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Measuring Phospholipase D Enzymatic Activity Through Biochemical and Imaging Methods

F. Philip, **E.E. Ha**, **M.A. Seeliger**, and **M.A. Frohman**[1]

Center for Developmental Genetics, Stony Brook University School of Medicine, Stony Brook, NY, United States

Abstract

The phospholipase D (PLD) enzymatic superfamily regulates a wide range of cell biological and physiological pathways, including platelet activation, immune responses, cancer, and spermatogenesis. The three main enzymatic actions of the superfamily entail (i) hydrolyzing membrane phospholipids (phosphatidylcholine (PC) and cardiolipin) to generate choline and the second messenger signaling lipid phosphatidic acid (PA), (ii) using ethanol to transphosphatidylate PC to generate the long-lived metabolite phosphatidylethanol, and (iii) hydrolyzing RNA transcripts to generate piRNAs, the third form of endogenous RNAi. We discuss briefly previously published methods for *in vitro* and *in vivo* detection and imaging of PA, and focus on production, purification, and *in vitro* endonuclease activity analysis for human PLD6, a mitochondrial-tethered isoform with roles in fertility, cancer, and neuronal homeostasis.

1. Introduction

The phospholipase D (PLD) superfamily of enzymes is defined by a catalytic site HxKxxxxD(6×)GG/SAN motif that mediates its trans-phosphalytic/hydrolytic actions on phosphodiester bonds in selected lipids and nucleic acid substrates (reviewed in Jenkins & Frohman, 2005). The canonical mammalian PLD1 (Hammond et al., 1995) and PLD2 (Colley et al., 1997) enzymes are best known for using water as the nucleophile to hydrolyze the abundant phospholipid phosphatidylcholine (PC) to generate the second messenger signaling lipid phosphatidic acid (PA).

Short-chained primary alcohols such as ethanol and 1-butanol can also serve as the nucleophile to generate phosphatidylethanol (PtdEtOH) and phosphatidylbutanol (PtdBut) (reviewed in McDermott, Wakelam, & Morris, 2004). Measuring PtdBut production in the presence of low amounts of 1-butanol has long been a favored method to quantify PLD1 and PLD2 cellular activity, since PtdBut is produced only by PLD, whereas PA is generated by several other families of enzymes, and since PtdBut is metabolically relatively inert, whereas PA is rapidly turned over (Morris, Frohman, & Engebrecht, 1997). Measurement of PtdEtOH by mass spectrometry of blood cells has recently become of great interest (Schrock, Thierauf, Wurst, Thon, & Weinmann, 2014), since the PtdEtOH generated

[1]Corresponding author: michael.frohman@stonybrook.edu

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subsequent to alcohol consumption can persist *in vivo* for up to a month (Gnann, Thierauf, Hagenbuch, Rohr, & Weinmann, 2014), whereas ethanol itself is much more rapidly metabolized. PtdEtOH is endogenously produced from ethanol generated by intestinal bacteria and has been proposed to be linked to the development of colon cancer in the context of heavy alcohol consumption (Pannequin et al., 2007).

PLD1- and PLD2 (PLD1/2)-generated PA has been associated with a variety of subcellular functions, such as intracellular trafficking, cytoskeletal dynamics, cell proliferation, and membrane remodeling (Jenkins & Frohman, 2005; McDermott et al., 2004). There are many proteins thought to be direct effectors of PA action, including mTOR (reviewed in Foster, Salloum, Menon, & Frias, 2014), PI4P5kinase (Honda et al., 1999), myosin phosphatase (Du & Frohman, 2009), sphingosine kinase 1 (Delon et al., 2004), kinesin (Manifava et al., 2001), lipin 1 (Huang et al., 2011), and Drp1 (Adachi et al., 2016). PA, as a cone-shaped, negatively charged lipid, has also been proposed to promote lipid bilayer negative curvature to lower the activation energy for membrane fusion and fission during exocytosis, endocytosis, and vesicular trafficking (Kooijman et al., 2005; Tanguy et al., 2016). PA can further be metabolized to generate the lipid signaling molecules diacylglycerol (DAG) and LysoPA, which regulate many additional pathways (Frohman, 2015). Physiologically, mice lacking PLD1 or PLD2 are viable but have defects in platelet activation (Elvers et al., 2010; Stegner et al., 2013), immune responses (Ali et al., 2013; Gobel et al., 2014; Schonberger et al., 2014), and cancer pathways (Chen et al., 2012).

PLD3 and 4 are transmembrane proteins that localize on the endoplasmic reticulum (ER) (Osisami, Ali, & Frohman, 2012; Yoshikawa et al., 2010). Catalytic activity has not been reported for either enzyme (Osisami et al., 2012; Yoshikawa et al., 2010). Mutations in PLD3 have been linked to Alzheimer's disease (Giri, Zhang, & Lu, 2016), whereas PLD4 mutations trigger autoimmune diseases such as systemic sclerosis (Terao et al., 2013). Mice lacking PLD5 have been generated, but no significant pheno-types were observed in a standardized phenotypic screen involving 25 tests, nor is it known whether PLD5 is catalytically active (http://www.mousephenotype.org/). PLD6 (also known as MitoPLD, and in *Drosophila*, Zucchini) is tail anchored into the outer surface of the mitochondrial membrane (Choi et al., 2006) and has been reported to have two catalytic functions, acting as a lipase to cleave the mitochondrial-localized lipid cardiolipin to generate PA (Baba et al., 2014; Choi et al., 2006; Huang et al., 2011; von Eyss et al., 2015; Zhang et al., 2016), and functioning as an endonuclease to cleave long RNAs during spermatogenesis to generate piRNAs (Ipsaro, Haase, Knott, Joshua-Tor, & Hannon, 2012; Nishimasu et al., 2012). piRNAs are small interfering RNAs that silence transposons to protect host germ lines during meiosis. PLD6's lipase function has been linked to mito-chondrial fusion and fission (Adachi et al., 2016; Baba et al., 2014; Choi et al., 2006; Huang et al., 2011; von Eyss et al., 2015; Zhang et al., 2016), neuronal homeostasis (Zhang et al., 2016), and breast cancer progression (von Eyss et al., 2015).

PLD1 and PLD2 are regulated by signaling processes ensuing from G protein-coupled receptor and receptor tyrosine kinase activation events (Jenkins & Frohman, 2005; McDermott et al., 2004). Regulation of PLD6 is not currently understood, although it has

been proposed that interaction with the protein Mitoguardin facilitates the lipase activity over the nuclease activity (Zhang et al., 2016).

Measurement of PLD activity has been key to advances in the field. Measurement approaches have involved *in vitro* and *in vivo* measurements of PA or PtdBut production, imaging of PA *in vivo* in cells, and analysis of oligonucleotide hydrolysis *in vitro* or piRNA production *in vivo*. PLD1 and PLD2 methods of analysis have been published and will be summarized here; biochemical, cellular, and organismal analysis of PLD6 lipase activity (Choi et al., 2006; Huang et al., 2011) and biochemical measurement of mouse and *Drosophila* PLD6 nuclease activity have been described as well (Ipsaro et al., 2012; Nishimasu et al., 2012). Here, we present a detailed method for purification and biochemical analysis of human PLD6 nuclease activity, which differs in detail from the published protocols for PLD6 analysis in other species.

2. Methods

2.1 PLD1 and PLD2 Biochemical Measurement Approaches

There are several considerations in choosing a PLD1/2 assay approach (reviewed in Morris et al., 1997). PLD1/2 activity can be measured *in vitro* using exogenously provided substrates or in intact or broken cell systems that employ endogenous phospholipid substrates, and there are different options for what is quantified (choline, PA, or Ptd-alcohol). Some approaches involve using a radiolabeled PC substrate. In the *in vitro* assay procedure, the substrate is incorporated into mixed phospholipid vesicles (alternately, short-chained PCs (e.g., di-C10 PC) that are soluble in aqueous solution can be used). In the *in vivo* assay, PLD1/2 activity can be measured in intact cells by prelabeling the endogenous PC through feeding the cells with ^{3}H-palmitic acid. PLD1/2 activity is then determined by measuring conversion of the radiolabeled lipid substrate to PA, or Ptd-alcohol if ethanol or 1-butanol is included in the assay medium. PLD1/2 can also be assayed using nonradioactive approaches, which has become more popular in recent years. One method involves a coupled assay that measures choline; another employs mass spectrometry analysis to measure PtdEtOH.

2.1.1 *In Vitro* Measurement of PLD1/2 Activity Using Liposomes Containing ^{3}H-PC—

This approach is ideal for assessing the effects of activators and inhibitors on PLD1/2 activity in isolation from cellular regulation. In the presence of the activators phosphatidylinositol 4,5-bisphosphate (PI4,5P_2), protein kinase C, and the small GTPases Rho and Arf, PLD1 activity increases 6000-fold over its basal activity. In contrast, PLD2 is activated only by PI4,5P_2 in this setting. Note that assay of cell lysates can be complicated, since varying amounts of the PLD1/2 activators will be present in stimulatory forms in the lysates, depending on the activation state of the cells at the moment of lysis. Methods for production and purification of PLD1, PLD2, and their activators, and the *in vitro* assay conditions using thin layer chromatography (TLC) to quantify PA and PtdBut have been published earlier in this series (Brown, Henage, Preininger, Xiang, & Exton, 2007; Frohman, Kanaho, Zhang, & Morris, 2000).

2.1.2 *In Vivo* Measurement of PLD1/2 Activity Using ^{3}H-Palmitic Acid Cell Labeling—This approach is ideal for assessing regulated activation of PLD1/2 in cells. In brief, cells are cultured with the labeled lipid for a day, during which time the palmitic acid is converted in part to ^{3}H-PC. PLD1/2 activation by signaling events accordingly generates ^{3}H-PA, or in the presence of primary alcohols, ^{3}H-Ptd-alcohol, which can again be quantified using TLC. Detailed methods for this assay have also previously been published in this series (Brown et al., 2007; Du, Morris, Sciorra, & Frohman, 2002).

2.1.3 *In Vivo* Measurement of PLD1/2 Activity in Organisms—These approaches are classically based on PLD1/2's transphosphatidylation activity. Introduction of ethanol into mice (and humans) results in the production of PtdEtOH, which can be detected immunologically in tissue sections (Pannequin et al., 2007) and quantitatively by mass spectrometry (Oliveira et al., 2010; Schrock et al., 2014; Scott et al., 2013). Sensitivity can be increased for LC–MS using labeled alcohol (Brown et al., 2007).

2.1.4 Measurement of PLD1/2 Activity Using a Coupled Assay—In this assay, PLD1/2 activity is monitored indirectly. PLD cleavage of PC generates choline (in addition to PA); the choline is then metabolized by choline oxidase to betaine and H_2O_2; finally, in the presence of horseradish peroxidase, the H_2O_2 reacts with 10-acetyl-3,7-dihydrophenoxazine to generate the highly fluorescent product resorufin, which is quantified in a plate reader. Commercially available as “Amplex Red” (Invitrogen), this approach has become widely used. The assay was initially targeted to *in vitro* analysis of purified PLD protein, since cellular factors that affect generation, turnover, or utilization of choline or H_2O_2 will alter the assay readout. However, we examined this approach in a side-by-side comparison with the traditional ^{3}H-palmitic acid cell labeling approach for platelets and found reasonable agreement between the assays (Elvers et al., 2010). Nonetheless, this may not apply to all tissues, in particular neuronal tissues that metabolize choline rapidly, or cells under oxidative stress that are generating H_2O_2. Appropriate controls should be used to establish the validity of the coupled assay approach on a case-by-case basis.

2.1.5 Measurement of Cellular Generation of PA Using Fluorescent Sensors—A growing number of PA sensors fused to fluorescent proteins (e.g., EGFP) have been developed over the past 15 years, starting with the isolated PA-binding domains of the Raf1-kinase (Rizzo, Shome, Watkins, & Romero, 2000) and the yeast t-SNARE Spo20 (Nakanishi et al., 2006; Su et al., 2009), to modified ones that employ FRET (Ferraz-Nogueira, Diez-Guerra, & Llopis, 2014; Nishioka, Frohman, Matsuda, & Kiyokawa, 2010) or have increased sensitivity (Zhang et al., 2014). PA-binding domains are generally short (20–40 amino acid), nonstructured, polybasic sequences that do not exhibit stereospecificity for PA and seem to bind through charged interaction. Genetic analysis established specificity to PA for the yeast sensor Spo20 (Nakanishi et al., 2006), which became puzzling in connection to a subsequent report that showed that affinity of the PA-binding motif for membrane surfaces appeared responsive to other anionic lipids (i.e., “integrating” the lipid environment; Horchani, de Saint-Jean, Barelli, & Antonny, 2014). However, this was recently resolved with the finding that all of the known PA sensors are sensitive to membrane curvature, with negative curvature enhancing binding and positive curvature eliminating binding of the

sensor to PA-containing membrane surfaces (Putta et al., 2016). It is now clear that the anionic lipids studied in (Horchani et al., 2014) affected membrane curvature. This finding should be taken into consideration in interpreting translocation of the sensor in the *in vivo* setting, and in particular, suggests that analysis of candidate PA-binding proteins to PA should be conducted using liposomes with physiological lipid composition and degrees of curvature, rather than the often employed lipid "dot blots."

2.1.6 General Comments on PLD1/2 Activity Assays—All of the approaches used to assay PLD1/2 activity require careful interpretation of the results, based on the use of appropriate controls. Measurement of PA is problematic, whether performed by TLC or by *in vivo* fluorescent sensor imaging, for several reasons. First, PA can be generated by other sources, specifically any of the many isoforms of DAG kinase, or LysoPA acetyltransferase enzymes (reviewed in Frohman, 2015). It has been compel-lingly demonstrated that pharmacological elimination of PLD1/2 activity (Ali et al., 2013; Su et al., 2009) or genetic ablation of PLD1/2 (Sato, Hongu, Sakamoto, Funakoshi, & Kanaho, 2013), in *some* settings, has little to no effect on PA generation at subcellular sites at which PLD1/2 normally generate PA during signaling events, due to a dominant or compensatory contribution of DAG kinase isoforms. Thus, in analysis of PA generation and localization, *it is critical to use pharmacological and/or genetic controls to enable reaching a conclusion that PLD1/2 are the source of the PA in that setting*. Second, PA is rapidly turned over, via the actions of PA phosphatases (Lipins) (Pascual & Carman, 2013) and PA-specific PLA enzymes (Baba et al., 2014). Third, radioactive labeling of lipid substrates may preferentially label membranes/membrane lipids in subcellular regions undergoing active remodeling. Ultimately, quantification or immunovisualization of Ptd-alcohol may be the most accurate means for assessment of PLD1/2 activity—but in all cases, every effort to employ the best possible set of negative controls will lead to the highest degree of *reproducibility* and *rigor* in the experimental outcomes. It should also be noted that although PLD1 and PLD2 become phosphorylated and undergo varied types of subcellular translocation during signaling and activation events, the phosphorylation and translocation, which can be monitored with immunostaining or other approaches, are in themselves are not accurate indicators of the degree of PLD1 or PLD2 activity.

2.2 Generation and Purification of Human PLD6

The PLD6 catalytic site is positively charged with a narrow groove that can accommodate a single-stranded but not double-stranded RNA or DNA oli-gonucleotide (Nishimasu et al., 2012). The conserved active site residues include the histidine and lysine of the HKD motif and are critical for PLD6 lipase (Choi et al., 2006) and nuclease (Nishimasu et al., 2012) activity. The N-terminus of PLD6 (amino acids 1–39) encodes a mitochondrial targeting sequence that anchors the enzyme into the mitochondrial outer membrane; this portion of the protein needs to be excluded from expression constructs to generate a soluble protein for biochemical assay (Choi et al., 2006).

2.2.1 Materials—pET-His6-GST-TEV (2-GT) bacterial expression vector (Addgene plasmid # 29707) or expression vector of choice.

2×YT media for growth of *Escherichia coli* BL21 (DE3) cells.

Buffer A: 50 m*M* Tris–HCl, pH 8, 300 m*M* NaCl, 3 m*M* β-ME, 20 m*M* imidazole, and protease inhibitors.

Buffer B: 50 m*M* Tris–HCl, pH 8, 300 m*M* NaCl, 3 m*M* β-ME, 500 m*M* imidazole, and protease inhibitors.

Buffer C: 50 m*M* Tris–HCl, pH 8, 300 m*M* NaCl, and 1 m*M* DTT.

2.2.2 Construct Preparation—A coding sequence for amino acids 36–252 of human PLD6 (Choi et al., 2006; available from multiple cDNA repositories or from the authors upon request) should be cloned in to the expression vector of choice. We chose to use pET-His6-GST-TEV (2-GT), cloning the PLD6 N-terminal-deleted fragment downstream of the His6-GST-TEV protease site leader sequence. The construct was transformed into *E. coli* BL21 (DE3) cells to enable inducible expression under control of the T7 promotor.

2.2.3 Protein Expression

1. A liter of 2×YT media containing ampicillin (100 μg/mL) is inoculated with bacteria harvested from a densely streaked 10-cm plate and incubated at 37°C at 220 rpm until an OD of 0.6 is attained (approximately 2–3 h).
2. The cells are then cooled to 20°C and protein expression induced using 0.5 m*M* IPTG (final concentration) and the incubation continued overnight at 20°C.
3. The following day, the cells are harvested by centrifugation at 4°C and the cell pellet homogenized twice at 15,000 psi in a cell homogenizer (Emulsiflex C3, Avestin) in 150 mL of Buffer A on ice.
4. The lysate is centrifuged at 18,000 ×*g* at 4°C for 1 h to pellet insoluble proteins and cell debris and the supernatant recovered.
5. The supernatant is loaded onto an FPLC column containing Ni-NTA resin (5 mL HisTrap, GE Healthcare) previously equilibrated with Buffer A. After washing the column with Buffer A, the protein is eluted with an imidazole gradient over 15 column volumes from 20 to 500 m*M* using Buffers A and B at 4°C. Elution fractions are analyzed by SDS-PAGE and the ones that contain GST-PLD6 pooled.
6. The protein is then further purified through a Glutathione agarose affinity chromatography column (5 mL, GSTrap, GE Healthcare) equilibrated in Buffer C, washed with 10 column volumes of Buffer C, and eluted with 10 mL of Buffer C plus 20 m*M* reduced L-glutathione, pH 7.2. Fractions are analyzed by SDS-PAGE, and the ones that contain PLD6 pooled. The concentration of GST-PLD6 is estimated by the Bradford assay (e.g., BioRad).
7. To cleave the GST tag, the protein is incubated with 0.1 mg of 6×His-tagged TEV protease per 2 mg of GST-PLD6 overnight at 4°C.

8. The cleaved tag and TEV protease are removed by adsorption to Ni-NTA. PLD6 remains in the flow through, for which the fractions are collected, analyzed by SDS-PAGE (Fig. 1), and quantified using Bradford reagent.

9. The protein is concentrated to 16 μ*M* and buffer exchanged to 20 m*M* Tris, pH 8.0, 150 m*M* NaCl, and 1 m*M* DTT through an Amicon filter before storing in aliquots at −80°C. A typical yield is 4 mg of PLD6 protein/liter of bacterial culture.

2.3 *In Vitro* PLD6 Assay

The nuclease activity of human PLD6, which is sequence independent, can be visualized using nonlabeled, radiolabeled, or fluorescent-labeled oligonucleotide substrates. The activity of the protein is inhibited by NaCl at concen-trations >30 m*M.* The protein activity is not affected by EDTA or Mg^{2+}, consistent with prior descriptions of *Drosophila* and mouse PLD6 (Ipsaro et al., 2012; Nishimasu et al., 2012). Mouse PLD6 activity was also reported to be enhanced in the presence of the divalent cations Ca^{2+}, Mn^{2+}, and Zn^{2+}. For human PLD2, we have observed that Mn^{2+}, Zn^{2+}, Cu^{2+} have little effect on protein activity, but a concentration of Ca^{2+} of at least 2 m*M* is essential to observe activity. Whether this *in vitro* observation is physiologically relevant (i.e., human PLD6 may be activated by Ca^{2+} release from the ER and mitochondria into the cytoplasm) is unknown at present.

2.4 ^{32}P Assay

2.4.1 Materials—A DNA oligonucleotide, e.g., 5′-TTATCCACTTCCAATGTTATTA TTAGGTTTGGCTTTCGCTGGAGC-3′.

Buffer D: 20 m*M* Hepes, 2 m*M* $CaCl_2$, pH 7.0.

2.4.2 Protocol

1. Label the 5′ end of the ssDNA with γ^{32}P-ATP using T4 polynucleotide kinase.
2. Purify the labeled oligonucleotide using a microspin G25column (GE).
3. Incubate 200 n*M* oligonucleotide with 800 n*M* purified PLD6 for 1.5 h in Buffer D at 37°C.
4. Resolve the products on a 15% polyacrylamide urea gel (Fig. 2).

3. Fluorescent-Based PLD Activity Assays

3.1 Polyacrylamide Gel-Based Assay

3.1.1 Materials

Substrate: A DNA oligonucleotide of length >40 nt, fluorescently labeled with an infrared (IR) dye with emission wavelength at 700 or 800 nM (IDT).

3.1.2 Protocol

1. Incubate 200 n*M* oligonucleotide with 800 n*M* purified PLD6 for 1.5 h in Buffer D at 37°C.

2. Resolve the products on a 15% polyacrylamide urea gel.
3. Image using an Odyssey CLx IR imager (Fig. 3).

Plate assay: A 96-well plate assay format for quantitative biochemical analyses of the protein activity can be used and is described as follows. This format is more sensitive than the gel-based activity assays.

3.1.3 Materials—A single-stranded DNA oligo labeled with biotin on the 5′ end and Alexa488 on the 3′ end (IDT).

Black well Neutravidin-coated plate (Life Technologies, catalog number 15217) pretreated with BSA to minimize nonspecific binding is used for the assay. Neutravidin is a deglycosylated form of Avidin with a near-neutral p*I* (6.3) that is responsible for its low nonspecific interactions. Round-bottom black 96-well plate (Eppendorf, catalog number 951040102).

0.02 mg/mL BSA (Pierce, catalog number 23209).

3.1.4 Protocol

1. Wash the wells of the Neutravidin-coated plate twice with 200 μL of Buffer D.
2. Add 100 μL of 30 n*M* Biotin-ssDNA-Alexa488 to the Neutravidin-coated plate and incubate for 1.5 h at RT with gentle shaking.
3. Measure the initial fluorescence of the Biotin-ssDNA-Alexa488 and the empty wells (background) on a plate reader (Molecular Devices, Model—FilterMax F5).
4. Remove unbound substrate and wash wells with 200 μL of Buffer D twice for 20 min of gentle shaking each time.
5. Add 100 μL of 120 n*M* PLD6 from a 16 μ*M* stock (133.3-fold dilution) to the well and incubate for 1.5 h at 37°C.
6. Pretreat the wells of the round-bottom black 96-well plate with 200 μL of 0.02 mg/mL BSA for 30 min at RT.
7. Transfer supernatant (containing the cleaved substrate) to the round-bottom black 96-well plate.
8. Measure fluorescence of both plates to quantify released substrate (Fig. 4A) and the amount remaining bound to the Neutravidin plate (Fig. 4B).

3.1.5 Notes

1. Since the fluorescence of the Biotin-ssDNA-Alexa488 substrate decays over time and is sensitive to temperature variations and Neutravidin coating, the substrate is also added to two additional wells in the Neu-travidin and round-bottom plate at the beginning of the assay, and the fluorescence quantified at the beginning and end of the assay. We found that Neutravidin substantially quenches the

Alexa488 fluorescence emission. Hence, the intensity values obtained for the cleaved fractions are larger than that originally bound to the wells. The intensity values obtained are background subtracted and corrected for the changes in the fluorescence that occur over time.

2. Optimal conditions for PLD6 activity: [NaCl] in the assay buffer should be kept to a minimum since [NaCl] >30 m*M* inhibits PLD6 activity. The protein is stored in 1 m*M* DTT/20 m*M* Tris (pH 8.0) containing 150 m*M* NaCl and is diluted using NaCl-free assay buffer for use, resulting in final assay conditions of 20 m*M* Hepes, pH 7, 1.1 m*M* NaCl, and 2 m*M* $CaCl_2$. Ca^{2+} is required for activity of human PLD6; pH 7.0 is the optimal pH for the assay.

3. This assay requires transfer of the cleaved substrate from the Neu-travidin plate to a second plate for fluorescence quantification. However, since Neutravidin quenches the fluorescence of Alexa488 on the bound, uncleaved substrate, measurement of the fluorescence of the total substrate (cleaved and uncleaved) in the Neutravidin plate after the enzymatic reaction yields an output almost as large as measuring just the cleaved substrate in a second plate after transfer (Fig. 4B).

4. Summary

We describe here a variety of approaches for assaying PLD6 nuclease activity *in vitro,* which suffice for experimental purposes and medium-throughput screens. PLD6 lipase activity (Choi et al., 2006), which can be readily assessed in cells using TLC to follow conversion of cardiolipin into PA (Choi et al., 2006) or fluorescent PA sensors to visualize PA formation on the mitochondrial surface (Baba et al., 2014; Choi et al., 2006; Huang et al., 2011; von Eyss et al., 2015; Zhang et al., 2016), has been much more difficult to reproducibly demonstrate *in vitro;* possibly the inclusion of the protein Mitoguardin (Zhang et al., 2016) in combination with an appropriate membrane surface will assist in development of an assay to measure this catalytic function.

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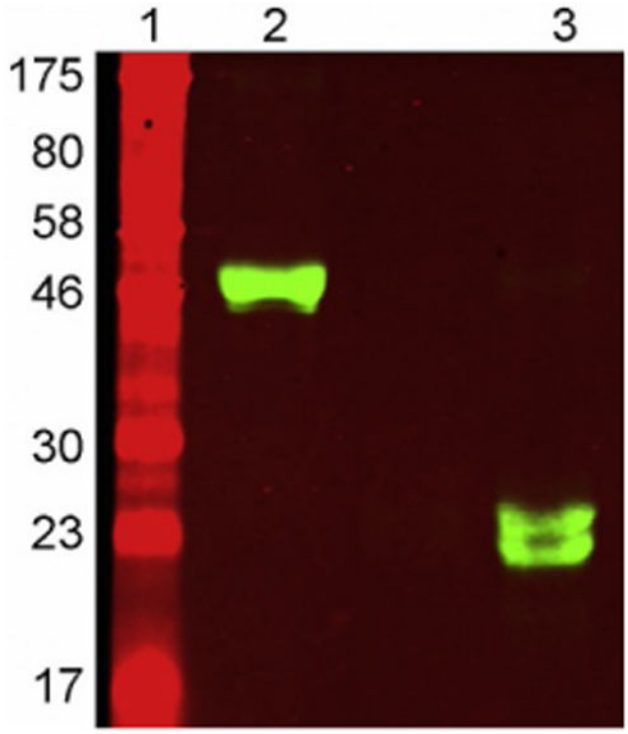

Fig. 1.

PLD6 expression and purification. Lane 1, molecular markers; lane 2, a sample of the His6-GST-TEV-PLD6 protein after enrichment on an Ni-NTA column. Lane 3, PLD6 protein after cleavage at the TEV site, purification, and concentration. It is not known why the purified PLD6 protein sometimes runs as a doublet.

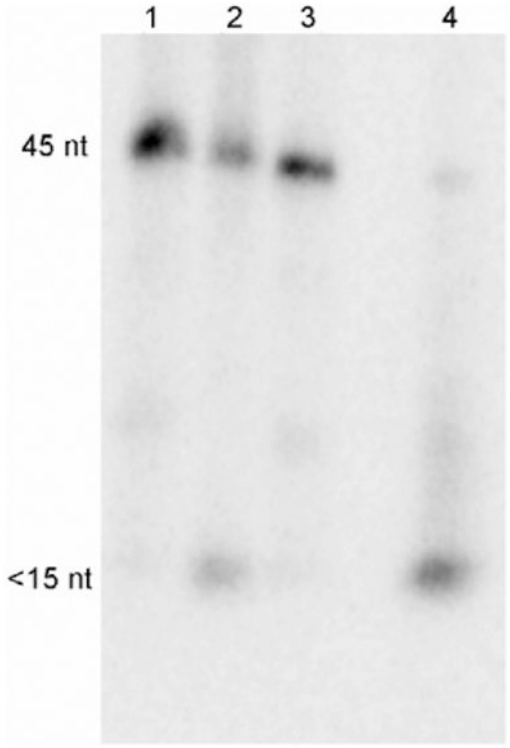

Fig. 2.

Cleavage of a radioactive oligonucleotide substrate by PLD6 and analysis on a polyacrylamide urea gel. Lane 1, kinased oligonucleotide; lane 2, incubation with C3PO, a positive control single-stranded endoculease; lane 3, incubation with PLD6-H156N, a catalytically inactive mutant allele prepared in parallel to the wild-type protein; lane 4, incubation with wild-type PLD6.

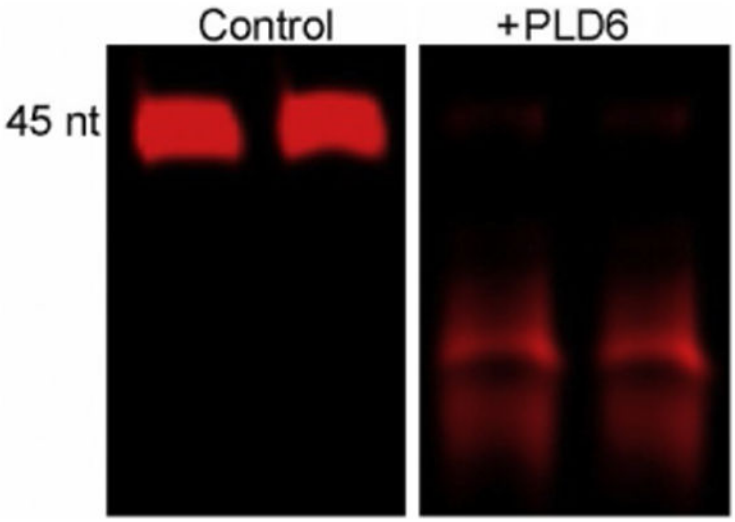

Fig. 3.

Cleavage of a fluorescent oligonucleotide substrate by PLD6 and analysis on a polyacrylamide urea gel. Assay performed in duplicate. The control incubation lacked PLD6 protein.

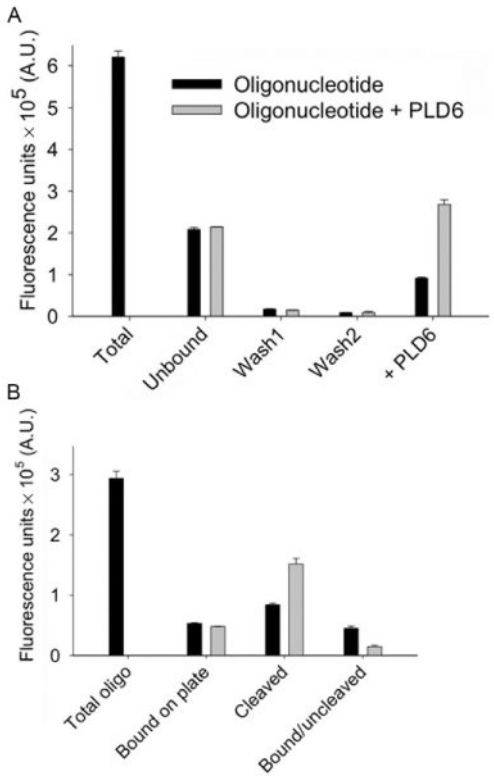

Fig. 4.

A plate assay for PLD6 activity. (A) The five columns show the total fluorescence added to the wells, the amount of fluorescence remaining unbound at 1 h at RT; the amount released in two washes; and the amount released in the assay, in the presence or absence of PLD6 protein. All of the measurements in (A) were made on soluble fluorescence transferred to a round-bottom black well plate. (B) Fluorescence measurements of bound and released fluorescence in the Neutravidin plate. The fluorescence values increase in the presence of PLD6 (lane 3, “cleaved”) since cleavage of the oligo substrate and release into the medium dequenches the fluorescence. Correspondingly, once the assay buffer is removed (lane 4), there is a weaker signal in the wells that contained PLD6, since less substrate remains on the walls of the wells. Experiment preformed in triplicate.