Activation of phospholipase D1 by direct interaction with ADP-ribosylation factor 1 and RalA

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Abstract Phospholipase D1 (PLD1) is known to be activated by ADP-ribosylation factor 1 (ARF1). We report here that ARF1 co-immunoprecipitates with PLD1 and that the ARF1-dependent PLD activation is induced by the direct interaction between ARF1 and PLD1. We found that RalA, another member of the small GTP-binding proteins, synergistically enhances the ARF1-dependent PLD activity with an EC_{50} of about 30 nM. Using in vitro binding assay, we show that ARF1 and RalA directly interact with different sites of PLD1. The results suggest that the independent interactions of RalA and ARF1 with PLD1 are responsible for the synergistic activation.

Key words: Phospholipase D1; Adenosine diphosphate-ribosylation factor 1; RalA; Phosphatidylinositol 4,5-bisphosphate

1. Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to produce choline and phosphatidic acid (PA). PA appears to be a second messenger in multiple signaling events, and its production can be regulated by a variety of extracellular agonists including hormones and growth factors [1,2].

It has been suggested that agonist-dependent PLD activation is mediated by protein kinase C (PKC), calcium ion, low molecular weight GTP-binding proteins (small G proteins) of the Ras superfamily, and protein-tyrosine kinases [3]. Several studies have demonstrated the involvement of the ADP-ribosylation factor, a member of the small G protein family, in PLD activation [4,5]. ARF was reported to synergistically stimulate PLD activity in concert with small G proteins of the Rho family including RhoA, Cdc42, and Rac1 [6]. In addition to the small G proteins, PKC-α has been shown to stimulate PLD activity, and PLD activation by PKC-α occurs synergistically with ARF and RhoA [7,8]. The molecular nature of ARF-, RhoA-, and PKC-dependent PLD has been recently elucidated and designated PLD1 [9,10].

RalA, another member of the small G protein family, was reported to play a role in v-Src, a non-receptor protein-tyrosine kinase, induced PLD activation [11]. RalA was reported to interact with the v-Src-dependent PLD, however, RalA did not directly stimulate PLD activity [11]. Thus, the role of RalA in v-Src-induced PLD activation is still ambiguous.

Previously, we demonstrated the stimulation of PLD activity by direct interaction of PLD1 with PKC-α in a phorbol 12-myristate 13-acetate (PMA)-dependent manner [12]. In contrast, it is still unknown whether the small G protein-dependent PLD activation is mediated by direct interaction with PLD1. In the present study, we show evidence for the first time that ARF1 and RalA directly interact with different sites on PLD1, that RalA synergistically stimulates PLD1 activity in concert with ARF, and we suggest a role for PLD1 as a point of convergence of diverse PLD-activating signals.

2. Materials and methods

2.1. Materials

Dioleoyl-phosphatidylethanolamine (dioleoyl-PE), dipalmitoyl-phosphatidylcholine (dipalmitoyl-PC), and phosphatidylinositol 4,5-bisphosphate (PIP_2) were purchased from Sigma. The enhanced chemiluminescence kit and dipalmitoylphosphatidyl-[*methyl*-^{3}H]choline were purchased from Amersham International (Buckinghamshire, UK). GTPγS, GDPβS, and thrombin were obtained from Boehringer Mannheim (Mannheim, Germany). SP-Sepharose and glutathione-Sepharose were from Pharmacia Biotech Inc. (Uppsala, Sweden). Anti-RalA antibody was obtained from Transduction Lab (Lexington, KY, USA). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Pansorbin cells and β-octyl glucopyranoside were obtained from Calbiochem (San Diego, CA, USA).

2.2. Preparation of GST-RalA fusion protein and ARF1

The cDNA for RalA was isolated by polymerase chain reaction (PCR) and inserted into the appropriate site of the PGEX-4T vector (Pharmacia Biotechnology). The glutathione-*S*-transferase-RalA (GST-RalA) fusion protein was induced in *Escherichia coli* by adding 100 μM isopropyl-1-thio-β-D-galactopyranoside followed by growth at 37°C for 5 h. The GST-RalA fusion protein was purified using glutathione-Sepharose, and RalA was released from the immobilized GST-RalA fusion protein with thrombin essentially as described [13]. Thrombin was removed by incubation with benzamidine-Sepharose 6B beads (Pharmacia). Myristoylated recombinant ARF1 was expressed in *Escherichia coli* and purified as described [14]. All recombinant proteins were purified to greater than 95% homogeneity as judged by Coomassie Blue staining of SDS gels.

2.3. Purification of phospholipase D1 from bovine brain

PLD1 was purified from the membrane fraction of bovine brain employing the anti-PLD1 antibody coupled to protein A-Sepharose resin according to a slightly modified procedure reported previously

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Abbreviations: PLD, phospholipase D; PC, phosphatidylcholine; PA, phosphatidic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; Small G proteins, low molecular weight GTP-binding proteins; ARF, ADP-ribosylation factor; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiotriphosphate); ECL, enhanced chemiluminescence

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[12]. In brief, PLD1 was partially purified from one bovine brain by Sephadex G-50 and SP-Sepharose column chromatography as described [15], further purified to homogeneity from this partially purified PLD1 fraction with the help of anti-PLD1 antibody coupled to protein A-Sepharose, and eluted by treatment with buffer A containing 0.4 M NaCl, 1% β-octyl glucopyranoside, and 400 μM peptide (TKLVPMEVWT) as described [12]. The final PLD1 preparation was homogeneous and contained about 4 μg of PLD1 as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining (data not shown).

2.4. Phospholipase D assay

PLD activity was measured by choline release from phosphatidylcholine as essentially described [5] with a slight modification. In brief, aliquots of the purified PLD1 preparation were added to a standard assay mixture containing 125 μl of buffer B (50 mM Tris-HCl, pH 7.3, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 3 mM EGTA, 80 mM KCl, and 1 mM dithiothreitol) and 25 μl of phospholipid vesicles composed of dioleoyl-PE, PIP_2, and dipalmitoyl-PC in a molar ratio of 16:1.4:1 and dipalmitoyl-[*methyl*-^{3}H]choline (total 200 000 cpm/assay). Final concentration of PC was 3.3 μM. In some experiments, ARF1, RalA, and guanine nucleotides were added to the reaction mixture to activate PLD1.

2.5. Coimmunoprecipitation of ARF1 with PLD1

Purified PLD1 (0.1 μg) was precipitated by immobilized anti-PLD1 antibody (1 μg of affinity-purified antibody/30 μl of Pansorbin cells). The immune complexes were incubated with 0.5 μM ARF1 and guanine nucleotides in 150 μl buffer B containing 0.5% β-octyl glucopyranoside at 37°C for 15 min. The resulting immune complexes were washed twice with buffer B containing 0.5% β-octyl glucopyranoside, further washed twice with buffer B, and resuspended in 50 μl of buffer B. The resuspended immune complexes were analyzed by SDS-PAGE in an 8–16% gradient polyacrylamide gel and transferred onto a nitrocellulose filter. The upper and lower parts of the nitrocellulose filter were probed with anti-PLD1 or anti-ARF1 antibody, respectively.

2.6. Binding of ARF1 and PLD1 to phospholipid vesicles

Mixed phospholipid vesicles composed of 53 μM PE, 3.3 μM PC, and various concentrations of PIP_2 were incubated with 10 ng PLD1, 0.5 μM ARF1, 10 μM GTPγS, and 150 μg bovine serum albumin (BSA) in 150 μl buffer B. After incubation at 37°C for 15 min, the reaction mixtures were centrifuged at 300 000 × *g* for 20 min in a TL-100 ultracentrifuge (Beckman). By inclusion of phosphatidyl-[^{3}H]inositol 4,5-bisphosphate or dipalmitoylphosphatidyl-[*methyl*-^{3}H]choline into the phospholipid vesicles, the recovery of precipitated phospholipids was estimated to be above 95% and the recovery was not affected by the various concentrations of PIP_2 and PLD1 (data not shown). The pellets obtained after ultracentrifugation containing phospholipid vesicles were resuspended and subjected to 8–16% SDS-PAGE and immunoprobing using anti-PLD1 or anti-ARF antibodies.

2.7. Binding of PLD1 to immobilized GST-RalA fusion protein

Immobilized GST-RalA fusion protein was pre-incubated with GTPγS or GDPβS according to the following procedure: the immobilized protein was incubated with of 50 μM GTPγS or GDPβS in buffer C (20 mM HEPES-NaOH pH 7.5, 10 mM EDTA, 5 mM $MgCl_2$, and 1 mM DTT) and washed twice with buffer B. Aliquots of PLD1 (0.1 μg) and 500 μg BSA were incubated with the nucleotide-preloaded immobilized proteins (10 μg) in 500 μl of buffer B containing 0.5% β-octyl glucopyranoside at 4°C for 1 h. The immobilized proteins were then washed twice with buffer B containing 0.5% β-octyl glucopyranoside, resuspended in 50 μl of 1× electrophoresis sample buffer, and analyzed by SDS-PAGE and immunoprobing using the anti-PLD1 antibody.

2.8. Western blotting

After electrophoresis, proteins were transferred to nitrocellulose by electroblotting. After transfer, the nitrocellulose was blocked with TTBS buffer (50 mM Tris-HCl, pH 7.4, 0.05% Tween 20, 150 mM NaCl) containing 5% skimmed milk. Subsequently, the blots were incubated with the first antibody (anti-PLD1 or anti-ARF1 in TTBS containing 5% skimmed milk) for 2 h. The nitrocellulose membranes were washed 4 times for 10 min with TTBS before incubation with horseradish peroxidase conjugated anti-rabbit IgG for 1 h. Visualization of immune complexes was performed by a horseradish peroxidase-dependent chemiluminescence (ECL) kit.

3. Results

3.1. Association of ARF1 with PLD1

To find out whether ARF can directly bind to PLD1, PLD1 was immunoprecipitated with anti-PLD1 antibody, and the immune complexes were incubated with ARF1 and guanine nucleotides in the standard assay mixture. 0.5% β-octyl glucopyranoside was added to the reaction mixtures to prevent non-specific interaction between the immune complexes and ARF1. The resultant immune complexes were resuspended and added into an assay mixture that measured PLD activity without or with additional inclusion of GTPγS. As shown in Fig. 1A, the ARF1- and GTPγS-pre-treated immune complex showed PLD activities which were not affected by additional inclusion of 10 μM GTPγS, suggesting the presence of GTPγS-bound ARF1 in the immune complex. By probing Western blots of the immune complexes with anti-ARF1 antibody, we found that ARF1 indeed binds to PLD1 in the presence of

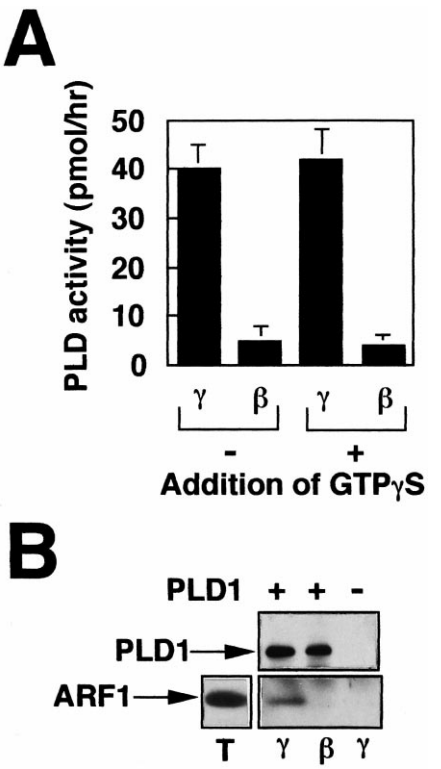

Fig. 1. Association of ARF1 with PLD1. A: PLD1 (0.1 μg)-coupled immune complexes were incubated with 0.5 μM ARF1 as described in Section 2. As a negative control, anti-PLD1 antibody (1 μg) coupled to Pansorbin cells (30 μl) was also incubated with 0.5 μM ARF1. 10 μM GTPγS (γ) or GDPβS (β) were included in the reaction mixtures as indicated. To measure PLD activity, aliquots (10 μl) of the resuspended immune complexes were added into the standard assay mixtures with (+) or without (−) additional inclusion of 10 μM GTPγS. The data shown are the means ± S.D. of three independent experiments. B: Aliquots (40 μl) of the resuspended immune complexes were subject to immunoblot analysis with antibodies against PLD1 and ARF1 and visualized by ECL exposure for 5 min. ARF1 (T) was subjected to immunoblot analysis without prior immunoprecipitation and represent 10% (150 ng) of the ARF1 used for the immunoprecipitation. The data are representative of three separate experiments.

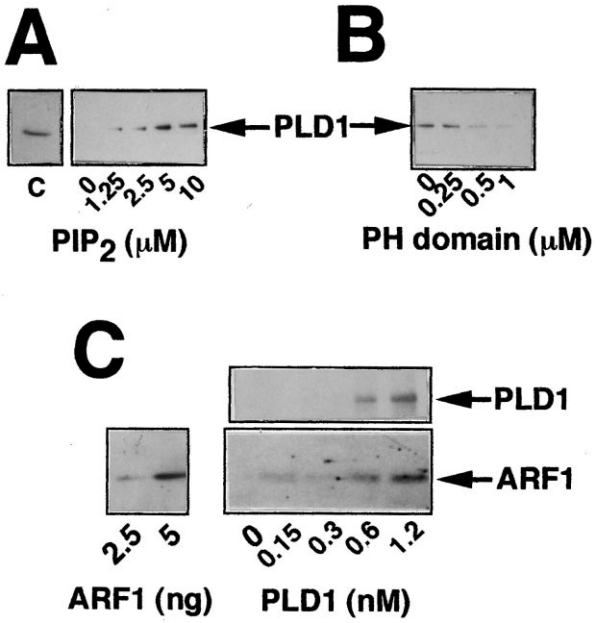

Fig. 2. Association of PLD1 and ARF1 with PIP_2-containing phospholipid vesicles. A: Aliquots (10 ng) of purified PLD1 were added into standard assay mixtures containing phospholipid vesicles containing the indicated concentrations of PIP_2 as described in Section 2. B: Aliquots (10 ng) of PLD1 were reconstituted with phospholipid vesicles containing 5 μM PIP_2 and the indicated concentrations of the pleckstrin-homology domain of PLC-δ1 fused to GST. C: 0.5 μM ARF1 and 10 μM GTPγS were added to assay mixtures containing the indicated concentrations of PLD1. The resultant pellets of phospholipid vesicles, 10 ng PLD1 (C), and the indicated amounts of ARF1 were analyzed by 8% SDS-PAGE, and probed with anti-PLD1 or anti-ARF1 antibodies. PLD1 and ARF1 were visualized by ECL exposure for 5 and 30 min, respectively. The data are representative of three separate experiments.

GTPγS but not GDPβS (Fig. 1B). These results suggest that ARF1 interacts with PLD1 with high affinity in the presence of GTPγS, thus activating PLD activity.

3.2. Association of PLD1 and ARF1 with PIP_2-containing phospholipid vesicles

Recent reports implicated PIP_2 in ARF-dependent PLD activation as a cofactor and in guanine nucleotide exchange of ARF1 through interaction with ARF1 [16–18]. Moreover, interaction between PIP_2 and partially purified rat brain PLD has also been demonstrated [19]. Thus, it seemed promising to examine the effect of interactions between PLD1, ARF1, and PIP_2 on PLD activity. To find out whether PLD1 and ARF1 interact with PIP_2, we added PLD1, ARF1, and PIP_2-containing mixed phospholipid vesicles into reaction mixtures to measure the amounts of PLD1 and ARF1 that would associate with PIP_2-containing phospholipid vesicles as described in Section 2. As seen in Fig. 2A, PLD1 maximally binds to the mixed phospholipid vesicles in the presence of 5 μM PIP_2. We found that PLD1 does not bind to phospholipid vesicles containing PI instead of PIP_2 (data not shown). The interaction between PLD1 and the PIP_2-containing phospholipid vesicles was decreased by incubation with the pleckstrin-homology domain of phospholipase C-δ1 fused to GST (Fig. 2B), suggesting that PLD1 binds to phospholipid vesicles through interaction with PIP_2. In support of our current observations, Yokozeki et al. [19] have shown that pleckstrin homology domain of β-adrenergic receptor kinase fused to GST inhibits the interaction of rat brain PLD and PIP_2. The amount of ARF1 associated with phospholipid vesicles was augmented by increased concentrations of PLD1 (Fig. 2C). Compared to the level of ARF1 seen in Fig. 1B, the levels of ARF1 are very low, thus prolonged exposure (30 min) for an ECL is required. The amount of ARF1 associated with about 0.18 pmol (1.2 nM) PLD1 is estimated to be more than 0.13 pmol (2.5 ng) but less than 0.26 pmol (5 ng), suggesting that ARF1 may interact with PLD1 in an approximate ratio of 1:1. Taken together, these results provide evidence for direct interaction between PLD1 and ARF1.

3.3. Effects of RalA on basal and ARF1-dependent PLD activities of PLD1

To determine the effect of RalA on the enzymatic activity of PLD1, we measured the basal and the ARF-dependent activities of PLD1 in the presence of GDPβS or GTPγS-preloaded RalA. As can be seen in Fig. 3, GTPγS-preloaded RalA had a small effect on the basal activity of PLD1, whereas it increased the ARF1-dependent PLD activity approximately 2.5-fold with an EC_{50} of 30 nM. In our study, we found that preloading with GTPγS is critical for the RalA-induced PLD activation (data not shown). Based on these results we suggest that RalA is a separate PLD1-activating factor which synergistically can stimulate PLD1 activity in concert with ARF.

3.4. Independent association of ARF1 and RalA with PLD1

The synergistic PLD1 activation by ARF1 and RalA suggested that PLD1 activity may be independently regulated by the two separate signals. Thus, we examined whether the synergistic activation of PLD1 is mediated by direct interaction between PLD1, RalA, and ARF1. As shown in Fig. 4A, PLD1 specifically bound not to immobilized GST but to the immobilized GST-RalA fusion protein. PLD1 bound to the immobilized GST-RalA fusion protein in a GTPγS-dependent manner. Next, we examined whether PLD1 interacts with

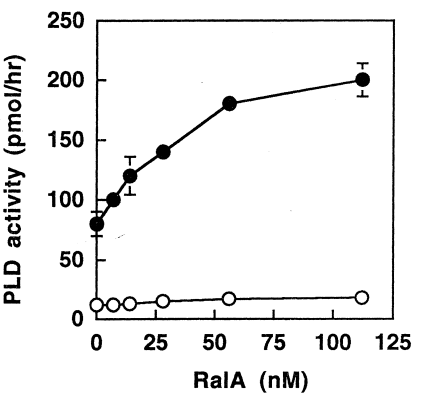

Fig. 3. Effects of RalA on basal and ARF1-dependent activity of PLD1. The indicated concentrations of RalA were preincubated with 50 μM GTPγS in buffer C in a final volume of 30 μl at room temperature for 30 min. The preincubated mixtures were added to standard assay mixtures containing 0.5 μM ARF1 (closed circle) and without ARF1 (open circle). The reaction was started by addition of aliquots (1 ng) of purified PLD1 to the assay mixtures and incubated at 37°C for 15 min. PLD activity was determined by measuring the formation of [^{3}H]choline as described in Section 2. Data are the means ± S.D. of three independent experiments.

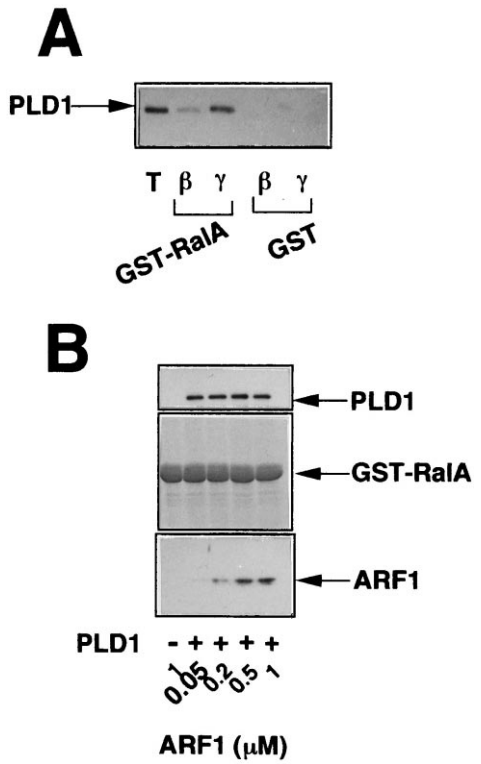

Fig. 4. Independent interaction of RalA and ARF1 with PLD1. A: Immobilized GST-RalA fusion protein was pre-incubated with GTPγS (γ) or GDPβS (β) as described in Section 2. Aliquots (0.1 μg) of purified PLD1 were incubated with the guanine nucleotide-preloaded immobilized proteins. The resulting beads and 40 ng PLD1 (T) were analyzed by 8% SDS-PAGE, and Western blots probed with anti-PLD1 antibody. B: The PLD1-pre-bound immobilized GST-RalA fusion protein was incubated with the indicated concentrations of ARF1 under standard assay conditions containing 0.5% β-octyl glucopyranoside. The beads were washed twice with buffer B containing 0.5% β-octyl glucopyranoside and subjected to 8–16% SDS-PAGE. The resolved proteins were transferred onto a nitrocellulose membrane and stained with 2% Ponceau S to visualize the GST-RalA fusion protein. The upper and lower parts of the transfer filter were probed with anti-PLD1 and ARF antibody, and visualized by ECL exposure for 5 and 30 min, respectively.

ARF1 and RalA through different binding sites. PLD1 was pre-bound to the immobilized GST-RalA fusion protein, and the resultant complexes were then further incubated with ARF1 protein. As seen in Fig. 4B, ARF1 bound to the PLD1-pre-bound GST-RalA fusion protein in a dose-dependent manner but not to the GST-RalA fusion protein alone. These results suggest that the synergistic activation of PLD1 is mediated by the simultaneous interaction of RalA and ARF1 with different sites on PLD1.

4. Discussion

Several signaling molecules, PKC-α, protein tyrosine kinases, and small G proteins including ARF and RhoA have been implicated in the regulation of PLD activity [20]. However, the mechanism of PLD activation by the PLD-activating factors is still unknown. In this study, we show evidence for the first time that ARF1 and RalA directly interact with PLD1 and that RalA synergistically stimulates ARF1-dependent PLD activity.

We show that GTP-bound ARF1 interacts with PLD1 with higher affinity than GDP-bound ARF1 (Fig. 1). Recent evidence suggested that PLD activation requires GTP-dependent translocation of ARF1 from the cytosol to the membrane [20,21]. Based on these observations, it is plausible that the GTP-dependent translocation of ARF1 to the membrane may give a function to the interaction between PLD1 and ARF1.

It has been reported that ARF1 requires PIP_2 as a cofactor for PLD activation [5,9]. PIP_2 has been known to stimulate the guanine nucleotide exchange activity of ARF1 [17]. In addition, Randazzo [18] suggested that ARF1 binds to PIP_2 and that PIP_2 binding to ARF1 may direct the interaction between ARF1 and ARF-interacting proteins, including the ARF-GTPase-activating protein and PLD. In this study, we show that PLD1 directly interacts with PIP_2-containing phospholipid vesicles, whereas the binding of ARF1 to the phospholipid vesicles requires the presence of PLD1 (Fig. 2). These results support the conclusion that ARF1-induced PLD activation may be mediated by direct association between PLD1 and ARF1.

We and other research groups demonstrated that PKC, RhoA, and ARF are involved in PLD activation by growth factors such as platelet-derived growth factor and epidermal growth factor [22–25]. However, Jiang et al. [11] have implicated RalA in v-Src, a non-receptor tyrosine kinase, induced PLD activation via Ras-induced activation of Ral-GDS. In our study, we found that RalA synergistically stimulates ARF-induced PLD activity of PLD1. In keeping with our observations, Luo et al. [26] demonstrated the direct interaction of RalA with PLD1. Taken together, these results suggest that PLD1 is regulated by different activating signals including receptor- and non-receptor-tyrosine kinases through ARF, RhoA, PKC, and RalA.

In order to elucidate the mechanism of the synergistic activation of PLD1, we asked whether the PLD activation is mediated by the direct interaction of the PLD-activating factors with PLD1. We previously demonstrated the stimulation of PLD1 activity by direct interaction with PKC-α in a PMA-dependent manner [12], suggesting that PLD1 activity may be regulated by the direct interaction with PLD1-activating factors. In this report, we show how the synergistic activation by ARF1 and RalA is derived from direct interaction with PLD1, suggesting an independent interaction of ARF1 and RalA with different sites on PLD1. Previous reports by several research groups [10,27] indicating possible interactions of PLD1 with ARF1 or RalA are consistent with our results. We therefore propose that PLD1 plays a key role in PLD activation by receiving different activating signals including receptor- or non-receptor-tyrosine kinase activities.

In summary, our data show that RalA synergistically stimulates the ARF-dependent PLD activity of PLD1 and that the synergistic activation of PLD1 is based on the independent interactions of PLD1 with PLD-activating factors. It will be of great interest to characterize the sites of interaction between PLD1 and the PLD1-activating factors in their independent association with the enzyme.

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