Role of Rho Family Proteins in Phospholipase D Activation by Growth Factors

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Treatment of fibroblasts with growth factors results in activation of phospholipase D (PLD). In order to determine the role of the Rho family of small GTPases in growth factor-mediated PLD activation, we used cells transfected with wild type and mutant Rac1. In response to epidermal growth factor (EGF), PLD activity was greatly increased in Rat1 fibroblasts expressing wild type Rac1 (wtRac1), and completely abrogated in cells expressing dominant negative N17Rac1, consistent with Rac1 mediating the action of this growth factor. In contrast, in cells treated with platelet-derived growth factor (PDGF) or phorbol ester, the wtRac1 cells showed little or no enhancement of PLD activity, and the response was not affected in the N17Rac1 cells, implying that Rac1 played a minimal role in the activation of PLD by PDGF or protein kinase C. Both growth factors produced an attenuated PLD response in cells expressing constitutively active V12Rac1, but these cells showed other changes, including altered morphology, increased basal PLD, and decreased growth factor receptor auto-phosphorylation. The effects of EGF and PDGF on phosphoinositide phospholipase C activity were not enhanced in cells expressing wtRac1 or inhibited in those expressing N17Rac1. In cells expressing constitutively active V12Rac1, basal phosphoinositide phospholipase C was elevated, but there were no significant effects of EGF or PDGF. We used C3 transferase of Clostridium botulinum, which ADP-ribosylates and inactivates RhoA, to investigate the involvement of RhoA in the activation of PLD by PDGF. Cells expressing wtRac1 and N17Rac1 showed a decreased PLD in response to PDGF when treated with C3 transferase, indicating a role for RhoA. In summary, these data indicate a major role for Rac1 in the activation of PLD by EGF, but not PDGF or protein kinase C.

Phospholipase D (PLD)1 is the enzyme responsible for the hydrolysis of phosphatidylcholine, which leads to the generation of choline and phosphatidic acid (1, 2). Phosphatidic acid can be further hydrolyzed by phospholipase A₂ to form the purported second messenger lysophosphatidic acid, or it can be hydrolyzed by phosphatidate phosphohydrolase to form 1,2-diacylglycerol (3). It has been suggested that the hydrolysis of phosphatidylcholine by PLD, and the subsequent action of phosphatidate phosphohydrolase on phosphatidic acid, are responsible for the long term increase in diacylglycerol in cells stimulated by growth factors (4). This diacylglycerol can activate Ca²⁺-independent isomers of protein kinase C (PKC) (5). In fibroblasts, the activity of PLD can be increased to different extents by platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (6–8). In some cases, this increase is dependent on PKC, since down-regulation of PKC or treatment of the cells with PKC inhibitors leads to an inhibition of growth factor-stimulated PLD activity (6–8). However, there are reports that PKC is not required for PLD activation, suggesting an alternate pathway(s) (9, 10).

Earlier reports showing that GTPαS activated PLD in membranes or permeabilized cells suggested the involvement of G proteins (11–14). Subsequent reports have shown that members of the Rho and ARF families of GTPases activate PLD (15–18). The Rho family of GTPases includes RhoA and Rac1. These proteins show 58% homology and are involved in alterations of the cytoskeleton (19, 20). In membranes from rat liver, neutrophils, and HL60 cells, recombinant RhoA stimulates PLD activity in the presence of GTPαS (15, 18, 21, 22). It has also been reported that treatment of Rat1 fibroblasts with C3 transferase from Clostridium botulinum, which ADP-ribosylates and inactivates Rho, results in decreased activation of PLD in response to lysophosphatidic acid and endothelin-1 (23).

In order to test whether Rac1 also played a role in the pathway leading to PLD activation, we used Rat1 fibroblasts stably expressing wild type Rac1 (wtRac1), the dominant negative form of Rac1 (N17Rac1), and the constitutively active form of Rac1 (V12Rac1) (24). The results indicate that Rac1 plays a major role in the activation of PLD by EGF, but not PDGF.

EXPERIMENTAL PROCEDURES

Materials—EGF (mouse receptor grade) was from Upstate Biotechnology Inc. (UBI), PDGF B/B (human recombinant) from Boehringer Mannheim, and 12-O-tetradecanoylphorbol-13-acetate (TPA) from Sigma. C3 exoenzyme from C. botulinum was purchased from List Biologicals. Phosphatidylbutanol standard was from Avanti Polar Lipids. [9,10-³H]Myristate and myo-[2-³H]Hiniositol were from DuPont NEN. Antibodies to the EGF receptor (sheep anti-human) were from UBI, antibodies to the PDGF receptor were from Oncogene Science, and those to Tyr(p) (PY20) were from ICN.

Expression of Rac Proteins in Rat1 Fibroblasts—Rat1 fibroblasts expressing Rac proteins are described elsewhere (24). Briefly, a plasmid expressing the tetracycline-sensitive transactivator was co-transfected with a neomycin resistance plasmid into Rat1 fibroblasts. Cells which showed repression to tetracycline were selected and co-transfected with...
Myc-tagged wtRac1, V12Rac1, N17Rac1, or empty vector controlled by an operator and a cytomegalovirus minimal promoter, along with a puromycin resistance plasmid. The presence of exogenous or expressed Rac proteins was confirmed with Western blotting using an anti-Myc antibody (M. Symons, Onyx Pharmaceuticals) for an anti-Rac antibody (C-14, Santa Cruz Biotechnology).

**Cell Culture**—Rat1 fibroblasts were grown in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal bovine serum, 0.4 mg/ml G415, 1.2 μg/ml puromycin, and the cells transfected with wtRac1 or mutant Rac1 were grown in the presence of 2 μg/ml tetra-cycline. The cells were grown at 37 °C in 5% CO2. Tetracycline was removed from the cells 48 h prior to experimentation.

All experiments were performed when cells were 70–80% confluent, with great care being taken to assure equal confluence between the different cell lines.

**Phospholipase D Assay**—Cells were plated in 100-mm tissue-culture plates or six-well plates. The cells were serum-starved in DMEM supplemented with 0.4 mg/ml puromycin and 0.5% fatty acid-free bovine serum albumin for 24 h before the start of the assay. For the final 16 h of serum starvation, the cells were labeled with 1 μCi/ml [9,10-3H]myristate (1 Ci/mmol, New England Nuclear). After the 16 h of serum starvation, 5% of the medium was removed, washed once with 5 ml PBS, and added 750 μl of ice-cold methanol. Cells were scraped off the plates, and the extracts separated with methanol/chloroform/methanol (2:1), and spotted onto silica gel 60A thin layer chromatography plates (Whatman). The plates were developed in the upper phase of the solvent system of ethyl acetate/isooctane/H2O/layer chromatography plates (Whatman). The plates were measured as described by Yeo and Exton (6). Briefly, cells were plated on six-well tissue culture plates at sub-confluent conditions. The cells were labeled with 1 μCi/ml [9,10-3H]myristate for 24 h. At the end of this incubation, cells were washed three times with 3 ml of 60 M ammonium formate, 5 M NaCl, 5.5 M MgCl2, either alone or in the presence of 5 μg/ml C3 transferase, using a rubber policeman. The cells were then aliquoted equally onto a six-well plate, and allowed to grow overnight. The cells were then serum-starved for 18 h, and 1 μCi/ml [9,10-3H]myristate was added for the final 15 h. At the end of this incubation, PLD was assayed.

**Scrape-loading of C3 Transferase**—Cells were scrape-loaded according to the method of Malcolm et al. (23). Briefly, cells were grown to confluence on 100-mm tissue culture dishes. The cells were scraped in 500 μl of scrape-loading buffer (10 mM Tris-HCl, pH 7.2, 114 mM KCl, 25 mM NaCl, 5.5 mM MgCl2), either alone or in the presence of 5 μg/ml C3 transferase, using a rubber policeman. The cells were then aliquoted equally onto a six-well plate, and allowed to grow overnight. The cells were then serum-starved for 18 h, and 1 μCi/ml [9,10-3H]myristate was added for the final 15 h. At the end of this incubation, PLD was assayed.

**EGF Receptor and PDGF Receptor Autophosphorylation**—Sub-confluent cells grown on 100-mm tissue culture plates were serum-starved overnight, then treated with 50 ng/ml EGF, 25 ng/ml PDGF, or vehicle (serum-free medium) for 5 min at 37 °C. At the end of this incubation, the cells were lysed in 400 μl of RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na3VO4, 1 mM NaF) by rocking the plates at 4 °C for 15 min. The cells were scraped into Eppendorf tubes on ice, and the tubes spun at 14,000 × g for 3 min at 4 °C. The supernatant was preclarified by centrifugation at 10,000 × g for 1 h at 4 °C, and a protein assay performed on the cleared supernatant. Five hundred μg of protein at a concentration of 1 mg/ml was immunoprecipitated overnight at 4 °C with 4 μg of anti-human EGF receptor antibody (UBI) or 1 μg of anti-mouse PDGF receptor antibody (Onogene Science). The EGFR was collected with 2 μg of goat anti-sheep antibody (Sigma) and 20 μl of Protein A-Sepharose, while the PDGFR was collected with 50 μl of Protein A-Sepharose beads (Pharmacia Biotech Inc.). The beads washed three times with 0.4 ml of ice-cold RIPA buffer. The EGFR was eluted from the beads with 20 μl of 2 × SDS sample buffer (Pharmacia), the PDGFR was eluted with 20 μl of 4 × SDS sample buffer, and the proteins separated by SDS-PAGE on a 6% Novex gel. The proteins were transferred to Immobilon P, and the phosphorylation level of the immunoprecipitated EGFR and PDGFR was determined by probing the blot with PY20 antibody.

**Western Blotting of EGF Receptor**—Subconfluent cells on a six-well plate were washed once with 5 ml PBS and scraped in 200 μl of 1 × SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.0025% bromphenol blue, 10% glycerol, 2.5% β-mercaptoethanol). Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce). Equal concentrations of cellular protein (2.5 μg) were separated by SDS-PAGE on a 6% gel, transferred to Immobilon-P, blocked in 1% bovine serum albumin, and probed with the antibody EGFR1005 (Santa Cruz). The blot was developed using ECL (Amersham).

**RESULTS**

**EGF-induced PLD Activation**—In order to investigate a role for Rac1 in the growth factor-induced PLD activity, we treated Rat1 fibroblasts expressing wild type or mutant forms of Rac1 with EGF. Western blotting showed that these forms of Rac1 were increased 5–10-fold over endogenous Rac1 in the cells (data not shown). The growth factor (100 nM) caused a small but reproducible increase (mean 27 ± 7%, n = 5, p < 0.05) in PLD activity in Rat1 fibroblasts transfected with the empty vector alone (Fig. 1A). The magnitude of the increase is similar to that seen in Swiss 3T3 fibroblasts (6). In contrast, EGF caused a much greater response (mean 340%, n = 5) in Rat1 fibroblasts transfected with wild type Rac1 (wtRac1) (Fig. 1A). Most strikingly, the PLD response to EGF was completely inhibited in the Rat1 fibroblasts expressing dominant negative Rac1 (N17Rac1) (Fig. 1A). N17Rac1 has a higher affinity for the guanine nucleotide exchange factor (GEF) and thus blocks the activation of endogenous Rac (19, 27, 28). For completeness, we also studied Rat1 fibroblasts transfected with constitutively active Rac1 (V12Rac1). In these cells, the basal level of PLD activity was increased 1.6-fold, and the response to EGF was similar to that seen in vector cells (Fig. 1A). However, these cells showed morphological changes when compared to vector cells. As found by another group (29), the cells were large and flat, and showed membrane ruffling, pinoctytic vesicles, and occasional multinucleation.

The PLD response to EGF in the wtRac1 cells occurred in a dose-dependent manner, and the activity was increased over vector alone at all concentrations of EGF tested (Fig. 1B). In contrast, the PLD response to EGF was completely lost in the N17Rac1 cells at all EGF concentrations tested. These data suggest that Rac1 plays a major role in the activation of PLD by EGF in Rat1 fibroblasts.

**PDGF-induced PLD Activity**—In contrast to the striking changes in EGF-induced PLD activation observed in the cells expressing wild type and mutant forms of Rac1, the changes in the PDGF response were much less. PDGF (50 ng/ml) caused a much larger (6-fold) increase in PLD activity in the vector cells than did EGF (Fig. 2A). Although PLD activity was enhanced in the wtRac1 cells upon treatment with PDGF, the increase over control (no EGF) was less than 2-fold greater than that in control cells.

**Phospholipase D Activity**—At the end of this incubation, PLD was assayed.
seen in vector cells, i.e. much less than the 12-fold increase in the increment due to 100 nM EGF seen when Rac1 and vector cells are compared (Fig. 1A). The V12Rac1 cells showed the expected increase in basal PLD activity and also an attenuated response to PDGF when compared to the vector cells. Surprisingly, the N17Rac1 cells showed no attenuation of PDGF-induced PLD activation. The mean response of the N17Rac1 cells to PDGF was 6-fold ($n = 5$) equal to that in vector cells.

The PLD response to PDGF occurred in a dose-response fashion in the vector, wtRac1, and N17Rac1 cells, with the wtRac1 cell response being greater than the vector cells’ response at all concentrations of PDGF (Fig. 2A). The V12Rac1 cells showed an attenuated PDGF response at all concentrations, whereas the response in the N17Rac1 cells was consistently unchanged (Fig. 2B). These data suggest that the PLD response to PDGF in Rat1 fibroblasts is not dependent on Rac1.

**Phorbol Ester-induced PLD Activity**—It has been reported that PKC is required for PLD activation in Swiss 3T3 fibroblasts (6). In order to determine whether PKC acted upstream of Rac1 in the regulation of PLD, we tested whether the TPA response differed in Rat1 cells transfected with wild type or mutant forms of Rac1. Vector, wtRac1, V12Rac1, and N17Rac1 cells all responded in a dose-dependent manner to TPA (Fig. 3), but the response did not differ in any of the transfected cells, implying that Rac1 did not lie “downstream” from PKC, in contrast to what has been shown for RhoA in Rat1 fibroblasts (23).

**Growth Factor-induced PI-PLC Activation**—In order to confirm that the enhanced PLD activity seen in the wtRac1 cells in response to the growth factors was specific for PLD and that expression of this G protein did not affect other pathways downstream of their receptors, we tested whether the ability of growth factors to stimulate PLC activity was changed. The basal activity of PI-PLC was not altered in the wtRac1 or
N17Rac1 cells, but was increased 2-fold in the V12Rac1 cells. Consistent with what is seen in Swiss 3T3 cells, none of the types of Rat1 cells showed a significant increase in PLC activity in response to EGF (Fig. 4 A) (6). In particular, there was no response in the wtRac1 cells. In contrast, all the cell types except the V12Rac1 cells showed increases in PI-PLC activity in response to PDGF. The responses in the wtRac1 and N17Rac1 cells did not differ significantly (Fig. 4 B). These data indicate that the PLD changes in the wtRac1 and N17Rac1 cells were not secondary to alterations in phosphoinositides or PI-PLC.

Level and Autophosphorylation of the EGF and PDGF Receptors—We hypothesized that the increased basal activities of PLD and PLC and the decreased PLD responses of the V12Rac1 cells to EGF and PDGF may be due to secondary changes occurring in response to the expression of constitutively active Rac1. In particular, we investigated whether the expression of Rac1 altered the level or autophosphorylation of their receptors (EGFR and PDGFR). Western blotting demonstrated that V12Rac1 cells did, in fact, have a decreased level of EGFR when compared to vector cells. In contrast, the wtRac1 and N17Rac1 cells showed comparable levels of EGFR when compared to vector cells (Fig. 5 A), supporting the view that the altered PLD responses were not due to changes in receptor level. The V12Rac1 cells also had decreased EGFR autophosphorylation levels in response to EGF when compared to the vector cells. The wtRac1 cells showed comparable autophosphorylation levels when compared to vector cells, and the N17Rac1 cells showed a slight decrease in EGFR autophosphorylation in response to EGF (Fig. 5 B), but this was insufficient to explain the total loss of PLD stimulation.

Autophosphorylation of the PDGFR was also measured (Fig. 5 C). The wtRac cells showed an attenuated PDGFR autophosphorylation in response to 25 ng/ml PDGF when compared to the vector cells, which cannot explain the increase in PLD activity seen in these cells. Interestingly, the V12Rac1 cells had the same level of PDGFR autophosphorylation in response to PDGF as the vector cells. The N17Rac1 cells also showed the same level of PDGFR autophosphorylation in response to PDGF as the vector cells, and the PLD response to PDGF was the same as the vector cells.

C3 Transferase Effect of PLD Activity—Because our data indicated that Rac1 played little or no role in the activation of PLD by PDGF, we investigated a role for RhoA. We introduced C3 transferase from C. botulinum into vector and N17Rac1 cells by the method of scrape-loading. Vector cells treated with C3 toxin show an 80% inhibition in PDGFR-induced PLD activity at two concentrations of PDGF (Fig. 6 A). N17Rac1 cells scrape-loaded with C3 transferase also showed a 70% inhibition of PLD activity in response to PDGF (Fig. 6 B). In vector cells scrape-loaded in the absence of C3 toxin and treated with

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*This effort to measure the level of PDGFR in the cells by Western blotting was unsuccessful due to the low and variable signal.*
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50 ng/ml PDGF, the increase in PLD activity ranged from 5.8- to 10.6-fold (n = 3). Although this increase was somewhat variable, we always observed inhibition by C3 transferase. These results suggest that RhoA mediates a large fraction of the PDGF-induced activation of PLD in Rat1 cells. We attempted similar experiments with EGFR-treated vector and wt-Rac1 cells. However, the C3 exoenzyme did not produce consistent effects in the vector cells. This was due to the small increase in EGF-induced PLD activity in these, which was rendered variable by scrape-loading. There was also a large decrease in the viability of C3-treated wtRac1 cells, rendering the results questionable. For the same reason, PDGF effects were also not tested in these cells.

DISCUSSION

Much evidence has accumulated in studies using cell-free systems to suggest that phospholipase D activity can be regulated by small G proteins (15–18, 21, 22), but there have been few reports on the role of the Rho family of small G proteins in activating PLD in intact cells. Recently, Malcolm et al. (23) reported a role for RhoA in agonist-stimulated PLD activity in whole-cell studies performed on Rat1 fibroblasts. The data presented here suggest a role for Rac1 in EGF-induced PLD activity in Rat1 fibroblasts. The PLD response of vector cells to PDGF was small, but consistently observed, and was greatly enhanced in wtRac1 cells (Fig. 1). In contrast to the findings with EGF, the vector cells showed a robust PLD response to PDGF, and this response was much less enhanced by Rac1 overexpression (Fig. 2). Most notably, Rac1 was necessary for the response of PLD to EGF, as cells expressing dominant negative N17Rac1 showed no PLD activity in response to EGF. On the other hand, Rac1 was not required for PDGF-induced PLD activity, as the response in N17Rac1 cells was not different from the vector cell response. These results indicate that the EGF and PDGF receptors are acting through different signaling pathways to activate PLD.3 Peppelenbosch et al. (30) using the same cells showed an EGF-induced increase in intracellular Ca\(^{2+}\) was dependent on Rac1, while the PDGF-induced increase in Ca\(^{2+}\) was only partly dependent on Rac1.

Studies in Rat1 cells and other types of fibroblasts show other differences in EGF and PDGF effects. For example, in Rat1 cells (Fig. 4) and Swiss 3T3 cells (6), the activation of PI-PLC is very much greater with PDGF than EGF. Furthermore, although the receptor autophosphorylation sites mediating the interaction of PI-PLC, RasGAP, and the p85 subunit of PI 3-kinase with the PDGF receptor are well defined (31, 32), those for the EGFR are not stringent (33, 34). There are also differences in the affinities of the two receptors for certain Src homology region 2-containing proteins (35, 36). The present findings provide further evidence of differences in the signal transduction pathways for the two receptors.

The cells expressing constitutively active V12Rac1 showed down-regulated responses of PLD and PI-PLC to PDGF. However, these cells showed many other changes. These include increased basal PLD and PI-PLC activities and morphological changes, such as a flattening of the cells, increased pinocytotic vesicles, and the appearance of multinucleated cells. Since autophosphorylation of the PDGFR was not decreased, these findings suggest that the coupling between the receptor and the two phospholipases was impaired. The mechanisms by which increased expression of V12Rac1 leads to such down-regulation are not immediately apparent and could be complex. It is of interest that Peppelenbosch et al. (30) showed that EGF-induced increase in intracellular Ca\(^{2+}\) is mediated by Rac1, and yet they did not observe elevated levels of Ca\(^{2+}\) in V12Rac cells. They also suggest this is due to efficient feedback mechanisms (30).

Since PDGF signaling to PLD did not appear to be dependent on Rac1, we examined the role of RhoA. Rat1 fibroblasts scrape-loaded with 5 μg/ml C3 transferase from C. botulinum showed a 60% decrease in the subsequent in vitro ADP-ribosylation of RhoA when the assay was performed 48 h subsequent to the scrape-loading (data not shown). In addition, when the cells were scrape-loaded with a fluorescent 70-kDa dextran, >90% of the cells showed fluorescence uptake (23). These results suggest that scrape-loading is a valuable technique for introducing proteins into these cells. The C3 transferase treatment resulted in an 80% inhibition in PDGF-induced PLD activity in vector and N17Rac1 cells, indicating that RhoA mediates a large fraction of the PDGF effect in Rat1 fibroblasts. RhoA may also act downstream of Rac1 to mediate EGF-induced PLD activity, as it does in mediating stress fiber formation (37). However, efforts to demonstrate a role for RhoA in the activation of PLD in Rac1-overexpressing cells were thwarted by a great reduction in viability in these cells when they were used.

3 A minor role for Rac1 in the activation of PLD by PDGF could have been obscured by the variation in the results with N17Rac1. Furthermore, the enhancement of the PDGF effect in the wtRac1 cells suggests that the PDGF signaling pathway interacts minimally with Rac1 when this is present at normal levels, but can do so when it is overexpressed.
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and mutant Rac cells with wortmannin did not alter the PDGF- or EGF-induced changes in PLD activity (S. Maclure, J. Hess, and J. H. Exton, unpublished results).

small G protein ARF (16, 17, 44). Much further work is required to delineate the pathways by which Rho, ARF, and PKC activate PLD and also the mechanisms by which growth factors and other agonists activate small G proteins.

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4 In preliminary experiments, treatment of vector cells, wtRac cells, and mutant Rac cells with wortmannin did not alter the PDGF- or EGF-induced changes in PLD activity (S. Maclure, J. Hess, and J. H. Exton, unpublished results).