# Colocalization of phospholipase D1 and GTP-binding-defective mutant of ADP-ribosylation factor 6 to endosomes and lysosomes

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**Abstract Phospholipase D (PLD) is involved in various aspects of cellular function. Two isoforms, PLD1 and PLD2, have been identified. PLD1, which has two splicing variants, is regulated by various factors, including ADP-ribosylation factor (ARF). We here show that both variants of PLD1 are predominantly localized to late endosomes and lysosomes, but not to the Golgi apparatus or endoplasmic reticulum in contrast to earlier studies. Furthermore, PLD1s show significant colocalization with an ARF6 mutant defective in GTP binding. The data suggest that PLD1, under the regulation of ARF6, plays a role in the function of endosomes and lysosomes.**



*Key words:* ADP-ribosylation factor; Endosome; Lysosome; Phospholipase D; Vesicular transport

## 1. Introduction

Phospholipase D (PLD) catalyzes hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) and choline. PA itself and its hydrolytic product, diacylglycerol, function as second messengers (for review, see [1–4]). Two mammalian PLD genes (PLD1 and PLD2) have recently been identified; PLD1 has two splicing variants, PLD1a and PLD1b. PLD1 is activated by several protein factors, including members of the ADP-ribosylation factor (ARF) family, while PLD2 is insensitive to the PLD1 activators and constitutively active in the presence of phosphatidylinositol 4,5-bisphosphate ([5–7], reviewed in [3,4]).

ARF was originally identified as a cofactor of the ADP-ribosyltransferase activity of the cholera toxin (for review, see [8]). Thereafter, ARF was shown to regulate a number of vesicular trafficking events, such as the formation of Golgi-derived COP I-coated and AP-1/clathrin-coated vesicles ([9,10]; reviewed in [11,12]). ARF may also regulate the endosome-endosome fusion event [13]. Of six mammalian ARFs (ARF1–ARF6) so far identified, ARF1, which has been most extensively studied, plays a pivotal role in vesicular trafficking (for reviews, see [11,12]). Another well characterized member of the ARF family is ARF6. Unlike ARF1, it appears to function in the plasma membrane and the endosomal system [14–16]. An ARF6 mutant restricted to the GTP-bound form, ARF6(Q67L), is localized to the plasma membrane and decreases the rate of transferrin uptake, while a GDP-bound mutant, ARF6(T27N), is localized to endosomal compartments and inhibits recycling of endocytosed materials to the cell surface [14–16].

Since ARF1, as well as ARF3, was first identified as a PLD activator [17,18], PLD is believed to be involved in the ARF-dependent vesicular trafficking. This notion is supported by the reports that ARF-dependent PLD activity is present in Golgi-enriched membranes and COP I binds to membranes upon production of PA by exogenous PLD [19,20]. In contrast, Brown et al. have demonstrated that green fluorescent protein (GFP)-tagged PLD1b overexpressed in RBL-2H3 cells localizes to secretory granules and lysosomes, but not to Golgi apparatus [21]. Analysis by Colley et al. of subcellular localization of epitope-tagged PLD1a revealed that it localizes to perinuclear regions including the endoplasmic reticulum (ER), Golgi apparatus and endosomes [6]. Although they did not unequivocally identify the subcellular compartment where PLD1a localizes, these observations, taken together, raise the possibility that an ARF-sensitive PLD isozyme of Golgi-enriched membranes is PLD1a. To examine this possibility, in this study we determined and compared the subcellular localization of PLD1a expressed in several types of mammalian cells with that of PLD1b. The results obtained show that both PLD1 variants are localized almost exclusively to late endosomes and lysosomes, but not to the Golgi apparatus or ER. Furthermore, PLD1 was found to be colocalized with ARF6(N122I), a mutant defective in GTP binding, suggesting that ARF6 is a physiological activator of PLD1.

## 2. Materials and methods

### 2.1. Plasmid construction

cDNAs of mouse PLD1b and human PLD1a were obtained by PCR of mouse brain and human HL60 cell cDNAs, respectively, using primers synthesized based on the published nucleotide sequences. Expression vectors for N-terminally hemagglutinin (HA)-tagged mouse PLD1b (pcDNA3-HA-PLD1b) and FLAG-tagged human PLD1a (pTB-FL-PLD1a) were constructed by subcloning of the cDNAs into pcDNA3-HAN and pTB701-FL, respectively. The construction of an expression vector for C-terminally HA-tagged ARF6 (pcDNA3-ARF6-HA) was described previously [22]. Q67L and N122I mutations of ARF6 were introduced into the ARF6 cDNA by a PCR-based strategy using mutagenic primers.

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*Abbreviations:* PLD, phospholipase D; PA, phosphatidic acid; ARF, ADP-ribosylation factor; GFP, green fluorescent protein; ER, endoplasmic reticulum; HA, hemagglutinin; Tfn-R, transferrin receptor; Man II, mannosidase II; BFA, brefeldin A


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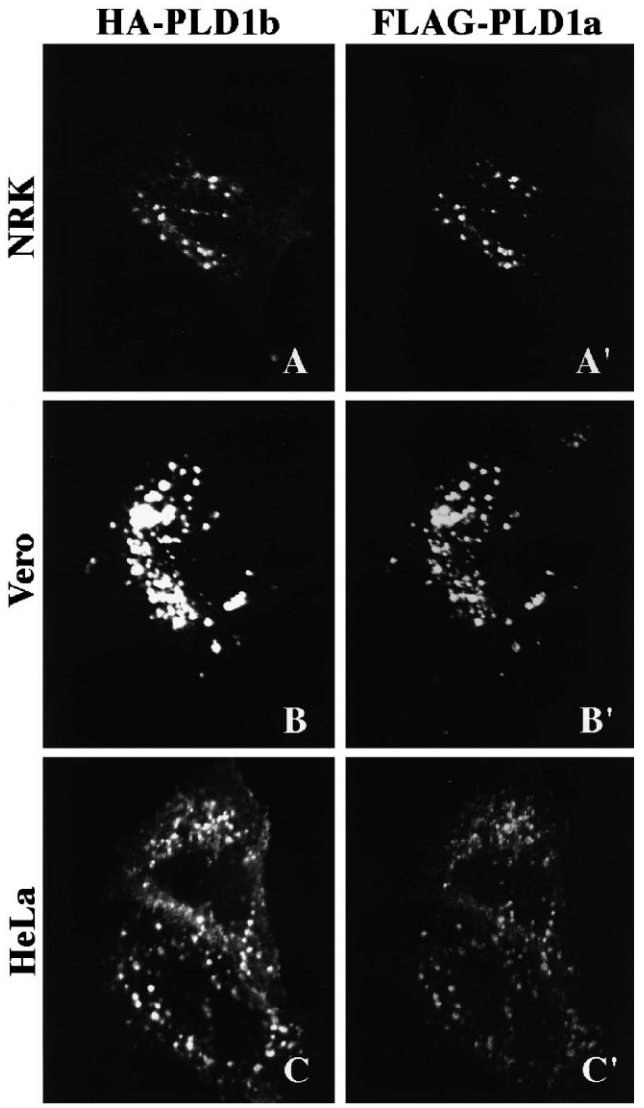


Fig. 1. Subcellular localization of PLD1a and PLD1b. NRK (A, A′), Vero (B, B′) or HeLa (C, C′) cells transiently cotransfected with pcDNA3-HA-PLD1b and pTB-FL-PLD1a were fixed, permeabilized, and double-stained with monoclonal rat anti-HA (A, B, C) and monoclonal mouse anti-FLAG M2 (A′, B′, C′) antibodies followed by FITC-labeled anti-rat and Cy3-labeled anti-mouse IgGs.

*2.2. Antibodies*

Polyclonal rabbit anti-rat lamp-1 antibody was kindly provided by Dr. K. Akasaki (Fukuyama University, Fukuyama, Japan) [23]. Monoclonal mouse anti-rat transferrin receptor (Tfn-R) antibody (OX-26) was purchased from Chemicon International. Monoclonal mouse anti-mannosidase II (Man II) antibody (53FC3) was from Berkeley Antibody Co. Monoclonal rat (3F10) and mouse (12CA5) antibodies against the HA epitope were from Boehringer Mannheim. Monoclonal mouse anti-FLAG M2 antibody was from Eastman Kodak Co. All secondary antibodies were from Jackson Immuno-Research Laboratories.

*2.3. Immunofluorescence analysis*

Clone 9 rat hepatocytes, monkey kidney Vero cells or HeLa cells grown in wells of 8-well Lab-Tek-II chamber slides (Nunc) were transfected with either pcDNA3-HA-PLD1b or pTB-FL-PLD1a alone, or in combination with an expression vector for either wild type or mutant ARF6 using TransIT LT1 (PanVera Corp.) or FuGENE 6 (Boehringer Mannheim) transfection reagent. The cells were cultured for 24–48 h and then processed for indirect immunofluorescence analysis as described previously [22,24,25]. The stained cells were observed with a laser-scanning confocal microscope (TCS-NT, Leica Lasertechnik).

## 3. Results and discussion

To determine and compare the subcellular localization of the PLD1a with that of PLD1b, we transiently expressed epitope-tagged PLD1a and PLD1b in several types of mammalian cells, including NRK, Vero and HeLa cells. As shown in Fig. 1, FLAG-tagged PLD1a and HA-tagged PLD1b were colocalized to large vesicular structures scattered in the cytoplasm in all the examined cells. Thus, there is no difference in the localization between the PLD1 splicing variants. Furthermore, the observations that PLD1 is localized to similar vesicular structures in many cell types exclude a possibility that transient overexpression of PLD1 resulted in its aberrant subcellular localization.

To unequivocally identify the intracellular compartment where PLD1 locates, cells expressing HA-tagged PLD1b were double-stained with antibodies to marker proteins for various organelles. When the HA-PLD1b-expressing cells were double-stained with antibodies to the HA epitope and Man II (a resident Golgi integral membrane protein), the staining for HA-PLD1b was not superimposed on that for Man II (Fig. 2A,A′). Furthermore, when the cells were treated with brefeldin A (BFA), which causes disintegration of the Golgi structure following dissociation of the COP I coat from Golgi membranes [26], the PLD1b localization was not altered, whereas Man II (Fig. 2B,B′) and β-COP (data not shown), a component of the COP I coat, were redistributed throughout the cytoplasm. These observations indicate that

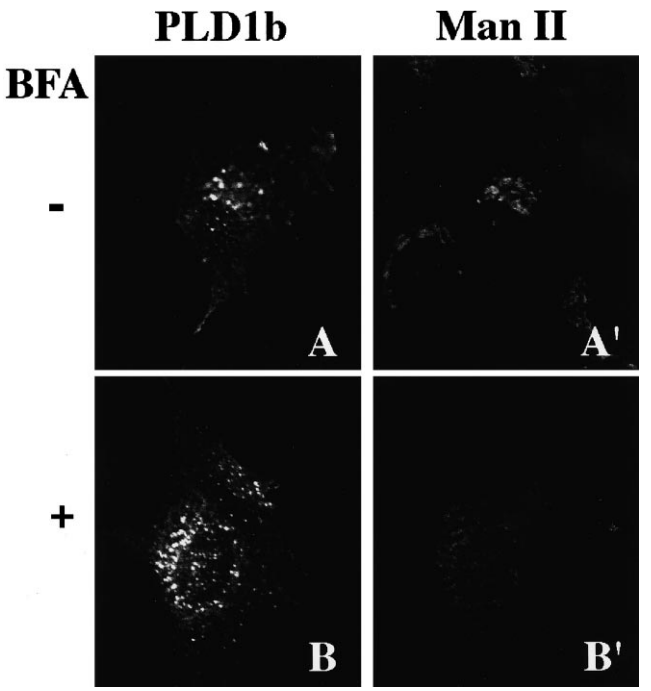


Fig. 2. Sensitivity to BFA of PLD1. NRK cells transiently transfected with pcDNA3-HA-PLD1b were incubated for 30 min in the presence (B, B′) or absence (A, A′) of 5 μg/ml BFA, fixed, permeabilized, and double-stained with monoclonal rat anti-HA (A, B) and monoclonal mouse anti-Man II (A′, B′) antibodies followed by FITC-labeled anti-rat and Cy3-labeled anti-mouse IgGs.

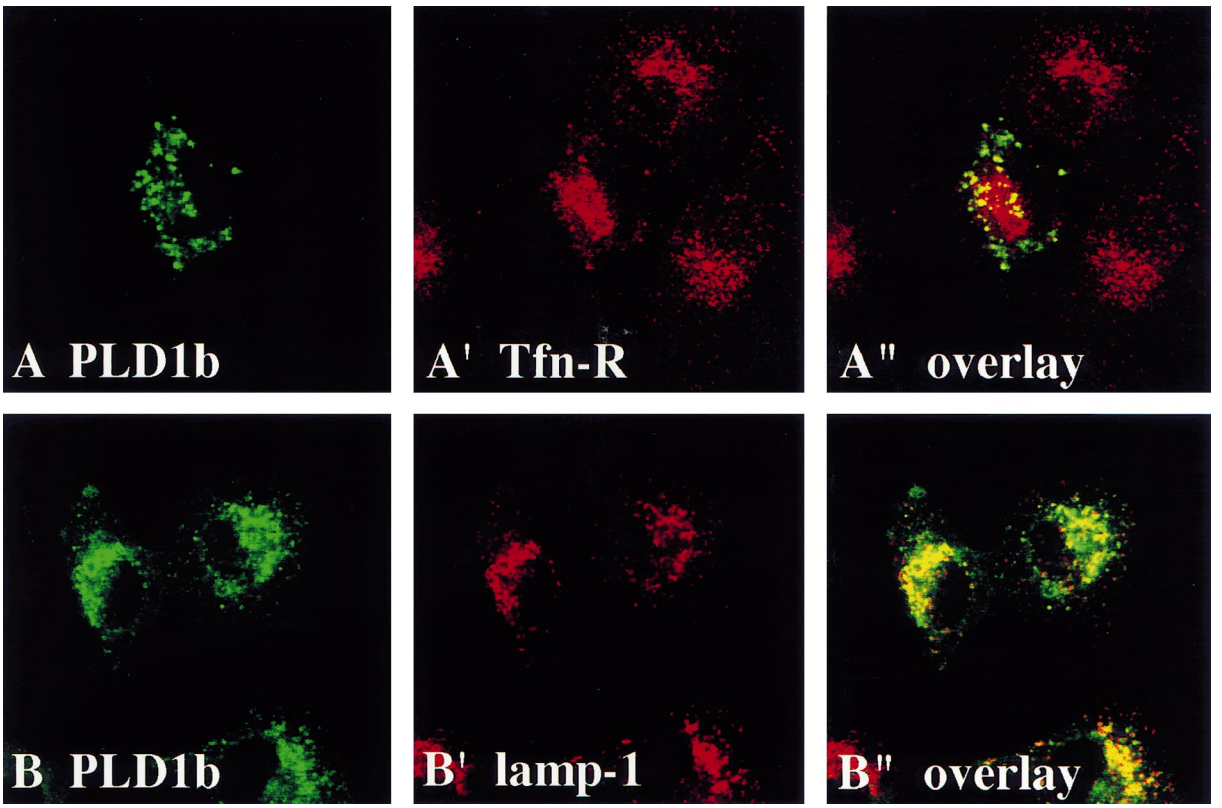


Fig. 3. Localization of PLD1 to late endosomes/lysosomes. NRK cells transiently transfected with pcDNA3-HA-PLD1b were fixed, permeabilized, and double-stained with a combination of monoclonal rat anti-HA (A) and monoclonal mouse anti-Tfn-R (A′) antibodies followed by FITC-labeled anti-rat and Cy3-labeled anti-mouse IgGs or a combination of monoclonal mouse anti-HA (B) and polyclonal rabbit anti-lamp-1 (B′) antibodies followed by FITC-labeled anti-mouse and Cy3-labeled anti-rabbit IgGs. Overlays are shown in the right panels.

PLD1 does not localize to the Golgi apparatus. We also double-stained the HA-PLD1b-transfected cells for HA and protein disulfide isomerase (an ER marker) but failed to observe their colocalization (data not shown), suggesting that PLD1 does not localize to the ER. Because the large vesicular structures containing PLD1 were reminiscent of lysosomes and endosomes, we then examined if PLD1 was colocalized with Tfn-R (an early and late endosomal marker) and lamp-1 (a marker for late endosomes and lysosomes). As shown in Fig. 3, a partial but significant overlap was observed between the staining for HA-PLD1b and Tfn-R (panels A and A″). Furthermore, the staining for HA-PLD1b was found to be largely superimposed on that for lamp-1 (panels B and B″). These observations clearly demonstrate that PLD1 is predominantly localized to late endosomes and lysosomes but not to the Golgi apparatus or ER, which is consistent with the previous report with GFP-tagged PLD1b [21].

Ktistakis et al. have previously reported that Golgi-enriched membrane fractions separated by sucrose gradients contain a high level of ARF-sensitive PLD activity [19], although we could not detect PLD1 on the Golgi apparatus. This discrepancy may be explained by a possible contamination of endosomes and lysosomes in the Golgi-enriched fraction. Alternatively, it is possible that there is an additional ARF-sensitive PLD isozyme(s) that is different from the already identified ARF-sensitive PLD1.

Because ARF6 is known to function in the recycling endocytic pathway [14–16] and, as well as ARF1 and ARF3, is able to activate PLD1 in vitro [17,18,27], it is possible to speculate that ARF6 physiologically functions as a PLD1 activator. To compare the localization of ARF6 with that of PLD1, cells were transiently cotransfected with expression vectors for FLAG-tagged PLD1a and HA-tagged ARF6 or its mutant, and double-stained with anti-FLAG and anti-HA antibodies. Because it is difficult to raise antibodies that specifically recognize ARF6 due to the high sequence similarity between ARF isoforms (ARF1–ARF6), we [22] and others [15] have used HA-tagged ARF6. As shown in Fig. 4A,A′, wild type ARF6, unlike PLD1a, was distributed throughout the cytoplasm. When HA-tagged ARF6(N122I) was coexpressed with PLD1a, the vesicular staining pattern for PLD1a was almost identical to that for ARF6(N122I) (Fig. 4C,C′). This is in good agreement with the previous report that ARF6(T27N) localizes to endosome/lysosomes [14–16]. Both ARF6(N122I) and ARF6(T27N) are thought to be GTP-binding-defective mutants, because it has been shown that equivalent ARF1 mutants, ARF1(N126I) and ARF1(T31N), are defective in GTP binding and similarly inhibit vesicular transport [28]. A GTP-bound active mutant, ARF6(Q67L), was localized to cytoplasm and the cell periphery and was not significantly colocalized with PLD1 (Fig. 4B,B′). We also analyzed the localization of wild type ARF1 and its mutants equivalent to ARF6(N122I) and ARF6(Q67L), but neither of them colocalized with PLD1 (data not shown).

Colocalization of PLD1 with the GTP-binding-defective mutant, ARF6(N122I), seems to be consistent with previous reports showing that another GTP-binding-defective mutant

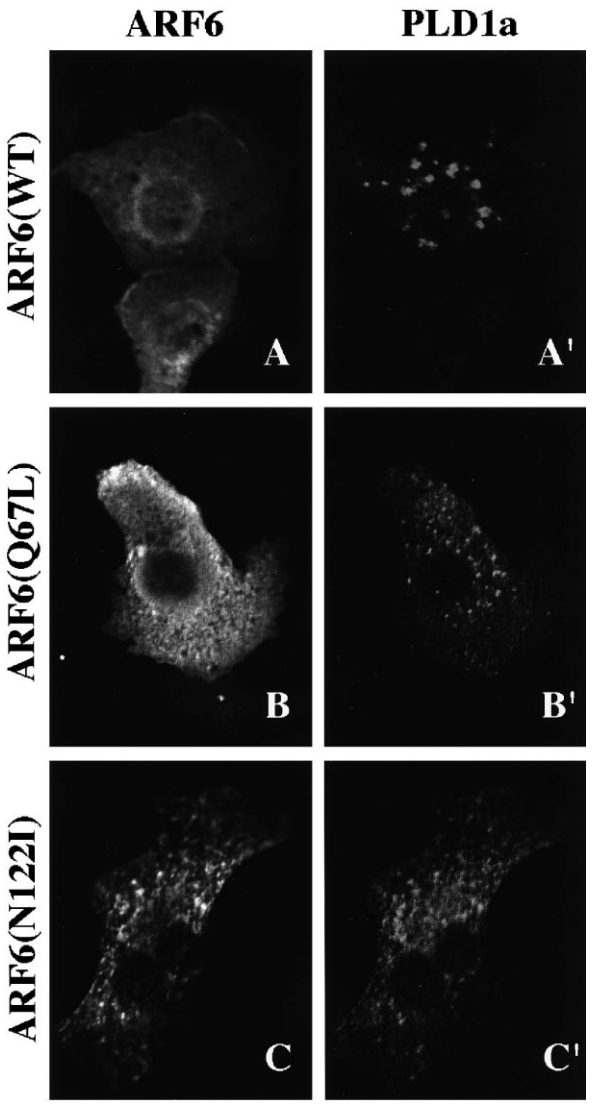


Fig. 4. Colocalization of PLD1 with ARF6(N122I). Vero cells transiently cotransfected with a combination of pTB-FL-PLD1a and either pcDNA3-ARF6-HA (A, A′), pcDNA3-ARF6(Q67L)-HA (B, B′) or pcDNA3-ARF6(N122I)-HA (C, C′) were fixed, permeabilized, and double-stained with monoclonal rat anti-HA (A, B, C) and monoclonal mouse anti-FLAG M2 (A′, B′, C′) antibodies followed by FITC-labeled anti-rat and Cy3-labeled anti-mouse IgGs.

ARF6(T27N) significantly colocalized with Tfn-R [14–16]. Localization of wild type ARF6 was, however, different from that of ARF6(N122I) and was distributed throughout the cytoplasm. At present, there is no clear explanation for the difference in subcellular localization between wild type ARF6 and its GTP-binding-defective mutant. Nevertheless, colocalization of PLD1 and the GTP-binding-defective ARF6 mutant, taken together with the observations that ARF6 functions in the recycling endocytic pathway [14–16] and is able to activate PLD in vitro [27], leads us to speculate that upon cell activation ARF6 activates PLD on lysosomes and/or endosomes, which, in turn, regulates the function of endosomes and lysosomes. This idea is consistent with the report by Brown et al. that activation of PLD1b overexpressed in RBL-2H3 cells by cross-linking of IgE receptors correlates well with the release of lysosomal contents [21].

Brown et al. have shown that stimulation of RBL-2H3 cells by cross-linking of IgE receptors causes translocation of GFP-tagged PLD1b to the plasma membrane [21], and Caumont et al. have shown that stimulation of chromaffin cells causes translocation of ARF6 to the plasma membrane fractions and concomitant activation of PLD in the plasma membrane fractions [29]. In the present study, however, we failed to show translocation of PLD1 from endosomes/lysosomes to the plasma membrane in cells expressing ARF6(Q67L), a constitutively active mutant. A possible explanation for the apparent discrepancy is that the constitutively active ARF6 mutant is directly located to the cell periphery without being trapped on endosomes/lysosomes where PLD1 is present, and thus unable to cause translocation and activation of PLD1. Cycling between GDP-bound inactive and GTP-bound active states may be required for ARF6 to activate and recruit PLD1 to the plasma membrane. To address this issue, experiments are under way in our laboratory.

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