Swiss 3T3 cells is stimulated by PDGF [1,4,32], inhibited by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin [32] and induced by expression of an active form of PI 3-kinase [34]. The observation that Tiam1 phosphorylation induced by PDGF is wortmannin-insensitive does not mean that Tiam1 is not involved in this effect because it is possible that PI 3-kinase is downstream from Tiam1 phosphorylation. It also remains possible that Rac1-mediated PMA-induced membrane ruffling [4] involves Tiam1 phosphorylation, since PMA treatment causes phosphorylation of this exchange factor in Swiss 3T3 cells [21].

PDGF-induced phosphorylation of Tiam1 may play an important regulatory role in some of the other signalling cascades that do not involve PI 3-kinase. This is consistent with the fact that active PI 3-kinase does not induce Ras/Rac/Rho pathways that can regulate transcription of c-Fos, c-Fos rerum response element or the transcription factors AP-1 or Elk-1 [34], and wortmannin does not affect Rac1-dependent phospholipase D activation [33]. Interestingly, PDGF stimulates both phospholipase D [35] and Tiam1 (Fig. 4) through PLC-γ1. Therefore, since Tiam1 acts as an exchange factor for Rac1, and Rac1 activates phospholipase D [33], it is possible that Tiam1 is involved in activation of this enzyme. Moreover, PDGF-stimulated Tiam1 phosphorylation and Rac1 translocation are both wortmannin-independent (McDonagh, C., Fleming, I.N. and Exton, J.H., unpublished observation) and Tiam1 phosphorylation (Fig. 2A) and Rac1 translocation [22] become detectable after 2.5 min, and maximum after 5 min PDGF treatment, suggesting that these findings may be connected. However, further work will be required to determine the biological function(s) of PDGF-induced phosphorylation of Tiam1.

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References

[1] Ridley, A.J. and Hall, A. (1992) Cell 70, 389–399.
[2] Miura, Y., Kikuchi, A., Musha, T., Kurodi, S., Yaku, H., Sasaki, T. and Takai, Y. (1993) J. Biol. Chem. 268, 510–515.
[3] Nobes, C.D. and Hall, A. (1995) Cell 81, 53–62.
[4] Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A. (1992) Cell 70, 401–410.
[5] Olson, M.F., Ashworth, A. and Hall, A. (1995) Science 269, 1270–1272.
[6] Hill, C.S., Wynne, J. and Treisman, R. (1995) Cell 81, 1159–1170.
[7] Coso, O.A., Chiariello, M., Yu, J-C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J.S. (1995) Cell 81, 1137–1146.
[8] Minden, A., Lin, A., Claret, F-X., Abo, A. and Karin, M. (1995) Cell 81, 1147–1157.
[9] Zhang, S., Han, J., Sells, M.A., Chernoff, J., Knaus, U.G., Ulevitch, R.J. and Bokoch, G.M. (1995) J. Biol. Chem. 270, 23934–23936.
[10] Bagrodia, S., Derijard, B., Davis, R.J. and Cerione, R.A. (1995) J. Biol. Chem. 270, 27995–27998.
[11] Qiu, R-G., Chen, J., Kirn, D., McCormick, F. and Symons, M. (1995) Nature 374, 457–459.
[12] Khosravi-Far, R., Solski, P.A., Clark, G.J., Kinch, M.S. and Der, C.J. (1995) Mol. Cell Biol. 15, 6443–6454.
[13] Cerione, R.A. and Zheng, Y. (1996) Curr. Opin. Cell Biol. 8, 216–222.
[14] Michiels, F., Stam, J.C., Hordijk, P.L., van der Kammen, R.A., Ruuls-Van Stalle, L., Feltkamp, C.A. and Collard, J.G. (1997) J. Cell Biol. 137, 387–398.

[15] Stam, J.C., Sander, E.E., Michiels, F., van Leewen, F.N., Kain, H.E.T., van der Kammen, R.A. and Collard, J.G. (1997) J. Biol. Chem. 272, 28447–28454.
[16] Rameh, L.E., Arvidsson, A-K., Carraway, K.L., Couvillon, A.D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M.P., Ravichandran, K.S., Burakoff, S.J., Wang, D-S., Chen, C-S. and Cantley, L.C. (1997) J. Biol. Chem. 272, 22059–22066.
[17] Graziani, G., Ron, D., Eva, A. and Srivastava, S.K. (1989) Oncogene 4, 823–829.
[18] Horii, Y., Beeler, J.F., Sakaguchi, K., Tachibana, T. and Miki, T. (1994) EMBO J. 13, 4776–4786.
[19] Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S. and Bustelo, X.R. (1997) Nature 385, 169–172.
[20] Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A. and Broek, D. (1998) Science 279, 558–560.
[21] Fleming, I.N., Elliott, C.M., Collard, J.G. and Exton, J.H. (1997) J. Biol. Chem. 272, 33105–33110.
[22] Fleming, I.N., Elliott, C.M. and Exton, J.H. (1996) J. Biol. Chem. 271, 33067–33073.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Claesson-Welsh, L. (1996) Int. J. Biochem. Cell Biol. 28, 373–385.
[25] Rankin, S. and Rozengurt, E. (1994) J. Biol. Chem. 269, 704–710.
[26] Olivier, A.R. and Parker, P.J. (1992) J. Cell. Physiol. 152, 240–244.
[27] Yeo, E-J. and Exton, J.H. (1995) J. Biol. Chem. 270, 3980–3988.
[28] Takuwa, N., Takuwa, Y., Yanagisawa, M., Yamashita, K. and Masaki, T. (1989) J. Biol. Chem. 264, 7856–7861.
[29] Issandou, M. and Rozengurt, E. (1990) J. Biol. Chem. 265, 11890–11896.
[30] Ji, Q-S., Winnier, G.E., Niswender, K.D., Horstman, D., Wisdom, R., Magnuson, M.A. and Carpenter, G. (1997) Proc. Natl. Acad. Sci. USA 94, 2999–3003.
[31] Michiels, F., Habets, G.G.M., Stam, J.C., van der Kammen, R.A. and Collard, J.G. (1995) Nature 375, 338–340.
[32] Nobes, C.D., Hawkins, P., Stephens, L. and Hall, A. (1995) J. Cell Sci. 108, 225–233.
[33] Hess, J.A., Ross, A.H., Qiu, R-G., Symons, M. and Exton, J.H. (1997) J. Biol. Chem. 272, 1615–1620.
[34] Reif, K., Nobes, C.D., Thomas, G., Hall, A. and Cantrell, D.A. (1996) Curr. Biol. 6, 1445–1455.
[35] Yeo, E-J., Kazlauskas, A. and Exton, J.H. (1994) J. Biol. Chem. 269, 27823–27826.