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Arf1-dependent PLD1 is localized to oleic acid-induced lipid droplets in NIH3T3 cells ☆

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Abstract

Phospholipase D (PLD) is known to play a role in vesicle transport through the hydrolysis of phosphatidylcholine (PC) to produce the bioactive lipid, phosphatidic acid. Lipid droplets (LDs) are surrounded by a monolayer of phospholipids, including PC and its lyso derivative, and exhibit a number of signaling proteins. Our recent report suggests that the association of adipose differentiation-related protein (ADRP) to LDs is regulated by an ADP-ribosylation factor 1 (Arf1)-dependent mechanism. In the present study, we found an increase in PLD activity accompanied with LD formation in oleic acid-treated NIH3T3 cells. Brefeldin A, an inhibitor of ARF-GEFs, suppressed both PLD activation and LD formation in oleic acid-treated cells. PLD1, but not PLD2, was found to exist in LDs by immunocytochemical analysis. Furthermore, co-existence of PLD1, Arf1, and ADRP was observed in the LD-enriched subcellular fractions obtained from oleic acid-treated NIH3T3 cells by Western blot analysis. PLD1 activity in the LD-enriched fractions was stimulated by exogenously added Arf1. Although LDs were induced in either PLD1- or PLD2-overexpressing CHO cells by oleic acid treatment, the stimulation of PLD activity was observed only in PLD1-CHO cells. Taken together, the data suggest that the activation of Arf1-dependent PLD1 occurs in LDs and may be involved in their physiological function.

Keywords: Phospholipase D; ADRP; Arf1; Lipid droplets; NIH3T3 cells

Phosphatidylcholine (PC)-specific phospholipase D (PLD) catalyzes the hydrolysis of PC to release choline and phosphatidic acid (PA). In mammalian cells, there are two PLD isozymes, PLD1 and PLD2. PLD1 is known to be activated by small molecular weight GTP-binding proteins (G-proteins), such as Arf, Ral, and Rho family G-proteins, and protein kinase C (PKC). Conversely, PLD2 activity is constitutively active and unaffected by G-proteins or PKC in vitro. The agonist-induced PLD activation, which generates PA or lyso-PA, has been reported to be responsible for the regulation of various biological functions: vesicle trafficking and cytoskeletal reorganization, cell growth, and differentiation [1–5]. However, the cellular regulation and function specific to each isoform have yet to be fully established.

Lipid droplets (LDs) are covered with a monolayer of phospholipids, including PC, and package neutral lipids, such as triacylglycerol and cholesterol esters [6–9]. In

☆ *Abbreviations:* ARF, ADP-ribosylation factor; LDs, lipid droplets; PA, phosphatidic acid; BFA, brefeldin A; PLD, phospholipase D; CHO, Chinese hamster ovary; PBut, phosphatidylbutanol; DPPC, dipalmitoylphosphatidylcholine; GFP, green fluorescence protein.

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in vivo experiments, the induction of LDs occurs in oleic acid-treated NIH3T3 cells at high efficiency. Simultaneously, the expression of adipose differentiation-related protein (ADRP) is dramatically increased and the translocation of ADRP to LDs occurs [10–12]. We recently reported that ADP-ribosylation factor (Arf) 1 was identified as a full-length ADRP-binding protein through a yeast two-hybrid screening [13].

Here, we report that PLD1 co-exists with Arf1 and ADRP in LDs induced by cell treatment with oleic acid, and that a bioactive PA generated by the activation of PLD1 on the surface of LDs may play a role in the physiological function of LDs.

Materials and methods

Reagents. [9,10-^{3}H(*N*)]Palmitic acid (50 Ci/mmol) and [choline-methyl-^{3}H]dipalmitoyl phosphatidylcholine (DPPC) (60 Ci/mmol) were purchased from NEN Life Science Products. Brefeldin A (BFA) was purchased from CalBiochem.

Antibodies. Anti-ARF antibody (1D9) was purchased from Affinity Reagents (Golden, CO). Polyclonal anti-PLD (224) and -PLD2 (58) antibodies were prepared as previously described [14]. The anti-PLD antibody (224) reacts with both PLD1 and PLD2, while the anti-PLD2 antibody (58) recognizes only PLD2. A rabbit polyclonal anti-ADRP antibody was raised against recombinant mouse ADRP.

Cell culture and the induction of lipid droplets. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (Nihon Pharmaceutical Co., Ltd.) containing 10% calf serum (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. CHO-vector, CHO-PLD1, and CHO-PLD2 cells which stably overexpress empty vector, wild-type human PLD1b and mouse PLD2, respectively, were prepared as described previously [15]. Each CHO cell was cultured in Ham's F-12 medium (Invitrogen) containing 10% FCS. Transfection of expression plasmids was done using calcium phosphate.

To induce lipid droplets (LDs) formation, oleic acid and bovine serum albumin (OA/BSA) complexes were prepared as previously described [16,17]. One day after culture, 300 μM OA/BSA was added to the culture medium. The cells were cultured for 24 h, harvested, and examined for LDs formation and PLD activity.

Plasmids and DNA transfection. Plasmids and DNA transfection were performed as described previously [13].

The isolation of LD-enriched subcellular fractions. NIH3T3 cells treated with oleic acid were cultured until 100% confluent. LD-enriched fractions were isolated as described by Yu et al. [18] with minor modifications. Briefly, NIH3T3 cells treated with oleic acid were washed with ice-cold PBS, and were resuspended in 3 ml of disruption buffer (25 mM Tris–HCl, pH 7.4, 100 mM potassium chloride, 1 mM EDTA, 5 mM EGTA, and proteinase inhibitor cocktail). The cells were disrupted with 50 strokes of a Dounce homogenizer (Kontes), and were mixed with an equal volume of 1.08 M sucrose in disruption buffer. After centrifugation at 1500 rpm for 10 min to pellet the nuclei, the supernatant fractions were transferred to a 12 ml-ultracentrifugation tube, and layered onto 2.0 ml of 0.27 M sucrose buffer, 0.135 M sucrose buffer, and top buffer (25 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 1 mM EGTA). After ultracentrifugation at 30,000 rpm for 2 h, eight fractions and microsomal pellet were separated. LD-enriched fractions were identified by Western blotting using anti-ADRP antibody.

Immunoprecipitation and Western blot analysis. The harvested NIH3T3 cells were washed with ice-cold PBS and resuspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 50 mM NaF, 1% Triton X-100, and 0.5% NP-40), and were spun at 15,000 rpm for 30 min at 4 °C. The supernatant was collected and used for subsequent immunoprecipitation.

The supernatant from NIH3T3 cell lysate and the LD-enriched fractions described above were immunoprecipitated with Immunopure immobilized protein G (Pierce) or protein A–Sepharose CL-4B (Amersham Biosciences) following standard protocol. The beads were washed three times with lysis buffer, and heated at 95 °C for 5 min in 2× sample buffer (50 mM Tris–HCl at pH 6.8, 5% 2-mercaptoethanol, 10% glycerol, 2% SDS, and 0.005% bromophenol blue) to remove the immune complexes. The samples were then subjected to Western blot analysis.

Immunofluorescence staining. The cells were fixed for 30 min in 3% paraformaldehyde in PBS, followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min. The permeabilized cells were blocked in 3% BSA (Wako Purechemicals Co., Ltd.) in PBS for 10 min, and incubate with the anti-PLD antibody (224), anti-PLD2 antibody (58) or anti-ADRP antibody in PBS containing 1% BSA for 60 min. The bound antibody was detected using a FITC-labeled anti-rabbit IgG antibody (Jackson Immuno Research Laboratory). The LDs were stained with Sudan III (Chroma). The stained cells were observed using a confocal laser microscope (Carl Zeiss Jena GmbH).

PLD assay. NIH3T3 cells, CHO-vector, CHO-PLD1, and CHO-PLD2 cells were each seeded at a density of 1×10^5 cells/35 mm dishes. The sub-confluent cells were harvested and labeled with [^{3}H]palmitic acid (1μCi/ml) in the medium for 16 h. The labeled cells were treated with oleic acid for 24 h, then with 0.3% of 1-butanol for 1 h, and subjected to the extraction of lipids by the method of Bligh-Dyer. [^{3}H]Phosphatidylbutanol (PBut) was separated by thin-layer chromatography, and measured as described by Banno et al. [14]. To investigate the effect of brefeldin A (BFA) on PLD activity, BFA was incubated with the cells for 30 min after oleic acid treatment.

For assay of Arf-dependent PLD activity in LD-enriched fractions, mixed lipid vesicles (phosphatidylethanolamine/phosphatidylinositol-4,5-bisphosphate/phosphatidylcholine, 160/14/10 molar ratio) containing [^{3}H]DPPC to yield 400,000 dpm/assay were prepared as described previously [15]. The LD fractions were incubated for 60 min at 37 °C with or without 5 μM recombinant ARF1 and 30 μM GTPγS. After the reactions were terminated, the released [^{3}H]choline was measured.

Results

Co-localization of Arf1-GDP and ADRP on LDs induced by oleic acid in NIH3T3 cells

We recently identified Arf1-GDP form as an ADRP-binding protein by yeast two-hybrid screening [13]. Furthermore, we have demonstrated that expression of ADRP was increased by oleic acid-induced lipid droplets (LDs) formation inNIH3T3 cells [19]. Then, we examined the presence of Arf1 in the LDs induced by oleic acid treatment of NIH3T3 cells. As shown in Fig. 1, the Arf1-GDP form, Arf1 (T31N), was detected on the surface of LDs. Furthermore, Arf1-GDP co-localized with ADRP on LDs in NIH3T3 cells as shown in the merged images.

Activation of PLD and LD formation in oleic acid-treated NIH3T3 cells

Arf1 is known to be an effector of PLD. To examine the effect of oleic acid treatment on PLD activity in

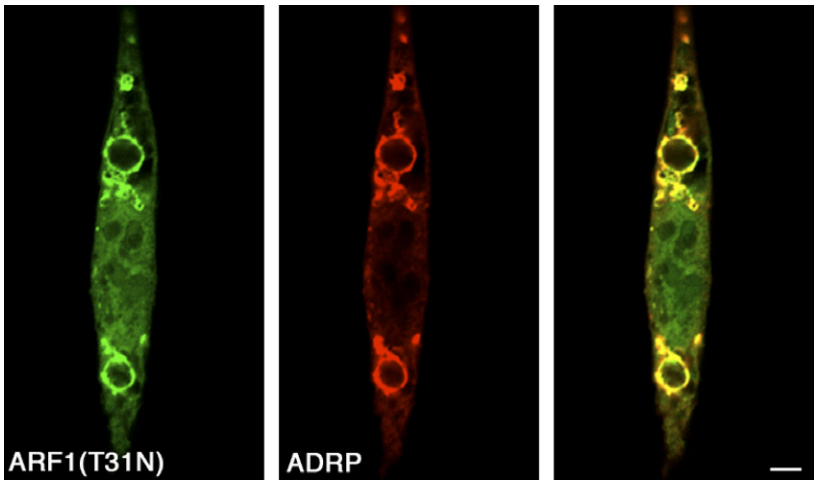

Fig. 1. Arf1-GDP exists in oleic acid-induced LDs. NIH3T3 cells transfected with GFP-tagged Arf1 (T31N) (green) were treated with oleic acid for 24 h and incubated with an anti-ADRP antibody (red). Antibody localization was observed using a confocal microscope. The merged image is shown in the right panel (Bar: 5 μm).

NIH3T3 cells, we measured PBut production in the presence of 0.3% butanol in [^{3}H]palmitic acid-prelabeled NIH3T3 cells with or without oleic acid treatment. The production of PBut in oleic acid-treated cells increased 6-fold over that of non-treated cells (Fig. 2A). At the same time, the formation of LDs, accompanied by the expression of ADRP, was observed in oleic acid-treated cells (Fig. 2B). We previously reported that brefeldin A (BFA), which is a fungal metabolic inhibitor of some of the guanine nucleotide-exchange factors for Arfs [20], reduced the total volume of LDs induced by oleic acid, due to the binding of ARF-GDP to ADRP resulting in the dissociation of ADRP from the LDs. Interestingly, incubation with BFA after oleic acid treatment of [^{3}H]palmitic acid-prelabeled NIH3T3 cells resulted in the suppression of the oleic acid-induced enhancement

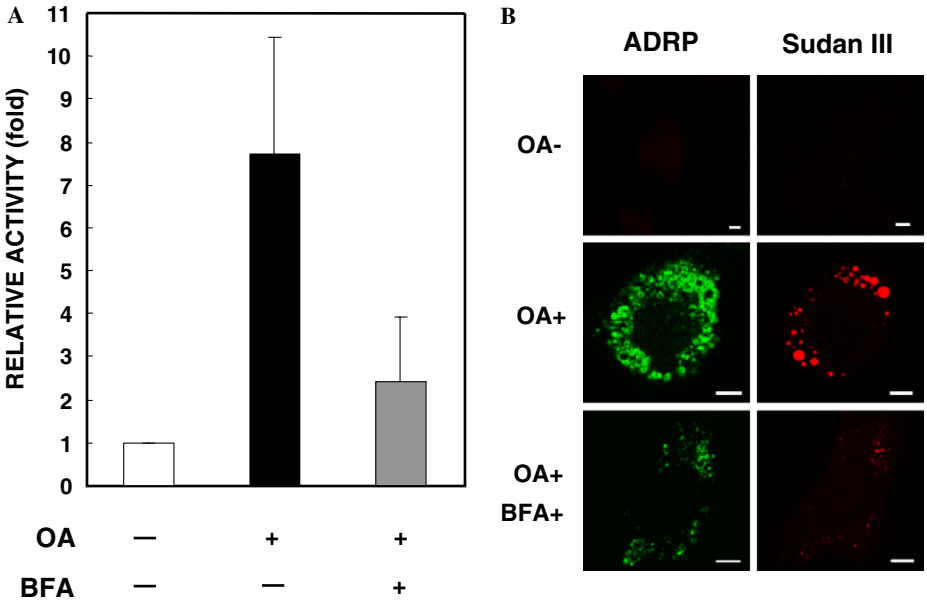

Fig. 2. The activity of PLD is stimulated in oleic acid-treated NIH3T3 cells, accompanied by an increase in the expression of ADRP and the formation of LDs. (A) NIH3T3 cells pre-labeled with [^{3}H]palmitic acid were incubated with and without oleic acid for 24 h, and then, with 0.3% of 1-butanol for 10 min. PLD activity was measured by the amount of [^{3}H]PBut production as described under Materials and methods. In order to examine the effect of BFA on PLD activity, oleic acid-treated cells were incubated with BFA (5 μg/ml) for 30 min before the addition of 0.3% of 1-butanol. The PLD activity in non-treated NIH3T3 was designated as 1. Data are expressed as means ± SEM of four different experiments. (B) NIH3T3 cells treated with (OA+) or without (OA−) oleic acid for 24 h were harvested, fixed with 3% paraformaldehyde, and immunostained with an anti-ADRP antibody and a Alexa-labeled anti-rabbit secondary (left panels) or with Sudan III to detect LDs (right panels). The expression of ADRP and the formation of LDs were observed using a confocal microscope. The effects of BFA on ADRP expression and LD formation were investigated by the incubation with BFA for 60 min after oleic acid-treatment (OA+ BFA+) (Bars: 5 μm).

of PBut production (Fig. 2A). Simultaneously, the amount of LDs and the expression of ADRP were reduced (Fig. 2B). These results show that an intracellular stimulation of PLD activity in oleic acid-treated cells closely correlates with LD formation and probably Arf1 activation in LDs.

Co-existence of PLD1 with ADRP and Arf1 in LD-enriched subcellular fractions prepared from oleic acid-treated NIH3T3 cells

To investigate the location of PLD on the surface of LDs, we prepared LD-enriched subcellular fractions from oleic acid-treated NIH3T3 cells according to the method of Yu et al. [18] and examined the presence of ADRP, Arf1, and PLD in each fraction. As shown in Fig. 3A, ADRP localized to only fractions 1 and 2. Thus, these fractions were identified to be the LD-enriched fractions. Arf1 also localized to the same fractions as ADRP (Fig. 3B).

To determine which PLD isoforms existed in the LD-enriched fractions, we examined using two types of PLD antibodies. Antibody 224 cross-reacts with both PLD1 and PLD2 while antibody 58 is specific for PLD2. Antibody 224 was used to immunoprecipitate PLD from the LD-enriched fractions, fractions 1 and 2. The immunoprecipitates were then analyzed by Western blotting using either 224 or 58. Only a 120 kDa spot, corresponding to PLD1, was detected in LD-enriched fractions with antibody 224 (Fig. 3C, upper left panel). Antibody 58, specific to PLD2, did not detect PLD2 at 100 kDa (Fig. 3C, lower left panel). Although both PLD1 and PLD2 were found in non-treated NIH3T3 cells (Fig. 3C, right panels), we found that only PLD1 was present in the LD-enriched fractions. These results indicate that PLD1, and not PLD2, co-exists with ADRP and Arf1 in the LD-enriched fractions.

To investigate the Arf1-dependency on PLD1 activity in LD-enriched fractions, each fraction was incubated

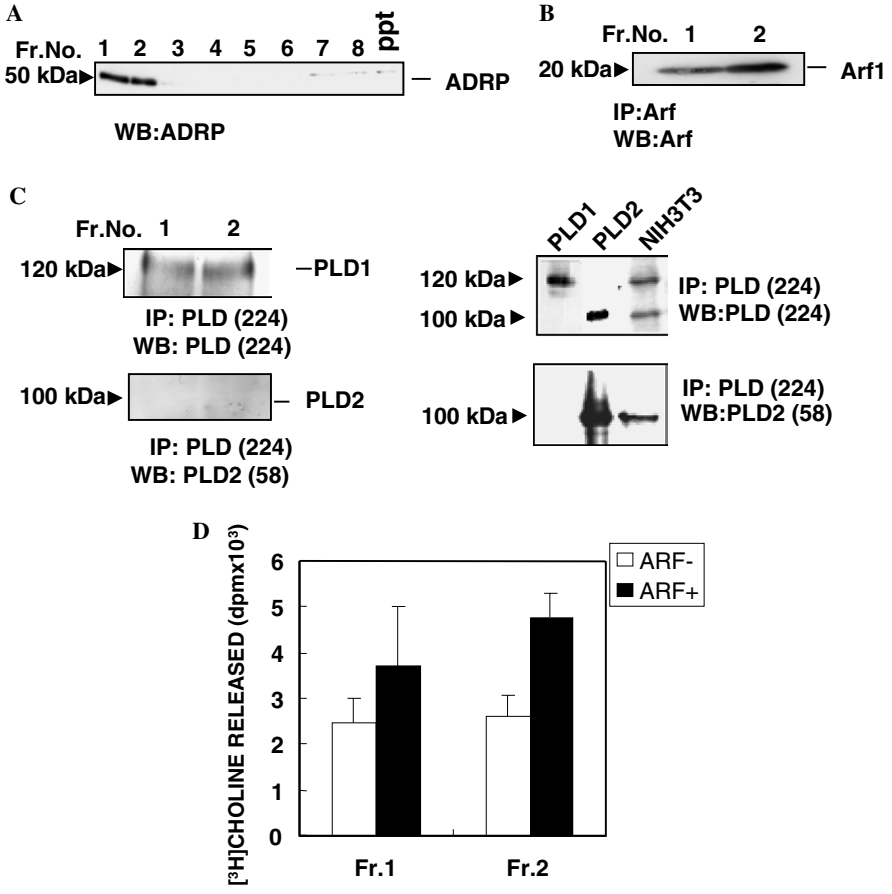

Fig. 3. PLD1 co-exists with Arf in LD-enriched subcellular fractions prepared from oleic acid-treated NIH3T3 cells. Oleic acid-treated NIH3T3 cells were homogenized and fractionated as described under Materials and methods. Fractions 1 and 2 were identified to be LD-enriched fractions by Western blot analysis (WB) using an antibody against ADRP (A). Fractions 1 and 2 were subjected to WB using an ARF1 antibody (B). PLD antibody 224 was used to immunoprecipitate (IP) PLD from fractions 1 and 2. The immunoprecipitates were then subjected to WB using either PLD antibody 224 or PLD2 antibody 58 (C, left panels). The lysates of PLD1 and PLD2-overexpressing NIH3T3 cells were immunoprecipitated with PLD antibody 224 and the precipitates were analyzed by WB with PLD antibody, 224 or 58. The recombinant PLD1b and PLD2 were used as standards (C, right panels). PLD activities in fractions 1 and 2 were measured with and without Arf1 using lipid vesicles containing [^{3}H]DPPC as described under Materials and methods (D). Data are expressed as means ± SEM of three different experiments.

with the lipid vesicles containing dipalmitoyl phosphatidylcholine-methyl [^{3}H]choline ([^{3}H]PC) with and without exogenous Arf1, and the amount of released [^{3}H]choline was measured. PLD1 in both fractions 1 and 2 significantly hydrolyzed [^{3}H]PC without exogenous Arf1. These activities seem to be due to endogenous Arf1 because the exogenously added Arf1 induced an approximate 2-fold stimulation of PLD1 activity in each fraction (Fig. 3D). These results suggest that Arf1-stimulated PLD1 exists in LDs.

PLD1 activation in oleic acid-treated and PLD1-overexpressing CHO cells

In order to confirm the activation specific to PLD1 in oleic acid-treated cells, we used CHO cells overexpressing PLD1 (CHO-PLD1) or PLD2 (CHO-PLD2). As shown in Fig. 4, the PBut production in CHO-PLD1 cells was markedly stimulated by oleic acid treatment, accompanied with LD formation, while an increase in PLD activity was not observed in CHO-PLD2 cells or in control (vector only) cells, in spite of the formation of LDs (Figs. 4A and B). Furthermore, the stimulation of PBut production and the formation of LDs in oleic acid-treated CHO-PLD1 cells were suppressed by the addition of BFA (Fig. 4A and data not shown), as observed in NIH3T3 cells (Fig. 2A). Thus, we identified PLD1 activation to be dependent on LD formation.

Presence of PLD1 on the surface of LDs induced by oleic acid

PLD1 exists primarily in intracellular organelles including the Golgi apparatus and endoplasmic reticulum, whereas PLD2 localizes to the plasma membrane [3,21]. We confirmed a similar pattern of PLD localization in non-treated CHO-PLD1 and CHO-PLD2 cells by immunocytochemical analyses (Figs. 5A and B). Additionally, we tried to immunocytochemically confirm the existence of PLD1 on the surface of LDs in oleic acid-treated cells. The merged images presented in Fig. 5E of LDs stained by Sudan III (red) and with the antibody against PLD (green) were consistent with what was observed in CHO-PLD1 cells, but not in CHO-PLD2 cells. From these in vivo and the above-mentioned in vitro results, we conclude that Arf1-stimulated PLD1 exists on the surface of LDs.

Discussion

The interaction site on Arf1 for PLD has been reported to exist in the N-terminus [2,3], but the exact site is still obscure. In the present study, we showed the existence of Arf and PLD1 on the LDs of oleic acid-treated cells by immunocytochemical methods (Figs. 1 and 5), and further, the co-existence of Arf1 and PLD1 in LD-enriched subcellular fractions by Western blot analysis (Fig. 3). Our previous report described the specific binding of Arf1-GDP to ADRP in LDs. Therefore, it may be suggested that Arf1 on the LDs might be responsible for the recruitment of PLD1 to the LDs and for the activation of PLD1 at the surface of the LDs, although it remains to be elucidated whether Arf-GEFs or similar systems are present in LDs [22,23].

One preliminary experiment showed that the addition of 1-butanol, but not of *tert*-butanol, to oleic acid-treated cells in order to convert PA to PBut induced the

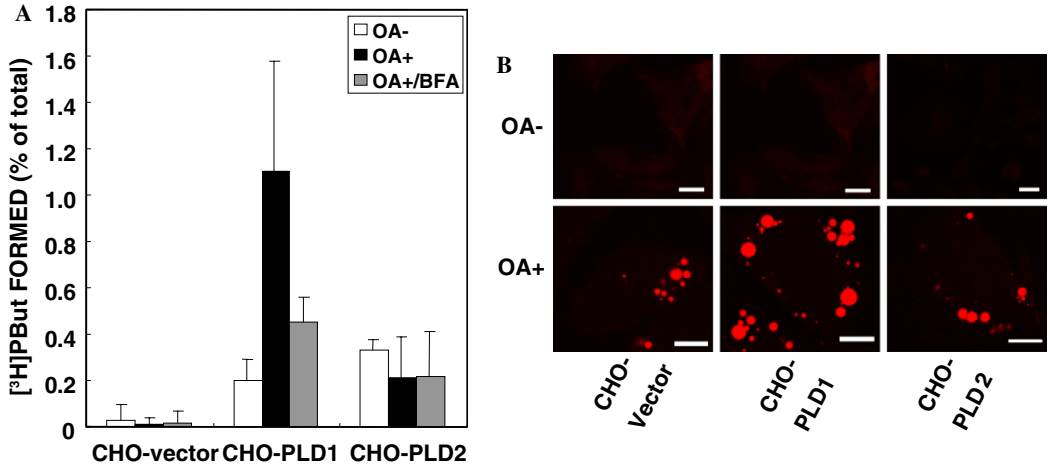

Fig. 4. PLD1 is activated in PLD1-overexpressing CHO cells (CHO-PLD1) by oleic acid treatment. (A) Oleic acid- and BFA-treatments, and the determination of PLD1 activity were carried out in empty vector-, PLD1- and PLD2-overexpressing CHO cells (CHO-vector, CHO-PLD1, and CHO-PLD2, respectively), as described in Fig. 2. White column, non-treated cells; black column, oleic acid-treated cells; and gray column, oleic acid and BFA-treated cells. Data are expressed as means ± SEM of three different experiments. (B) Oleic acid-treated CHO-vector (left panels), CHO-PLD1 (middle panels), and CHO-PLD2 (right panels) cells were stained with Sudan III to detect LDs. The formation of LDs was observed using a confocal microscope (Bars: 10 μm).

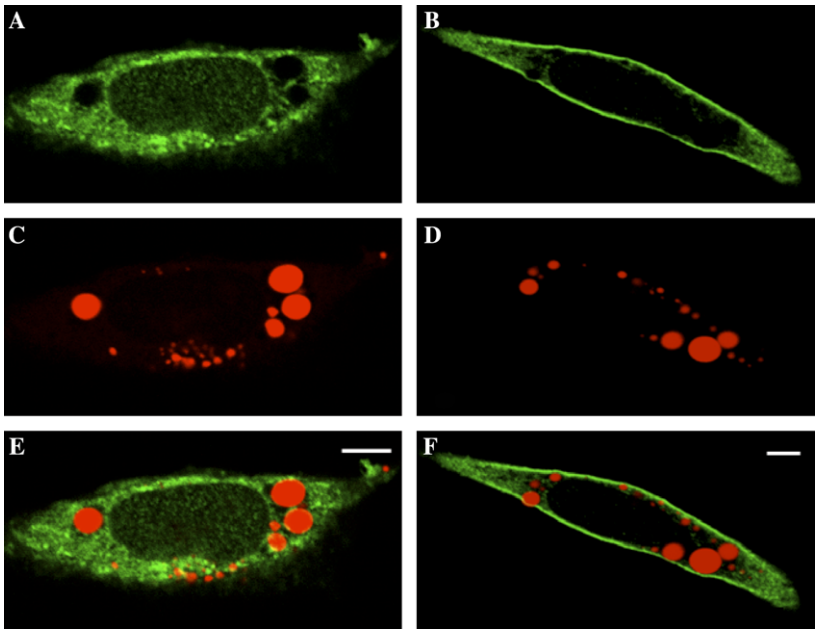

Fig. 5. PLD1 exists in oleic acid-induced LDs. CHO-PLD1 (A,C,E) and CHO-PLD2 (B,D,F) cells treated with oleic acid were fixed in 3% paraformaldehyde and stained with Sudan III (C,D) to detect LDs, or immunostained with an anti-PLD antibody (224) and Alexa-labeled anti-rabbit secondary (A). CHO-PLD2 cells treated by oleic acid were immunoreacted with the anti-PLD2 antibody (58) (B). The cells were observed using a confocal microscope. The merged images are shown in the lower panels (E,F) (Bars: 5 μm).

reduction of large LDs and resulted in the increase of small and shape-changed LDs (unpublished data). This result implies that PA generated by PLD activation on the surface of LDs may play a role in the sequential fusion of LDs with other intracellular membranes, as suggested in the differentiation of phagosomes to phagolysosomes by Iyer et al. [24].

Marchesan et al. [25] developed a microsome-based cell-free system that assembles newly formed triglycerides into spherical LDs. LD formation in this system requires an activator present in the 160,000*g* supernatant of rat adipocytes which induces PLD activation in the microsomes of the cell-free system. Interestingly, this activator can be replaced by a constitutively active PLD prepared from cabbage or by PA. The supernatant used as activator may contain ARF1, which is expected to activate PLD1 in a cell-free system.

In conclusion, PLD1, Arf1, and ADRP co-exist in LDs, which suggests a new physiological role of Arf1-stimulated PLD1 on the surface of LDs through the production of PA.

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