Dependence of Phospholipase D1 Multi-monoubiquitination on Its Enzymatic Activity and Palmitoylation*

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Phospholipase D (PLD) is an important lipase in many cellular processes, including vesicular trafficking, cell survival, and cell migration. In the present study, we show that PLD1, but not PLD2, is posttranslationally modified by multi-monoubiquitination. Intriguingly, suppression of lipase activity either by mutation of the HKD motif (PLD1 H896R, K898R, or D903A) or the phosphatidylinositol 4,5-bisphosphate binding motif (PLD1 R691G, R695G) or through use of PLD-selective inhibitors impaired the ubiquitination of PLD1, although stimulation of lipase activity by phorbol 12-myristate 13-acetate did not enhance its ubiquitination. A palmitoylation-deficient mutant PLD1 allele, which exhibits altered patterns of vesicular trafficking, had significantly lower levels of monoubiquitination. In addition, the expression of ubiquitin-fused PLD1 induced aberrantly enlarged vesicles partially co-localized with the Golgi complex but not with early endosomes. The altered localization was reduced by the K898R mutation, suggesting a role of multi-monoubiquitination in PLD1 subcellular localization. Surprisingly, the degradation of PLD1, but not of PLD1 K898R or PLD2, was blocked by inhibitors of proteasomes but not by inhibitors of lysosomes or other proteases, suggesting a role of the ubiquitination in proteasomal degradation of PLD1. In summary, our studies show that PLD1, but not PLD2, is multi-monoubiquitinated. The ubiquitination modification might represent a novel regulatory mechanism in PLD1 functioning, particularly in the context of subcellular trafficking between different membrane compartments.

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) and choline (1). PA may directly affect membrane curvature and in doing so facilitate fusion and fission of vesicular membranes (1), or it may act as a lipid second messenger, binding and activating varied signaling proteins, including Raf-1 (2), mTOR (3), and phosphatidylinositol-4-phosphate kinase (4), and promote mitogenic, anti-apoptotic, and chemotactic signals. Two PLD isoforms, PLD1 and PLD2, have been identified in mammalian cells (1, 5). Both are membrane-associated, but they exhibit different patterns of subcellular localization and regulate different steps in membrane vesicle trafficking (6). PLD1 co-localizes with perinuclear small vesicles, the Golgi complex, and early endosomes in non-stimulated cells, translocates to plasma membrane (PM) upon stimulation, and then recycles to perinuclear vesicles through endocytosis (7), whereas PLD2 has been reported to localize at the plasma membrane (PM) under resting conditions and translocate to endosomes upon stimulation (8). This difference is believed to contribute to the different cell biological roles undertaken by PLD1 and PLD2. However, the basis for this difference in the localization and function of the PLD isoenzymes remains poorly understood.

Ubiquitination is a key posttranslational modification for many proteins (9, 10). Lys63-linked polyubiquitination mainly directs proteins to the 26 S proteasome to target them for degradation, but the roles of other ubiquitin linkages, especially monoubiquitination and Lys48-linked polyubiquitination, have been well documented to regulate the intracellular trafficking of a broad spectrum of proteins (9, 10). Of note, the monoubiquitination of PM proteins, such as tyrosine kinase receptors and G protein-coupled receptors, facilitates their endocytosis, endosomal sorting, and lysosomal degradation (11). Therefore, we became interested in whether ubiquitination is also involved in the regulation of PLD isoenzymes. Accordingly, in preliminary studies, we noted that a fraction of PLD1 protein exhibited retarded migration when examined by Western blotting analysis, suggesting a possibility that PLD1 was ubiquitinated, which we subsequently examined. In this report, we present evidence that PLD1, but not PLD2, is multi-monoubiquitinated and investigate the role of lipase activity and palmitoylation, another posttranslational modification of PLD isoenzymes, in the regulation of PLD1 ubiquitination.

EXPERIMENTAL PROCEDURES

Chemicals, Antibodies, and Reagents—All chemicals and antibodies except those mentioned below were obtained from Sigma. The pharmacological inhibitors of PLD compound 5WO (VU0155056) and 809 (VU0155069) were generous gifts from Dr. H. Alex Brown (Vanderbilt University School of Medicine, Nashville, TN). Antibody against HA tag (3F10) was pur-
chased from Roche Applied Science, antibody against Myc tag was from Cell Signaling Technology (Boston, MA), antibody against GM130 was from BD Biosciences, antibodies against PLD1 (H160) and ubiquitin (P4D1) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and antibody against early endosomal antigen 1 (EEA1) was a generous gift from Dr. Marvin J. Fritzler (University of Calgary). Ham’s F-12 medium, Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), tetracycline-reduced FBS, penicillin, and streptomycin were purchased from Invitrogen.

**Cell Culture, Transient Transfection, and Inducible Expression System**—Chinese hamster ovary (CHO) cells were cultured using Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1) supplemented with 10% (v/v) tetracycline-reduced FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml). HeLa and COS-7 cells were cultured using Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and the aforementioned antibiotics. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Myc-tagged ubiquitin and the empty vector were generous gifts from Dr. Daniel J. Finley (Harvard Medical School, Boston, MA). FLAG-tagged ubiquitin, Lys48 mutant, Lys63 mutant, and lysineless mutant were obtained from the University of Dundee (Scotland, UK). All PLD or PLD mutant vectors were constructed as previously described (7, 12). Cells were transiently transfected with these expression vectors using Lipofectamine 2000 as per the manufacturer’s specifications (Invitrogen). Protein expression was monitored using Western blotting analysis.

The cDNAs encoding HA-tagged PLD or PLD mutants were inserted into a tetracycline-inducible T-REx system (Invitrogen) in CHO cells (8, 13). Expression of PLD was induced in medium containing 1 µg/ml doxycycline (Dox). To avoid unintentional induction of PLD expression, the PLD T-REx CHO cells were cultured with tetracycline-reduced FBS.

**Immunoprecipitation and Western Blotting Analysis of Ubiquitination of PLD**—For immunoprecipitation-ubiquitination experiments, cells were lysed for protein extraction using radioimmunoprecipitation assay buffer (Sigma) supplemented with 1% (v/v) Triton X-100, protease inhibitor mixtures (Roche Applied Science), 1 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 5 µM MG132 was used to extract total cellular protein.

**PLD Activity Assay**—A PLD activity assay using 1-butanol was conducted as described previously with minor modifications (12). In brief, serum-starved cells were labeled with 1 µCi/ml [9,10-³H]myristic acid (GE Healthcare) for 24 h and then washed and pre-equilibrated for 1 h. Phorbol 12-myristate 13-acetate (PMA) (100 nM) and 1-butanol (0.5%, v/v) were added during the last 10 min. Lipid was extracted after incubation as specified in the figure legends, and phosphatidylbutanol was separated on a silica thin layer chromatography sheet. The PLD activity was calculated as the radioactivity counts of phosphatidylbutanol normalized to the counts of total lipids as measured using a liquid scintillation counter (Beckman).

**Construction of PLD1 Mutants**—Mutagenesis of PLD1 K898R, ΔN (lacking N-terminal 328 amino acid residues), and C240S,C241S mutants were constructed as previously described (7). Mutagenesis of PLD1 H896R, D903A, ΔPH (lacking PH domain), and R691G, R695G mutants was performed using the QuikChange™ kit (Stratagene, La Jolla, CA). A plasmid expressing HA-tagged PLD1 fused N-terminally to Myc-tagged ubiquitin (Ub-PLD1) was also constructed using the QuikChange™ kit. Briefly, the Myc-tagged ubiquitin fragment was amplified with Myc-tagged ubiquitin expression vector as template using conventional PCR with the primer 5’-CGG GTA TGG CTT CTA GCG CTG CTG AAC AAA AGC TTA TTT C-3’ (forward) and 5’-GTC AGC TCA TAA GGA TAT CCT AGT CTT AAG ACA AGA TG-3’ (reverse) and then subjected to agarose gel purification using the GeneJET™ gel extraction kit (Fermentas, Burlington, Canada) and inserted into the HA-tagged pcGN-PLD1 plasmid between the promoter and the HA-tagged PLD1 cDNA according to the manufacturer’s specifications. Note that two C-terminal glycine residues of ubiquitin were removed to avoid the potential cleavage by deubiquitinating enzymes. All intended mutations were confirmed by sequencing (University of Calgary Core RNA Services).

**Immunofluorescence Analysis of PLD1 Localization**—COS-7 cells grown on glass coverslips were briefly washed with PBS, fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.2% Triton X-100 for 5 min, and blocked in 2% skim milk for 30 min. Cells were then stained with GM130 or EEA1 antibody for 1 h, followed by incubation with rhodamine-conjugated antimouse secondary antibody (Invitrogen) for 1 h to label cis-Golgi complex and early endosome, respectively. HA-tagged PLD1 was stained with HA tag antibody and Alexa Fluor 488-conju-
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gated anti-rat secondary antibody (Invitrogen). In some experiments, Myc-tagged ubiquitin-PLD1 fusion protein was stained with Myc tag antibody and rhodamine-conjugated anti-mouse secondary antibody. The coverslips were then mounted in a drop of Prolong antifade reagent containing 4',6-diamidino-2-phenylindole (Invitrogen). Imaging and quantification of localization of PLD1 proteins were performed using a 4×0 dry lens and a Spot Insight Color (3.2.0) CCD digital camera (Diagnostic Instruments, Sterling Heights, MI) with a fluorescence microscope (BX50, Olympus). At least 200 cells were randomly selected and scored for each sample.

Data Analysis—Data are expressed as the mean ± S.D. The number of replicates (n) represents the number of independent experiments performed. Densitometry was conducted using ImageMaster (Amersham Biosciences). Differences between means were evaluated by Student’s t test or one-way analysis of variance. p < 0.05 was considered significant. All statistical analysis was performed using Instat3.0 (GraphPad Software).

RESULTS

Phospholipase D1, but Not Phospholipase D2, Is Ubiquitinated—To examine whether PLD isoforms undergo ubiquitination, we began by employing CHO cells harboring a tetracycline-inducible (T-REx) system to express HA-tagged PLD1 and PLD2 (PLD1/2 T-REx CHO cells) to facilitate our detection of ubiquitination (8, 13). Myc-tagged ubiquitin was transfected into PLD1 and PLD2 T-REx cells, followed by induction of PLD expression with doxycycline (Dox; 1 μg/ml) for 24 h. MG132 (1.5 μM), the inhibitor of 26 S proteasome, was added to prevent the potential rapid degradation of the PLD isoforms, which were immunoprecipitated from whole-cell lysates using anti-HA tag antibody and visualized by Western blotting analysis using anti-HA tag antibody and anti-Myc tag antibody. The expression level of PLD1 was consistently lower than that of PLD2. Upon overexpression of ubiquitin, a broad pattern of ubiquitinated higher molecular weight species was detected in cells induced to express PLD1 but not in cells induced to express PLD2, suggesting that PLD1, but not PLD2, is a target for ubiquitination in vivo (Fig. 1, A (top) and B).

We next sought to confirm the PLD1 ubiquitination and determine what percentage of PLD1 became ubiquitinated. To do so, PLD1 T-REx cells were transfected with FLAG-tagged ubiquitin, and the ubiquitin conjugates were immunoprecipitated using anti-FLAG tag antibody. The successful pull-down of ubiquitin conjugates was confirmed by Western blotting analysis using anti-FLAG antibody (Fig. 1C, right). Detection of the higher molecular weight PLD1 species in the immunoprecipitates using HA tag antibody confirmed the ubiquitination of PLD1. Approximately 3.3 ± 0.8% the total PLD1 protein induced by Dox using the T-REx system was ubiquitinated (n = 3).

We then examined the modification of endogenous PLD1 by endogenous ubiquitin using a rabbit polyclonal PLD1 antibody (H-160), which was confirmed to be capable of detecting PLD1 as well as its ubiquitinated species, and immunoprecipitated PLD1 protein (data not shown). PLD1 was immunoprecipitated from HeLa cell protein extracts using the anti-PLD1 antibody (and rabbit IgG as a specificity control), followed by Western blotting analysis with anti-ubiquitin antibody. A broad band of ubiquitin-labeled proteins was observed (Fig. 1D), which was similar in size to that seen when PLD1 and ubiquitin were overexpressed (Fig. 1A), suggesting that endogenous PLD1 also undergoes ubiquitination.

PLD1 Undergoes Multi-monoubiquitination—The type of ubiquitination that takes place on a given protein can result in distinct outcomes (9–11). We thus examined whether the ubiquitination of PLD1 was through Lys48- or Lys63-linked ubiquitination or monoubiquitination, by transfecting the PLD1 T-REx CHO cells with FLAG-tagged wild-type ubiquitin, a ubiquitin Lys48 mutant (K48R), a Lys63 mutant (K63R), or a lysineless mutant (K0). The induced PLD1 was immunoprecipitated with anti-HA tag antibody, and the ubiquitination was detected with anti-FLAG tag antibody. Transfection with K48R or K63R did not reduce the extent of PLD1 ubiquitination signals (Fig. 2A), indicating that PLD1 does not undergo Lys48- or Lys63-linked ubiquitination. Moreover, the ubiquitination signals were also not reduced in cells expressing the K0 mutant (Fig. 2B), indicating that PLD1 undergoes only monoubiquitination, presumably multiple times on individual PLD1 lysine residues.

PLD Lipase Activity Is Critical for the Ubiquitination of PLD1—Multi-monoubiquitination frequently correlates with activity of the target proteins, such as ligand-induced activation of tyrosine kinase receptors (11, 14). We thus examined whether the ability of PLD1 to perform its enzymatic lipase activity affected the extent of its monoubiquitination. To do so, we first examined the effects of inhibition of PLD activity on PLD1 multimonoubiquitination using two recently developed pharmacological inhibitors (15), compound 5WO (VU0155056, 10 μM, dual inhibitor of PLD1 and PLD2) and compound 809 (VU0155069, 10 μM, PLD1-specific). Our results showed that inhibition of PLD1 and PLD2 or PLD1 alone significantly reduced the ubiquitination of PLD1 (Fig. 3, A and B).

To further establish the requirement of PLD1 lipase activity for its ubiquitination, we took advantage of the lipase-inactive HKD motif mutant, PLD1 K898R (16). The mutant was stably expressed in CHO cells using the T-REx inducible system, as described earlier for PLD1 and PLD2 and shown in Fig. 1A. After transfection with Myc-tagged ubiquitin, the cells were induced by the addition of Dox. Thereafter, ubiquitination of the mutant PLD1 was detected using immunoprecipitation and Western blotting analysis as described before. Supporting the results obtained using pharmacological inhibitors, we observed significant reduction of the ubiquitination signal in PLD1 K898R-expressing cells as compared with cells expressing wild-type PLD1 (Fig. 1, A and B). Because lysine 898 could be a direct ubiquitin conjugation site, we generated several additional non-lysine lipase-inactive mutants to confirm the activity dependence of PLD1 ubiquitination, including two HKD motif mutants, H896R and D903A (16), and a phosphatidylinositol 4,5-bisphosphate non-interacting mutant, PLD1 R691G,R695G (7, 17). All three inactive mutants showed significant reduction in ubiquitination signals when transiently expressed in CHO cells together with Myc-tagged ubiquitin (Fig. 3, C–E), indicating that the lipase activity of PLD1 is critical for its monoubiquitination.
Because catalytic activity was important, but the basal activity of PLD1 is low (1), we employed PMA to stimulate PLD1 activity and determined if PMA increased the extent of ubiquitination. However, 100 nM PMA treatment, although enhancing PLD activity (Fig. 4C), did not stimulate the ubiquitination of either HA-tagged PLD1 in CHO cells (Fig. 4A and B) or endogenous PLD1 in HeLa cells (Fig. 4D).

Palmitoylation Is Critical for the Ubiquitination of PLD1—
Palmitoylation of PLD1 is required for its correct membrane localization and endocytosis after PMA-stimulated PM translocation (7, 18). Both palmitoylation and monoubiquitination play important roles in the vesicular trafficking of membrane-associated proteins, and palmitoylation has been reported as a prerequisite for the ubiquitination and trafficking of H-Ras.
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![Representative Western blots showing the effect of pharmacological inhibitors of PLD isoenzymes on the ubiquitination of PLD1.](image)

**A** Ctrl 5WO 809 | **B** IP: HA-PLD1 | **C** IP: HA-PLD1 | **D** IP: HA-PLD1 | **E** IP: HA-PLD1

**A–C** and **D–F**, representative Western blots showing the effect of pharmacological inhibitors of PLD isoenzymes on the ubiquitination of PLD1. After transfection with FLAG-tagged ubiquitin for 24 h, HA-tagged PLD1 T-REx CHO cells were treated with 1 μg/ml Dox for another 24 h and 1.5 μM MG132 during the last 6 h before cell lysis. PLD inhibitors were added 6 h before cell lysis as indicated. SWO, compound 5WO (VU0155056) (10 μM), a dual inhibitor for PLD1 and PLD2; 809, compound 809 (VU0155069) (10 μM), an isomeric specific inhibitor for PLD1; Ctrl, vehicle control, 0.1% (v/v) DMSO. PLD1 ubiquitination and expression were then detected as described in the legend to Fig. 1. The densitogram of PLD1 ubiquitination levels in A from three independent experiments. Ubiquitination level in the control group was set as 100%; *, p < 0.05 when compared with control group (n = 3). C and D, representative Western blots showing the effect of mutation in the HKD motif (C, H896R; D, D903A) or phosphatidylinositol 4,5-bisphosphate binding motif (D, R691G, R695G) on the ubiquitination of PLD1. Myc-tagged ubiquitin and HA-tagged wild-type PLD1 or three PLD1 mutants, PLD1 lacking the first 328 amino acids at the NH2 terminus (PLD1ΔN), PLD1 lacking the PH domain (PLD1ΔPH), and the palmitoylation-deficient PLD1 C240S, C241S (18, 20), all three of which lack palmitoylation and exhibit defects in poststimulation endocytosis (7). Dramatic reduction of ubiquitination was observed for all of the mutants (Fig. 5), indicating that palmitoylation of PLD1 is critical for its monoubiquitination and suggesting an association of monoubiquitination with vesicular trafficking of PLD1. In addition, we observed that the PLD1ΔN mutant showed a nearly complete loss of ubiquitination, whereas PLD1 ΔPH and C240S, C241S mutants had notable ubiquitination signals, suggesting that some other region or domain modifications in the N terminus may be necessary for ubiquitination.

Ubiquitinated PLD1 Induces Abruptly Enlarged Vesicles in COS-7 Cells—To determine whether the monoubiquitination of PLD1 plays any role in its subcellular localization, we constructed an N-terminally Myc-tagged ubiquitin-fused HA-tagged PLD1 expression plasmid and then examined the subcellular localization of ubiquitinated PLD1 (Ub-PLD1) in COS-7 cells. Consistent with a previous report (7), ~90% of cells transfected with wild-type PLD1 under basal conditions exhibited a perinuclear vesicular distribution for PLD1, as demonstrated by HA tag staining (Fig. 6, A–C and G–J), which partially co-localized with the Golgi complex and early endosomes, as indicated by overlap with GM130 (Fig. 6, A–C) and EEA1 (Fig. 6, G–J). Notably, ~50% cells transfected with Ub-PLD1 exhibited an aberrantly enlarged vesicular distribution for PLD1 as demonstrated by HA tag staining (Fig. 6, D–F and J–L) or Myc tag (Fig. 6O). In most Ub-PLD1-positive cells, Ub-PLD1 remained partially co-localized with Golgi complex (Fig. 6, D–F) but generally lost the co-localization with early endosomes (Fig. 6, J–L). To validate the protein expression of Ub-PLD1 in COS-7 cells, we found that Ub-PLD1 co-migrated with the upper band of PLD1 in urea-SDS-polyacrylamide gel when detected with HA tag antibody in Western blotting analysis (Fig. 6N), suggesting that the ectopic ubiquitin conjugation to the N terminus of PLD1 did not lead to its unscheduled polyubiquitination and proteasomal degradation. Additionally, when detected with Myc tag antibody in Western blotting assay, Ub-PLD1, but not wild-type PLD1, showed a positive band of similar size to the HA tag band (Fig. 6N), suggesting that the Myc-tagged ubiquitin fusion was not cleaved by deubiquitinating enzymes.

Because PA is involved in SNARE-mediated membrane fusion (21), we hypothesized that these aberrantly enlarged vesicles might be induced by accumulated PA in situ produced by ubiquitinated PLD1 trapped in some vesicular compartment. To test this possibility, we constructed an N-terminally ubiquitin-fused PLD1 K898R (Ub-PLD1 K898R) expression vector and confirmed that its protein expression and Myc-tagged ubiquitin conjugation are indistinguishable from that of Ub-PLD1 (data not shown). We observed that only 22 ± 12% (n = 3) cells transfected with Ub-PLD1 K898R showed aberrantly enlarged vesicles, and this percentage was significantly lower. (19) To test the possibility that loss of palmitoylation reduces the monoubiquitination of PLD1, we transfected CHO cells with Myc-tagged ubiquitin and HA-tagged wild-type PLD1 or three PLD1 mutants, PLD1 lacking the first 328 amino acids at the NH2 terminus (PLD1ΔN), PLD1 lacking the PH domain (PLD1ΔPH), and the palmitoylation-deficient PLD1 C240S, C241S (18, 20), all three of which lack palmitoylation and exhibit defects in poststimulation endocytosis (7). Dramatic reduction of ubiquitination was observed for all of the mutants (Fig. 5), indicating that palmitoylation of PLD1 is critical for its monoubiquitination and suggesting an association of monoubiquitination with vesicular trafficking of PLD1. In addition, we observed that the PLD1ΔN mutant showed a nearly complete loss of ubiquitination, whereas PLD1 ΔPH and C240S, C241S mutants had notable ubiquitination signals, suggesting that some other region or domain modifications in the N terminus may be necessary for ubiquitination.
than that of cells transfected with Ub-PLD1 (52 ± 5%, n = 3) (Fig. 6M). In addition, 4-h treatment with PLD inhibitor compound 809 (VU0155069; 10 μM) and 1-butanol (1%, v/v) decreased the percentage of vesicles containing Ub-PLD1-positive cells (23% for compound 809 versus 59% for DMSO vehicle; 27% for 1-butanol versus 47% for 2-butanol). Taken together, our data indicate that the aberrantly enlarged vesicles in Ub-PLD1-transfected cells are at least partially dependent on PLD1-generated PA, and they may represent some specific vesicular compartments enriched in ubiquitinated species of PLD1.

**PLD1 Monoubiquitination**

PLD1 Is Degraded through the Proteasomal Pathway but Not via Lysosomes—Monoubiquitination can serve as a sorting signal to lysosomal degradation for internalized proteins (11). To determine whether the monoubiquitination of PLD1 directs its degradation, we examined the effects of various protease inhibitors on the expression levels of PLD isoenzymes using the Dox-inducible system. In serum-starved cells, expression levels of PLD1 were significantly increased at 6 and 24 h (Fig. 7, A and D) (data not shown) only by the proteasome inhibitors MG132 (1.5 μM) and lactacystin (10 μM) but not by inhibitors of lysosomes (chloroquine (100 μM), NH₄Cl (10 mM), or leupeptin (10

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**FIGURE 4. PMA does not enhance the ubiquitination of PLD1.** A, representative Western blots showing the time-dependent effect of PMA on the ubiquitination of PLD1. After transfection with FLAG-tagged ubiquitin for 24 h, HA-tagged PLD1 T-REx CHO cells in the presence or absence of 10% FBS were treated with 1 μg/ml Dox for another 24 h and 1.5 μM MG132 during the last 6 h before cell lysis. PMA (100 nm) was added at various time points before cell lysis as indicated. PLD1 ubiquitination and expression were then detected as described in Fig. 1. The data are representative of 3 independent experiments. (B) The densitograph of PLD1 ubiquitination levels in the absence of serum as shown in A from 3 independent experiments. Ubiquitination level at 0 min was set as 100%, n = 3. (C) PLD activity assay showing the stimulation of PLD activity by PMA. Serum-starved HA-tagged PLD1 T-REx CHO cells after 24-h induction with Dox were treated with PMA (100 nm) or DMSO vehicle control for 10 min and then assessed for PLD activity as described under “Experimental Procedures.” PLD activity of the control group was set as 100%. *, p < 0.01 when compared with control group (n = 6). D, Western blots showing the time-dependent effect of PMA on the ubiquitination of endogenous PLD1. HeLa cells were treated with PMA (100 nm) for different time periods as indicated before cell lysis. PLD1 ubiquitination and expression were then detected as described in the legend to Fig. 1. IP, immunoprecipitation; IB, immunoblotting.

**FIGURE 5. Loss of palmitoylation impairs the ubiquitination of PLD1.** A, representative Western blots showing the ubiquitination of palmitoylation-deficient PLD1 mutants. Myc-tagged ubiquitin and HA-tagged wild-type PLD1 (PLD1 wt), PLD1 lacking the N-terminal 328 amino acids (PLD1 ΔN), PLD1 lacking the pleckstrin homology domain (PLD1 ΔPH), or palmitoylation-deficient PLD1 (PLD1 C240,241S) were co-transfected into CHO cells for 42 h and then treated with 1.5 μM MG132 for 6 h before cell lysis. PLD1 ubiquitination and expression were then detected as described in the legend to Fig. 1. B, the densitograph of PLD1 ubiquitination levels in A from three independent experiments. The ubiquitination level in the wild-type PLD1 group was set as 100%*. *, p < 0.01 when compared with wild-type group (n = 3). IP, immunoprecipitation; IB, immunoblotting.
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FIGURE 6. Ubiquitinated PLD1 induces aberrantly enlarged vesicles in COS-7 cells. COS-7 cells were transiently transfected with PLD1, Ub-PLD1, PLD1 K898R, or Ub-PLD1 K898R as indicated. Four hours after transfection, the cells were incubated with fresh growth medium for 16 h and then starved in serum-free medium for 24 h before lysing for protein extraction or fixing and immunostaining with anti-HA, GM130, EEA1, or Myc antibodies as indicated. Localization of PLD1 isoforms was visualized using an Alexa Fluor 488-conjugated secondary antibody (A, C, D, F, G, I, J; HA tag (green)) or a rhodamine-conjugated secondary antibody (O only; Myc tag (red)). Golgi complex (B and E; GM130) and early endosomes (H and K; EEA1) were visualized using a rhodamine-conjugated secondary antibody (red). A–C and G–I, representative images showing normal perinuclear vesicular localization of PLD1. D–F and J–L, representative images showing aberrantly enlarged vesicular localization of Ub-PLD1 as indicated by arrows. Note that the Ub-PLD1 staining signal rarely co-localized with EEA1. M, summarized data from three independent experiments showing the percentage of cells positive for aberrantly enlarged vesicular PLD1. *, p < 0.01 when compared with the PLD1 group; **, p < 0.05 when compared with the Ub-PLD1 group (n = 3). N, representative Western blots showing the expression of PLD1 and Ub-PLD1 in COS-7 cells. Note that Myc tag signal was only detected in Ub-PLD1-transfected cells. Glycerophosphate-3-phosphate dehydrogenase (GAPDH) was used as loading control. O, representative image showing aberrantly enlarged vesicular localization of Ub-PLD1 by immunostaining with Myc tag antibody. DAPI, 4′,6-diamidino-2-phenylindole. IP, immunoprecipitation; IB, immunoblotting.

DISCUSSION

Monoubiquitination is a critical regulatory mechanism in a variety of cellular processes, including endocytosis, lysosome sorting, transcription, and DNA repair (11). Our results reveal that PLD1, but not PLD2, undergoes multi-monoubiquitination, which may contribute to the differential trafficking behaviors and the roles in cellular signaling of both PLD isoenzymes.

The ubiquitination of PLD1 was confirmed through immunoprecipitation of PLD1 and immunoblotting with anti-ubiquitin antibody. We estimate that the amount of ubiquitinated PLD1 was about 3% of total PLD1 overexpressed in the CHO cells. Of note, this percentage of ubiquitinated species in the total protein population is not unusually low among those proteins undergoing vesicular trafficking facilitated by ubiquitination, such as H-Ras (~2% in CHO1 cells; the percentage is estimated based on densitometric analysis of published immunoblots) (19), epsin (~6% in 293T cells) (22), Gap1p (~7% in yeast) (23), and Ste2p (~8% in yeast) (24).

An intriguing finding is the role of lipase activity in the regulation of monoubiquitination of PLD1. The dependence on basal lipase activity of PLD1 ubiquitination was validated by the inhibitory effects on PLD1 ubiquitination of the newly developed PLD inhibitors and mutation of the PLD1 HKD motif (His896, Lys898, and Asp903) (16) or phosphatidylinositol 4,5-bisphosphate binding motif (Arg601 and Arg605) (7). On the other hand, the inability of PMA to enhance the ubiquitination of PLD1 suggested that enhanced activation by stimuli might not be required for ubiquitination. It is also possible that the ubiquitination has reached the maximal level even under resting conditions. PLD lipase activity normally results in the production of PA. However, the enzyme can also use primary alcohols, such as ethanol or 1-butanol, and when concentrations of the alcohols are sufficiently high, PA production is diminished in preference for generating the corresponding phosphatidylalcohol. Thus, although PLD1 enzymatic activity per se is unaltered, PA production is blocked. Interestingly, culture of CHO cells in 0.5% 1-butanol did not impair PLD1 ubiquitination,3 supporting the speculation that the active conformation of PLD1 and/or access to its catalytic site, but not production of PA, is critical for its ubiquitination. It is unknown

3 H. Yin and X.-L. Zheng, unpublished observation.
was found to be dependent on its palmitoylation. A previous study indicates that loss of palmitoylation abolishes the lipid raft association and poststimulation endocytosis of PLD1 (7, 18). This suggests that monoubiquitination of PLD1 may play an important role in its intracellular trafficking. Our data on PLD1 have echoed findings with H-Ras that palmitoylation is required for its mono- and diubiquitination and that the cooperation of palmitoylation and ubiquination modulates its trafficking and signaling (19). It remains unclear how palmitoylation promotes monoubiquitination of PLD1. Because the palmitoylation-deficient PLD1 mutant is still lipase-active (7), it is likely that palmitoylation regulates the monoubiquitination through altering the membrane localization of PLD1 (e.g. its lipid raft association (7)) to facilitate contact of PLD1 with the ubiquitination machinery or isolate PLD1 from deubiquitinating enzymes.

The biological relevance of PLD1 ubiquitination has been supported by the association of ubiquitinated PLD1 with an aberrantly enlarged vesicular compartment. Interestingly, this observation is reminiscent of membrane trafficking defects resulting from malfunction of ARF1 (26), a PLD1 activator, or endosomal sorting complex required for transport machinery, such as SKD1/VPS4 (27) and CHMP2B (28). The enlarged vesicle observed in our study is possibly post-Golgi but not early endosome, as demonstrated by a lack of overlapping with EEA1 staining. It remains to be determined whether these vesicles originate from endoplasmic reticulum, late endosome, multivesicular body, or lysosome. The reduced formation of enlarged vesicles upon Ub-PLD1 K898R expression or blockade of PA production by PLD inhibitor or 1-butanol suggests that in situ production of PA contributes to the enlargement of these vesicles, possibly through SNARE-dependent membrane fusion events (21). In addition, non-lipase PLD1 functions, such as interaction with lipids or proteins and recently identified dynamin GAP activity (29), could also contribute to this vesicle phenotype because K898R mutation showed an incomplete suppression effect on the vesicle enlargement. It is possible that the enlarged vesicle observed in the current study may represent an intermediate vesicular compartment enriched in ubiquitinated species of PLD1 during PLD1 trafficking. Since the ectopically ubiquitinated PLD1 mutant employed in our study is resistant to deubiquitination, our data suggest that an ubiquitination/deubiquitination cycle may regulate the trafficking of PLD1 at different membrane fractions. Of note, PLD1 plays a regulatory role in the vesicular trafficking of various membrane-associated proteins, such as endocytosis of EGFR (30), PM targeting of Rap1 (31) and GLUT4 (32), and exocytosis of

whether PLD1 undergoes conformational changes in the presence of its substrate, phosphatidylcholine, or upon stimulation. However, it is possible that the PLD1 protein requires a specific active conformation to be recognized by the ubiquitination machinery, which would be consistent with our findings. We also found that PLD2 does not become ubiquitinated. We thus examined whether the loop domain, one of the PLD1-unique regions, is the domain that is targeted by or responsible for the ubiquitination but found that PLD1 lacking the loop domain only showed a modest reduction (25 ± 11%, n = 3) in ubiquitination in comparison with wild-type PLD1,2 indicating that other regions of the PLD1 protein are responsible for the difference in ubiquitination between the PLD isoenzymes.

Both palmitoylation and monoubiquitination are important regulatory mechanisms in the intracellular trafficking of target proteins (11, 25). In our study, the monoubiquitination of PLD1

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PLD1 Monoubiquitination

noradrenalin (33). It will be interesting to assess whether the multi-monoubiquitinated species of PLD1 represents the subset of the PLD1 protein actively engaged in membrane trafficking. Ubiquitinated PLD1 could be localized in some specific membrane fractions and act as a membrane-processing component through local PA production. Consistent with this proposal, PA is enriched in some specific post-Golgi vesicles (34).

Monoubiquitination is widely recognized as a signal for the lysosomal sorting and degradation (11) and is also now known to play a role in proteasomal degradation, as has been reported for ferritin (35). In our study, PLD1 was degraded via proteasomes rather than lysosomes, whereas in contrast, PLD1 K898R and PLD2, both of which were defective in ubiquitination, did not undergo proteasomal degradation, suggesting that the proteasome degradation is dependent on PLD1 monoubiquitination. Because elevated PLD1 activity has been reported in many human diseases (36), it will be interesting to evaluate the pathophysiological roles of the ubiquitin-dependent degradation of PLD1.

The E3 ligase and deubiquitinating enzyme for PLD1 are unknown. Literature analysis suggests that the Nedd4 family might be candidate of E3 ligase for PLD1. Nedd4 may monoubiquitate PLD1 at the Golgi complex so as to regulate its endosomal sorting, as it does for the Gap1p amino acid permease (23). UBPY (37) or AMSH (associated molecule with ubiquitinate PLD1 at the Golgi complex so as to regulate its might be candidate of E3 ligase for PLD1. Nedd4 may monoubiquitate PLD1 at the Golgi complex so as to regulate its endosomal sorting, as it does for the Gap1p amino acid permease (23). UBPY (37) or AMSH (associated molecule with

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