学位論文

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香川大学大学院医学系研究科

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Smriti Sultana Binte Mustafiz

The role of intracellular anionic phospholipids in the production of *N*-acyl-phosphatidylethanolamines by cytosolic phospholipase $A_2\varepsilon$.

Smriti Sultana Binte Mustafiz[1], Toru Uyama[1], Zahir Hussain[1], Katsuhisa Kawai[2], Kazuhito Tsuboi[3], Nobukazu Araki[2], and Natsuo Ueda[1,*]

[1]Department of Biochemistry, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan

[2]Department of Histology and Cell Biology, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan

[3]Department of Pharmacology, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan

[*]Corresponding author:

Dr. Natsuo Ueda, Professor

Department of Biochemistry, Kagawa University School of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan

Tel.: +81-87-891-2102, Fax: +81-87-891-2105

E-mail address: nueda@med.kagawa-u.ac.jp (N. Ueda)

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Abbreviation: AEA, arachidonoylethanolamide; $cPLA_2$, cytosolic phospholipase A_2; DOX, doxycycline; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HEK293, human embryonic kidney 293; LC-MS/MS, liquid chromatography-tandem mass spectrometry; lysoNAPE, *N*-acyl-lysophosphatidylethanolamine; NAE, *N*-acylethanolamine; NAPE, *N*-acyl-phosphatidylethanolamine; OEA, oleoylethanolamide; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoylethanolamide; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP_2, PI 4,5-bisphosphate; PPAR, peroxisome proliferator-activated receptor; PS, phosphatidylserine; PSS, PS synthase; siRNA, small interfering RNA; TLC, thin-layer chromatography; TRPV1, transient receptor potential vanilloid 1.

Summary

N-Acyl-phosphatidylethanolamines (NAPEs) represent a class of glycerophospholipids and serve as the precursors of bioactive *N*-acylethanolamines, including arachidonoylethanolamide (anandamide), palmitoylethanolamide and oleoylethanolamide. NAPEs are produced in mammals by *N*-acyltransferases, the enzymes which transfer an acyl chain of glycerophospholipids to the amino group of phosphatidylethanolamine. Recently, the ε isoform of cytosolic phospholipase A_2 (cPLA$_2$ε, also called PLA2G4E) was identified as Ca^{2+}-dependent *N*-acyltransferase. We showed that the activity is remarkably stimulated by phosphatidylserine (PS) *in vitro*. In the present study, we investigated whether or not endogenous PS regulates the function of cPLA$_2$ε in living cells. When PS synthesis was suppressed by the knockdown of PS synthases in cPLA$_2$ε-expressing cells, the cPLA$_2$ε level and its *N*-acyltransferase activity were significantly reduced. Mutagenesis studies revealed that all of C2, lipase and polybasic domains of cPLA$_2$ε were required for its proper localization as well as the enzyme activity. Liposome-based assays showed that several anionic glycerophospholipids, including PS, phosphatidic acid and phosphatidylinositol 4,5-bisphosphate, enhance the Ca^{2+}-dependent binding of purified cPLA$_2$ε to liposome membrane and stimulate its *N*-acyltransferase activity. Altogether, these results suggested that endogenous PS and other anionic phospholipids affect the localization and enzyme activity of cPLA$_2$ε.

N-Acylethanolamines (NAEs) represent a class of lipid mediators and refer to ethanolamides of various fatty acids with different chain length and saturation. NAEs exert various biological functions through the interaction with their corresponding receptors (*1–3*). For example, arachidonoylethanolamide (AEA), also known as anandamide, has attracted attention as an endogenous agonist for cannabinoid receptor CB1 and transient receptor potential vanilloid 1 (TRPV1) (*2,4*). AEA shows a variety of biological activities including analgesia, neuroprotection, hypotension, reduction of anxiety and depression, and stimulation of appetite (*4*). Palmitoylethanolamide (PEA) has an agonist activity toward peroxisome proliferator-activated receptor (PPAR)-α and mediates anti-inflammatory, analgesic, neuroprotective and retinoprotective actions (*5,6*). Oleoylethanolamide (OEA) suppresses appetite through PPAR-α, GPR119 and TRPV1 (*7,8*).

NAEs are enzymatically produced from *N*-acyl-phosphatidylethanolamine (NAPE), a class of rare membrane glycerophospholipids having three acyl chains per molecule (Fig. 1). In mammals, NAPEs are formed by *N*-acyltransferases which transfer an acyl chain of glycerophospholipids to the amino group of phosphatidylethanolamine (PE) (*1,9,10*). Mammals express two types of *N*-acyltransferases distinguished by Ca^{2+} dependency. We have identified phospholipase A/acyltransferase (PLAAT)-1–5 as Ca^{2+}-independent *N*-acyltransferases (*1,11*). On the other hand, using the activity-based protein profiling of mouse brain, Ogura et al. identified the ε isoform of cytosolic phospholipase A_2 (cPLA$_2$ε, also called PLA2G4E) as Ca^{2+}-dependent *N*-acyltransferase (*12*). cPLA$_2$ε has been previously found as a member of the cPLA$_2$ family and shown to exhibit weak phospholipase A_2 and lysophospholipase activities in a Ca^{2+}-dependent manner (*13*).

Recently, we characterized mouse and human cPLA$_2$εs using their purified recombinant proteins and revealed that their Ca^{2+}-dependent *N*-acyltransferase activities *in vitro* are potently stimulated by an anionic glycerophospholipid, phosphatidylserine (PS) (*14*). For example, in the presence of 1 mM $CaCl_2$, 200 μM PS increased the *N*-acyltransferase activity of purified mouse cPLA$_2$ε 25-fold. Moreover, 200 μM PS significantly increased the affinity of mouse cPLA$_2$ε with Ca^{2+}, lowering the EC_{50} for Ca^{2+} around 8-fold. In agreement with the previous reports (*13,15*), we showed that recombinant cPLA$_2$ε expressed in mouse macrophage RAW264.7 cells is mainly present in lysosome and early endosome and on plasma membrane (*14*). This localization was similar to that of

PS, suggesting a role of PS as an endogenous stimulator of cPLA$_2\varepsilon$. However, it remains unclear whether intracellular PS is actually involved in the generation of NAPE by cPLA$_2\varepsilon$. In addition, it has not been determined whether or not other anionic glycerophospholipids, including phosphoinositides, can stimulate cPLA$_2\varepsilon$ activity.

Here, we first examined the role of intracellular PS in cPLA$_2\varepsilon$ function by suppressing the expression of PS synthases (PSSs) in cells expressing human cPLA$_2\varepsilon$. Next, we constructed several mutants of cPLA$_2\varepsilon$ and evaluated their enzyme activity and localization. Finally, we tested the effects of several anionic glycerophospholipids on the enzyme activity and membrane association of cPLA$_2\varepsilon$.

Materials and Methods

Materials

1,2-[1'-^{14}C]Dipalmitoyl-*sn*-glycero-3-phosphocholine was purchased from PerkinElmer Life Science (Boston, MA, USA). [^{14}C(U)]L-Serine was from Moravek Biochemicals (Brea, CA, USA). 1,2-Dioleoyl-*sn*-glycero-3-phospho-L-serine, 1,2-dioleoyl-*sn*-glycero-3-phosphate, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*myo*-inositol) and 1,2-dioleoyl-*sn*-glycero-(1'-*myo*-inositol-4',5'-bisphosphate) were from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, anti-FLAG M2-conjugated agarose affinity gel and FLAG peptide were from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG antibodies were from GE Healthcare (Piscataway, NJ, USA). Rabbit anti-FLAG (DYKDDDDK) monoclonal antibody was from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-green fluorescent protein (GFP) monoclonal antibody was from MBL International (Nagoya, Japan). Dulbecco's modified Eagle's medium, Lipofectamine 2000, Lipofectamine RNAiMAX, small interfering RNAs (siRNAs) directed against human PSS1 (siPSS1-a and siPSS1-b) and PSS2 (siPSS2-c and siPSS2-d), a control siRNA (siCont), fetal bovine serum, hygromycin B, blasticidin, Pierce® Western Blotting Substrate Plus, pEF6/Myc-His and pcDNA5/TO were from Invitrogen/Thermo Fisher Scientific (Carlsbad, CA, USA). pEGFP-C1 and doxycycline (DOX) were from Clontech (Mountain View, CA, USA). Nonidet P-40 was from Nacalai Tesque (Kyoto, Japan). *Ex*

Taq DNA polymerase and a PrimeScript RT reagent kit were from TaKaRa Bio Inc. (Ohtsu, Japan). The RNeasy mini kit was from QIAGEN (Hilden, Germany). KOD-Plus-Neo DNA polymerase was from TOYOBO (Osaka, Japan). Protein assay dye reagent concentrate was from Bio-Rad (Hercules, CA, USA). Dithiothreitol (DTT), 3(2)-*t*-butyl-4-hydroxyanisole, Tween 20 and silver staining MS kit were from Wako Pure Chemical (Osaka, Japan). Immobilon-P and precoated silica gel 60 F_{254} aluminum sheets (20 x 20 cm, 0.2 mm thick) for thin-layer chromatography (TLC) were from Merck (Darmstadt, Germany). Human embryonic kidney 293 (HEK293) cells were from Health Science Research Resources Bank (Osaka, Japan). pmCherry-LactC2 was generated by gene synthesis (GeneScript) according to the previous report (*16*).

cDNA cloning and construction of expression vectors

All the primers used for this purpose are shown in Table I. PCR was carried out with KOD-Plus-Neo DNA polymerase. The cDNAs encoding mutants (S412A, ΔC2 and ΔLip) of N-terminally FLAG-tagged cPLA$_2$ε (FL-cPLA$_2$ε) were prepared by megaprimer PCR, consisting of two sets of PCR reactions, using pEF6/Myc-His vector harboring FLAG-tagged isoform B of human cPLA$_2$ε (*14*) as a template. Primers used for two sets of PCR reactions are as follows: S412A, F1/R2 and F2/R1; ΔC2, F1/R3 and F3/R1; ΔLip, F1/R4 and F4/R1. The obtained two DNA fragments of each mutant were subjected to megaprimer PCR using primers F1 and R1. The cDNA encoding the mutant ΔPB was also constructed using the expression vector harboring FL-cPLA$_2$ε. Primers F1 and R5 were used as the forward and reverse primers, respectively. The obtained DNA fragments were finally subcloned into *Spe*I and *Not*I restriction sites of pEF6/Myc-His.

In order to construct cPLA$_2$ε fused to the C-terminus of EGFP (EGFP-FL-cPLA$_2$ε-WT) and their mutants (EGFP-FL-cPLA$_2$ε-S412A, -ΔC2, -ΔLip and -ΔPB), PCR was performed using pEF6/Myc-His vector harboring the corresponding cDNAs as a template and the obtained DNA fragment was subcloned into *Bgl*II and *Sal*I restriction sites of pEGFP-C1. Primers F6 and R6 were used for the construction of EGFP-FL-cPLA$_2$ε-WT, -S412A, -ΔC2 and -ΔLip, and F7 and R7 were for EGFP-FL-cPLA$_2$ε-ΔPB.

To construct pcDNA5/TO vector harboring FL-cPLA$_2\varepsilon$, pEF6/Myc-His vector harboring FL-cPLA$_2\varepsilon$ was digested with *Acc*65I and *Not*I, and the resultant DNA fragment was subcloned into the corresponding sites of pcDNA5/TO. To construct pcDNA5/TO vector harboring EGFP-FL-cPLA$_2\varepsilon$, pEGFP-C1 vector harboring EGFP-FL-cPLA$_2\varepsilon$ was digested with *Aor*51HI and *Sal*I, and the resultant DNA fragment was subcloned into *Eco*RV and *Xho*I sites of pcDNA5/TO. All constructs were sequenced in both directions using an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Expression and purification of recombinant proteins

HEK293 cells were grown at 37°C to 90% confluency in 100 mm plastic dishes containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a humidified 5% CO_2 and 95% air incubator. The pEF6/Myc-His vectors harboring FL-cPLA$_2\varepsilon$ and its mutants or insert-free pEF6/Myc-His vector were introduced into HEK293 cells using Lipofectamine 2000. Forty-eight hours after transfection, cells were harvested and sonicated twice each for 5 s in 20 mM Tris-HCl (pH 7.4). To establish Tet-on cell lines expressing FL-cPLA$_2\varepsilon$ (FL-cPLA$_2\varepsilon$/Tet-on) or EGFP-FL-cPLA$_2\varepsilon$ (EGFP-FL-cPLA$_2\varepsilon$/Tet-on), a HEK293 cell line expressing tetracycline repressor (*17*) was transfected with pcDNA5/TO vector harboring FL-cPLA$_2\varepsilon$ or EGFP-FL-cPLA$_2\varepsilon$ by the use of Lipofectamine 2000. Cells were selected in the medium containing 200 μg/ml hygromycin B and 6 μg/ml blasticidin. Clonal cell lines were isolated by colony lifting and maintained in the medium containing hygromycin B and blasticidin. One of the clones expressing cPLA$_2\varepsilon$ stably was used throughout this study.

For the purification of cPLA$_2\varepsilon$, FL-cPLA$_2\varepsilon$/Tet-on cells were grown in three 15 cm dishes and maintained in the medium containing 1 μg/mL DOX. Cells were harvested and sonicated twice each for 5 s in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl. A soluble fraction was prepared by ultracentrifugation of the homogenate containing 0.05% (w/v) Nonidet P-40 at 105,000 *g* for 30 min at 4°C. The purification was carried out with the aid of anti-FLAG M2-conjugated agarose affinity gel as described previously (*14*). The protein concentration was determined by the method of Bradford with bovine serum albumin as a standard (*18*).

Suppression of PSSs

The siRNAs of human PSS1 and PSS2, or a control siRNA (siCont) were introduced into cells at 40 nM as the final concentration, using Lipofectamine RNAiMAX. Forty-eight hours after transfection, the cells were used for experiments.

Total RNAs were isolated from cells using an RNeasy mini kit. cDNAs were prepared from the total RNAs using a PrimeScript RT reagent kit and subjected to PCR amplification by *Ex Taq* DNA polymerase. Oligonucleotides shown in Table II were used as primers. The PCR conditions used were as follows: denaturation at 98℃ for 10 s, annealing at 55°C for 30 s and extension at 68°C for 60 s with 29 cycles for cPLA$_2\varepsilon$, PSS1 and PSS2 and 26 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

FL-cPLA$_2\varepsilon$/Tet-on cells cultured in the presence of 1 μg/mL of DOX were plated in a 6-well plate and transiently transfected with 40 nM (as the final concentration) of siRNAs of PSS1 and PSS2 or siCont, using Lipofectamine RNAiMAX. After 48 h, the cells were labeled with [^{14}C]serine (0.2 μCi/well) for 3 h. Total lipids were then extracted from the cells by the method of Bligh and Dyer (*19*), spotted on a silica gel thin-layer plate (20 cm height), and developed at 4°C for 90 min in a mixture of chloroform/methanol/28% ammonium hydroxide (65:35:8, v/v). The resultant radioactive band corresponding to authentic PS on the TLC plate was quantified using an image reader FLA-7000 (Fujifilm, Tokyo, Japan).

Western blotting

Samples (30-40 μg of protein) were subjected to 8% SDS-PAGE and electrotransferred to a hydrophobic polyvinylidene difluoride membrane (Immobilon-P) at 100 volt for 75 min. The membrane was blocked with PBS containing 5% dried skimmed milk and 0.1% Tween 20 (buffer A) and then incubated with rabbit anti-FLAG monoclonal antibody (Fig. 2B) or mouse anti-GFP monoclonal antibody (Fig. 4B) (1:2000 dilution) in buffer A at room temperature for 1 h, followed by incubation with horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG (1:4000 dilution) in buffer A for 1 h at room temperature. The membrane was finally treated with Pierce® Western Blotting Substrate Plus, and the labeled proteins were visualized with the aid of a LAS1000plus lumino-imaging analyzer (FUJIX Ltd.,

Japan).

Enzyme assay

The cell homogenates (35 μg of protein) or purified cPLA$_2$ε protein (0.1 μg of protein) were incubated with 200 μM 1,2-[^{14}C]dipalmitoyl-phosphatidylcholine (PC) (45,000 cpm) and 100 μM dioleoyl-PE in 100 μl of 100 mM Tris-HCl buffer (pH 8.2), 200 μM of dioleoyl-PS, 5 mM (for homogenates) or 2.5 mM (for purified cPLA$_2$ε) of $CaCl_2$, 2 mM DTT and 0.1% Nonidet P-40 at 37°C for 30 min. In some assays dioleoyl-PS was replaced with other dioleoyl-glycerophospholipids. Reactions were terminated by the addition of 320 μl of a mixture of chloroform and methanol (2:1, v/v) containing 5 mM 3(2)-*t*-butyl-4-hydroxyanisole. After centrifugation, 100 μl of the organic phase was spotted on a silica gel thin-layer plate (10 cm height) with a calibrated capillary glass pipette connected to a rubber aspirator tube (Drummond Scientific Co., Broomall, PA, USA) and was dried under the airflow of a hair dryer. The plate was next developed at 4°C for 25 min in a mixture of chloroform/methanol/28% ammonium hydroxide (80:20:2, v/v). The distribution of radioactivity on the plate was visualized, and the radiolabeled products were quantified using an image reader FLA-7000. *N*-Acyltransferase activities were calculated by the amounts of produced *N*-[^{14}C]palmitoyl-PE.

For enzyme assay using liposomes, liposomes were prepared essentially in the same way as described in the section of "Liposome co-sedimentation assay" with the addition of 1,2-[^{14}C]dipalmitoyl-PC (22,500 cpm) to the phospholipid mixtures. The purified FL-cPLA$_2$ε (0.1 μg of protein) was incubated with 57 nmol of liposomes in 50 μl of 22 mM of HEPES buffer (pH 7.5), 2.5 mM $CaCl_2$ and 2 mM DTT at 37°C for 30 min. *N*-Acyltransferase activities were then determined as described above.

Microscopy

EGFP-FL-cPLA$_2$ε/Tet-on cells cultured in the presence of 1 μg/mL of DOX were plated onto 35 mm glass bottom dishes and transiently transfected with 40 nM (as the final concentration) of siRNAs of PSS1 (siPSS1-a) and/or PSS2 (siPSS2-c) or siCont, using Lipofectamine RNAiMAX. After 24 h, these cells were transiently transfected with the vector harboring mCherry-LactC2, a biosensor originally

derived from the C2 domain of lactadherin (a milk glycoprotein) (*16*), using Lipofectamine 2000. Twenty-four hours after transfection, the cells were placed in Ringer's buffer (155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM glucose, 0.5 mg/mL bovine serum albumin and 10 mM HEPES at pH 7.2) in a temperature-controlled chamber at 37°C and the fluorescent images of live cells were observed with an LSM 700 confocal laser microscope (Carl Zeiss, Germany).

HEK293 cells were transiently transfected with the expression vector harboring EGFP-FL-cPLA$_2\varepsilon$ or its mutants by the use of Lipofectamine 2000. Twenty-four hours after transfection, the fluorescent images of live cells were observed as described above.

Liposome co-sedimentation assay

Liposomes were prepared according to the protocol as reported earlier (*20*) with some modifications. Liposomes contained 75% (mol/mol) dioleoyl-PE, 20% dipalmitoyl-PC and 5% of one of different dioleoyl-glycerophospholipids. These phospholipid mixtures (65 nmol) were completely dried under nitrogen stream and hydrated in 50 μl of 25 mM HEPES (pH 7.5) containing 100 mM NaCl for 1 h at 37°C followed by vortexing for 1 min. The purified FL-cPLA$_2\varepsilon$ (0.2 μg of protein) was incubated with 14 nmol of the aforementioned liposomes in 25 μl of 22 mM HEPES (pH 7.5), 2 mM DTT and either 2.5 mM EDTA or 2.5 mM $CaCl_2$ at 37°C for 10 min. The mixture was then ultracentrifuged at 105,000 *g* for 30 min at 4°C, and the resultant supernatant and pellet were subjected to SDS-PAGE. The gels were stained with silver nitrate. Intensities of the individual cPLA$_2\varepsilon$ bands were quantified with an NIH Image J software (https://imagej.nih.gov/ij/). The band intensity in the pellet fraction of the samples without liposomes (b) was deducted from that of the samples containing liposomes (PPTi) to calculate the net amount of bound protein (PPTi–b). The percentage of bound protein was obtained by the following equation: %bound = 100 x (PPTi–b) / [SUPi + (PPTi–b)], where SUPi means the band intensity of supernatant fraction of the samples containing liposomes (*20*).

Results

In mammals two enzymes, PSS1 and PSS2, are responsible for the biosynthesis of PS by the serine-exchange reaction (*20,21*). PSS1 replaces choline of PC with serine, while PSS2 exchanges

ethanolamine of PE with serine, generating PS in both cases. To see a possible role of PS in the regulation of cPLA$_2\varepsilon$ activity at cellular level, we established a HEK293 cell line (FL-cPLA$_2\varepsilon$/Tet-on) which expresses FLAG-tagged isoform B of human cPLA$_2\varepsilon$ (FL-cPLA$_2\varepsilon$) in a DOX-dependent manner. When the cells were cultured in the presence of DOX and treated with siRNAs against PSS1 or PSS2, the expressions of the target mRNAs were largely suppressed as expected (Fig. 2A). Suppression of PSS1 did not increase the expression of PSS2 in a compensatory manner, and vice versa. To confirm the decrease in PS formation, we metabolically labeled PSS1 and PSS2–knockdown cells with [^{14}C]serine and found that total amount of radioactive PS was decreased by 24 ± 9.6% (mean values ± SD, n = 3) in the PSS-knockdown cells in comparison with siCont cells (data not shown). Interestingly, Western blotting revealed that the suppression of PSS1 and/or PSS2 caused a decrease in cPLA$_2\varepsilon$ levels as compared with control cells treated with siCont (Fig. 2B). In consistence, the *N*-acyltransferase activity of cPLA$_2\varepsilon$ in the cell homogenates concomitantly decreased with these suppressed cPLA$_2\varepsilon$ levels (Fig. 2C). The most profound effect was seen in the cells simultaneously treated with siRNAs of PSS1 and PSS2. These results suggested that the contents of cPLA$_2\varepsilon$ correlate to intracellular PS levels.

Next, we were interested whether intracellular PS regulates the localization of cPLA$_2\varepsilon$. To this end, we established a Tet-on HEK293 cell (EGFP-FL-cPLA$_2\varepsilon$/Tet-on) which expresses EGFP-fused FL-cPLA$_2\varepsilon$ in a DOX-dependent manner. In the DOX-treated cells, PS visualized by a fluorescent PS probe, mCherry-LactC2, was enriched on plasma membrane and in intracellular vesicles, and EGFP-FL-cPLA$_2\varepsilon$ was co-localized with these PS-rich membrane domains (Fig. 3). These results were consistent with our previous observations that majority of recombinant mouse cPLA$_2\varepsilon$, expressed in a macrophage cell line RAW264.7, was present in PS-rich membrane domains such as plasma membrane, lysosome and early endosome (*14*). When the cells were treated with siRNA against PSS1 or PSS2 or both, fluorescent signals of mCherry-LactC2 decreased in all cells. Especially, signals of intracellular vesicles became weak, while those at plasma membrane seemed to be unchanged or slightly decreased. Similar tendencies were observed with EGFP-FL-cPLA$_2\varepsilon$ signals. The cells subjected to co-suppression of PSS1 and PSS2 expression showed the most pronounced effects. These results suggested that PS helps cPLA$_2\varepsilon$ to anchor to certain sub-cellular structures and thus, functions as a stimulator for *N*-acyltransferase activity of cPLA$_2\varepsilon$.

Isoform B of human cPLA$_2\varepsilon$ is composed of 868 amino acids and has several domain structures such as a C2 domain, which is known to be required for the binding to Ca^{2+} in several proteins, a lipase domain containing a catalytic dyad, Ser-412 and Asp-700, and a polybasic domain rich in basic amino acids near C-terminus (*22*) (Fig. 4A). To examine the functions of these domains, we constructed mutants fused to EGFP; S412A, a catalytically inactive point mutant in which alanine was substituted for Ser-412 (*12,15*); ΔC2, ΔLip and ΔPB, deletion mutants lacking C2, lipase or polybasic domain, respectively. In reference to amino acid sequences, the molecular weights of EGFP-fused cPLA$_2\varepsilon$ and its mutants, Ser-412, ΔC2, ΔLip and ΔPB, were estimated to be 128, 128, 116, 100 and 126 kDa, respectively. When these proteins were transiently expressed in HEK293 cells and the cell homogenates were subjected to Western blotting with anti-GFP antibody, the immunopositive bands were detected at the positions of their estimated molecular weights (Fig. 4B). However, none of the mutants showed *N*-acyltransferase activity (Figs. 4C and D), suggesting that all the domains are required to keep the enzyme activity or correct steric structure. Next, intracellular localization of these EGFP-fused mutants was observed by confocal microscopy. In accordance with the image in Fig. 3, transient expression of wild-type cPLA$_2\varepsilon$ was localized on the plasma membrane and in intracellular vesicles (Fig. 5). Interestingly, none of the mutants (S412A, ΔC2, ΔLip or ΔPB) were localized at membrane structures but they were mainly found in cytosol (Fig. 5). The signals were neither seen in nuclei nor other organelles. This localization was also different from that of EGFP itself, which was ubiquitously distributed throughout the cells including nuclei (not shown). These results suggested that C2, lipase and polybasic domains were all required for the original intracellular localization of cPLA$_2\varepsilon$. In addition, the result with S412A mutant suggested that Ser-412, the catalytic nucleophile of cPLA$_2\varepsilon$ (*12*), is also involved in the correct localization.

We examined stimulatory effects of various phospholipids on the activity of cPLA$_2\varepsilon$. When various dioleoyl-phospholipids at 200 μM were tested under the standard assay conditions (Fig. 6A), not only PS but also phosphatidylinositol (PI) showed potent stimulatory effects. Phosphatidic acid (PA) and phosphatidylglycerol (PG) also showed moderate effects. Phosphatidylinositol 4,5-bisphosphate (PIP_2) was rather inhibitory as compared with PC (a negative control).

We also tested various phospholipids contained in liposomes composed of dioleoyl-

PE/dipalmitoyl-PC/a tested dioleoyl-glycerophospholipid (75:20:5, mol/mol) (Fig. 6B). Dioleoyl-PE and dipalmitoyl-PC (including [^{14}C]dipalmitoyl-PC) in the liposomes were expected to serve as an acyl acceptor and an acyl donor, respectively. Interestingly, not only PS but also PA and PIP_2 significantly stimulated the activity. A slight increase was observed with PI. These results showed that PS is not the sole stimulator but some other acidic glycerophospholipids also stimulate the activity. Notably, the stimulatory effect of PIP_2 was observed only when it exists in liposomes.

To determine whether or not cPLA$_2\varepsilon$ preferably binds to membrane containing specific phospholipid, we employed the liposome co-sedimentation assay (Fig. 7). The purified recombinant cPLA$_2\varepsilon$ was incubated with liposomes used in the assay of Fig. 6B. The results showed that even in the absence of Ca^{2+}, 29-50% of cPLA$_2\varepsilon$ bound to all the tested liposomes including a control composed of only PE and PC, suggesting a considerable Ca^{2+} -independent affinity of cPLA$_2\varepsilon$ protein for PE/PC (Fig. 7C). Ca^{2+} was found to significantly increase the binding rate of cPLA$_2\varepsilon$ to all the tested liposomes up to 65-84%. Furthermore, PA and PIP_2 as well as PS significantly increased the binding rate in the presence of Ca^{2+} while PI and PG did not. It should be noted that PS, PA and PIP_2 contained in liposomes also stimulated the *N*-acyltransferase activity as described above.

Discussion

PS is an acidic phospholipid accounting for 3–15% of the total phospholipids in eukaryotic cells and plays key roles in various physiological processes such as the coagulation cascade, the clearance of apoptotic cells, and the targeting of proteins to membrane through their PS-binding domains (*23*,*24*). PS is enriched in the inner leaflet of the membrane bilayer and is distributed unevenly in cells, being rich in specific organelles such as early endosome and the plasma membrane and less abundant in mitochondria (*23*). In the present study, with the aid of a PS-probe, mCherry-LactC2 (*16*), we revealed that in HEK293 cells PS is mainly present on the plasma membrane and in intracellular vesicles.

We recently demonstrated that PS stimulates Ca^{2+}-dependent *N*-acyltransferase activity of cPLA$_2\varepsilon$ *in vitro* (*14*). In agreement with an earlier report that cPLA$_2\varepsilon$ was detected in intracellular dot-like structures including lysosomes and endosomes (*13*), we showed that recombinant cPLA$_2\varepsilon$, expressed in mouse macrophage RAW264.7 cells, exists in lysosomes, endosomes and plasma

membrane and co-localizes with PS (*14*). These results suggested a certain role of endogenous PS in the function and localization of cPLA$_2\varepsilon$.

In this study, we first investigated the effect of knockdown of PS synthases, PSS1 and PSS2, which resulted in the decreased formation of PS. The knockdown of PSSs decreased both cPLA$_2\varepsilon$ levels and *N*-acyltransferase activity. Thus, it was suggested that PS affects not only the intracellular localization of cPLA$_2\varepsilon$ but also its content. Considering that recombinant cPLA$_2\varepsilon$ was subjected to the analysis, the decrease in its levels may be caused by destabilizing the produced cPLA$_2\varepsilon$ protein.

We were interested in the domain structure of cPLA$_2\varepsilon$. All of the deletion mutants of C2, lipase or polybasic domain (ΔC2, ΔLip and ΔPB) were enzymatically inactive and were distributed throughout cytoplasm. These results suggested that all these domains are required for the catalytic activity and proper localization of cPLA$_2\varepsilon$, although we cannot exclude the possibility that the deletion of these domains causes gross structural abnormality. Our results with ΔPB mutant were in agreement with the previous report that cPLA$_2\varepsilon$ was targeted to the clathrin-independent endocytic route and that the deletion of the polybasic domain resulted in its translocation to cytosol (*15*). S412A mutant also located in cytoplasm. As an example that the mutation of a single amino acid affects intracellular localization of a protein, it was earlier reported that a point mutant of phospholipase Cδ3, in which His-393 forming the catalytic center was changed to alanine, was localized to cytosol while its wild-type counterpart was localized to plasma membrane and nucleus (*25*).

Previously, we screened several acidic phospholipids (PS, PA, PI, PIP$_2$ and PG) for the stimulatory effect on *N*-acyltransferase activity of cPLA$_2\varepsilon$ at the concentration of 20 μM and found that only PS can stimulate the activity (*14*). However, at that time we did not test higher concentrations of acidic phospholipids other than PS. Therefore, in the present study we examined relatively high concentration (200 μM) of phospholipids. We also employed the liposome-based enzyme assay (*20*). Based on the results of Fig. 6A (liposome-free standard assay conditions) and B (liposome-containing assay conditions), PS, PA and PI showed stimulatory effects under both assay conditions. However, PIP$_2$ was stimulatory only under the liposome-containing conditions, while PG was significantly stimulatory only under the liposome-free conditions. Although the use of liposomes mimicking biomembrane and the removal of non-ionic detergent Nonidet P-40 from the reaction mix were likely

to affect the stimulatory effects of anionic phospholipids, it remains unclear why the effect differs by each phospholipid. Interestingly, in the liposome co-sedimentation assay, the binding of cPLA$_2\varepsilon$ to liposomes significantly depended on Ca^{2+}. Moreover, some acidic phospholipids (PS, PA and PIP_2) caused additional enhancement of the liposome binding. Thus, these acidic phospholipids may stimulate the enzyme activity by enhancing Ca^{2+}-dependent membrane association of cPLA$_2\varepsilon$.

Although it remains unclear which domain of cPLA$_2\varepsilon$ is responsible for the binding to these anionic phospholipids, the C2 domains of protein kinase C family members and δ isoform of phospholipase C were reported to be capable of binding to PA and PIP_2 in addition to PS (*26,27*). Moreover, Capestrano et al. reported that cPLA$_2\varepsilon$ interacts with PIP_2 through its C-terminal polybasic strech (KKRxR) and that the deletion of this strech, corresponding to ΔPB in the present study, redistributed cPLA$_2\varepsilon$ from membrane to cytoplasm (*15*). These results suggest that C2 and polybasic domains of cPLA$_2\varepsilon$ play important roles in its membrane association through binding to acidic phospholipids. Since PS is one of the most abundant acidic phospholipids in cells, it is likely that PS principally affects intracellular localization and activity of cPLA$_2\varepsilon$ among various phospholipids. However, PA and PIP_2 are also implicated in signaling pathway (*26,27*) and their local accumulation may lead to the activation of cPLA$_2\varepsilon$ together with an increase in the intracellular Ca^{2+} concentration.

In conclusion, our results suggest that the intracellular activity and localization of cPLA$_2\varepsilon$ are regulated by acidic phospholipids such as PS. However, further studies are needed to elucidate how acidic phospholipids interact with cPLA$_2\varepsilon$ and stimulate its enzyme activity.

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Conflict of Interest

None declared

Acknowledgments

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1 Table I. Primers used for the construction of expression vectors.

Name of construct (Accession number)	Primer[a]	Sequence (Restriction site and tag sequence contained)
FL-cPLA$_2$ε (NM_001206670)	F1	5'-cgcactagtggaaaatggattacaaggatgacgacgataagagtctccaggcctcggaaggctgtcctggc-3' (*Spe*I site followed by an in-frame FLAG sequence)
	R1	5'-cgcgcggccgcctaggagggacactggcccttcaggcgcttcttcttctc-3' (*Not*I site)
FL-cPLA$_2$ε-S412S	F2	5'-tacatcaccggtctagcaggggccacctgg-3'
	R2	5'-ccaggtggcccctgctagaccggtgatgta-3'
FL-cPLA$_2$ε-ΔC2	F3	5'-ctgtctccatgccacctcaacccgcagggc-3'
	R3	5'-gccctgcgggttgaggtggcatggagacag-3'
FL-cPLA$_2$ε-ΔLip	F4	5'-gacgaggtgccgctggagccgatcctgcct-3'
	R4	5'-ccaggtggcccctgctagaccggtgatgta-3'
FL-cPLA$_2$ε-ΔPB	R5	5'-cgcgcggccgcctactccactgcgagccgcagagcctg-3' (*Not*I site)
EGFP-FL-cPLA$_2$ε-WT, -S412S, -ΔC2 and -ΔLip	F6	5'-cgcagatctatggattacaaggatgacgacgataag-3' (*Bgl*II site)
	R6	5'-cgcgtcgacctaggagggacactggcccttcaggcg-3' (*Sal*I site)
EGFP-FL-cPLA$_2$ε-ΔPB	F7	5'-cgcagatctatggattacaaggatgacgacgataag-3' (*Bgl*II site)
	R7	5'-cgcgtcgacctactccactgcgagccgcagagc-3' (*Sal*I site)

2 [a]F, forward primer; R, reverse primer.

3

1 Table II. Primers used for reverse transcription-PCR.

Name (Accession number)	Direction	Sequence
Human cPLA$_2\varepsilon$ (NM_001206670)	Forward	5′-GAGGATCCCGGAGTCTCGAATCTGC-3′
	Reverse	5′-GGATTTCAGGCAGGATCGGCTCGTC-3′
Human PSS1 (NM_014754)	Forward	5′-CGATACGCCACAAGGGAAGCAGATG-3′
	Reverse	5′-CTACGGATCAGCAAGGCCTTCATGG-3′
Human PSS2 (NM_030783)	Forward	5′-AGCTGGGAGTCCCACTGCCAGAGAG-3′
	Reverse	5′-AAAGTGCGCGGGAACAAAGCCATCC-3′
Human GAPDH (NM_002046)	Forward	5′-CGCTGAGTACGTCGTGGAGTCCACT-3′
	Reverse	5′-AGCAGAGGGGGCAGAGATGATGACC-3′

2

3

Figure legends

Fig. 1 Outline of the NAE-biosynthetic pathway.

Fig. 2 Effect of the suppression of PSSs on cPLA$_2$ε levels. (A) FL-cPLA$_2$ε/Tet-on cells cultured in the presence of DOX were transiently transfected with a control siRNA (siCont) or siRNAs against PSS1 and/or PSS2 (a–d indicate different siRNAs). mRNAs of cPLA$_2$ε, PSS1, PSS2 and GAPDH (a control) were detected by reverse transcription-PCR. DOX(–), cells cultured in the absence of DOX. (B) The cell homogenates (40 μg of protein) were analyzed by Western blotting with anti-FLAG antibody to detect FL-cPLA$_2$ε. (C) The cell homogenates (35 μg of protein) were assayed for *N*-acyltransferase activity in the presence of 200 μM of dioleoyl-PS as described in Materials and Methods (mean values ± SD, n = 3). Tukey-Kramer's test showed significant differences among siCont-transfected cells and other cells (* $P < 0.05$).

Fig. 3 Effect of the suppression of PSSs on the intracellular localization of cPLA$_2$ε. EGFP-FL-cPLA$_2$ε/Tet-on cells cultured in the presence of DOX were transiently transfected with a control siRNA (siCont) or siRNAs against PSS1-a and/or PSS2-c, followed by transient transfection with the vector harboring mCherry-LactC2. The cells were then observed with a confocal laser-scanning microscope. mCherry-LactC2 and EGFP-FL-cPLA$_2$ε are shown in red and green, respectively, and all images are merged. Scale bar, 10 μm.

Fig. 4 *N*-Acyltransferase assay of cPLA$_2$ε and its mutants. (A) Schematic structures of EGFP-fused wild-type cPLA$_2$ε (WT) and its mutants (S412A, ΔC2, ΔLip and ΔPB) are shown. F, FLAG tag; C2, C2 domain; Lip, lipase domain; PB, polybasic domain. Amino acid numbers of native cPLA$_2$ε are shown. (B) The cell homogenates (35 μg of protein) were analyzed by Western blotting with anti-GFP antibody. Mock indicates HEK293 cells transfected with insert-free vector. (C) The cell homogenates (35 μg of protein) were assayed for *N*-acyltransferase activity in the presence of 200 μM of dioleoyl-PS as described in Materials and Methods (mean values ± SD, n = 3). Asterisks indicate significant differences

from siCont-transfected cells (* $P < 0.05$ compared by ANOVA followed by Dunnett's test). (D) A representative image of TLC obtained in the enzyme assay is shown.

Fig. 5 Intracellular localization of EGFP-fused cPLA$_2$ε and its mutants. HEK293 cells were transiently transfected with the expression vector harboring wild-type EGFP-cPLA$_2$ε (WT) or its mutants and were observed with a confocal laser-scanning microscope. Scale bar, 10 μm.

Fig. 6 Effect of anionic phospholipids on the enzyme activity of cPLA$_2$ε. The purified cPLA$_2$ε (0.1 μg of protein) was assayed for *N*-acyltransferase activity in the presence of the indicated dioleoyl-glycerophospholipids at 200 μM (A) or 57 nmol of liposomes containing the indicated dioleoyl-glycerophospholipids (5%, mol/mol) (B). Assays were performed in triplicate (mean values ± SD, n = 3). Asterisks indicate significant differences from PC as a control (* $P < 0.05$ compared by ANOVA followed by Dunnett's test).

Fig.7 Effect of anionic phospholipids on the binding of cPLA$_2$ε to liposomes. The purified cPLA$_2$ε (0.2 μg of protein) was incubated without liposomes (–) or with 14 nmol of liposomes containing the indicated dioleoyl-glycerophospholipids (5%, mol/mol) in the presence of 2.5 mM EDTA (A) or 2.5 mM $CaCl_2$ (B). The mixtures were then ultracentrifuged and the resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE followed by silver staining. (C) The intensities of individual bands were quantitated. The percentage of liposome-bound cPLA$_2$ε (%bound) is shown (mean values ± SD, n = 3). Asterisks indicate significant differences between PC and one of the indicated phospholipids while hashes indicate significant differences between EDTA and Ca^{2+} for each phospholipid. (* $P < 0.05$ compared by ANOVA followed by Dunnett's test and # $P < 0.05$ compared by two-sided *t*-test).

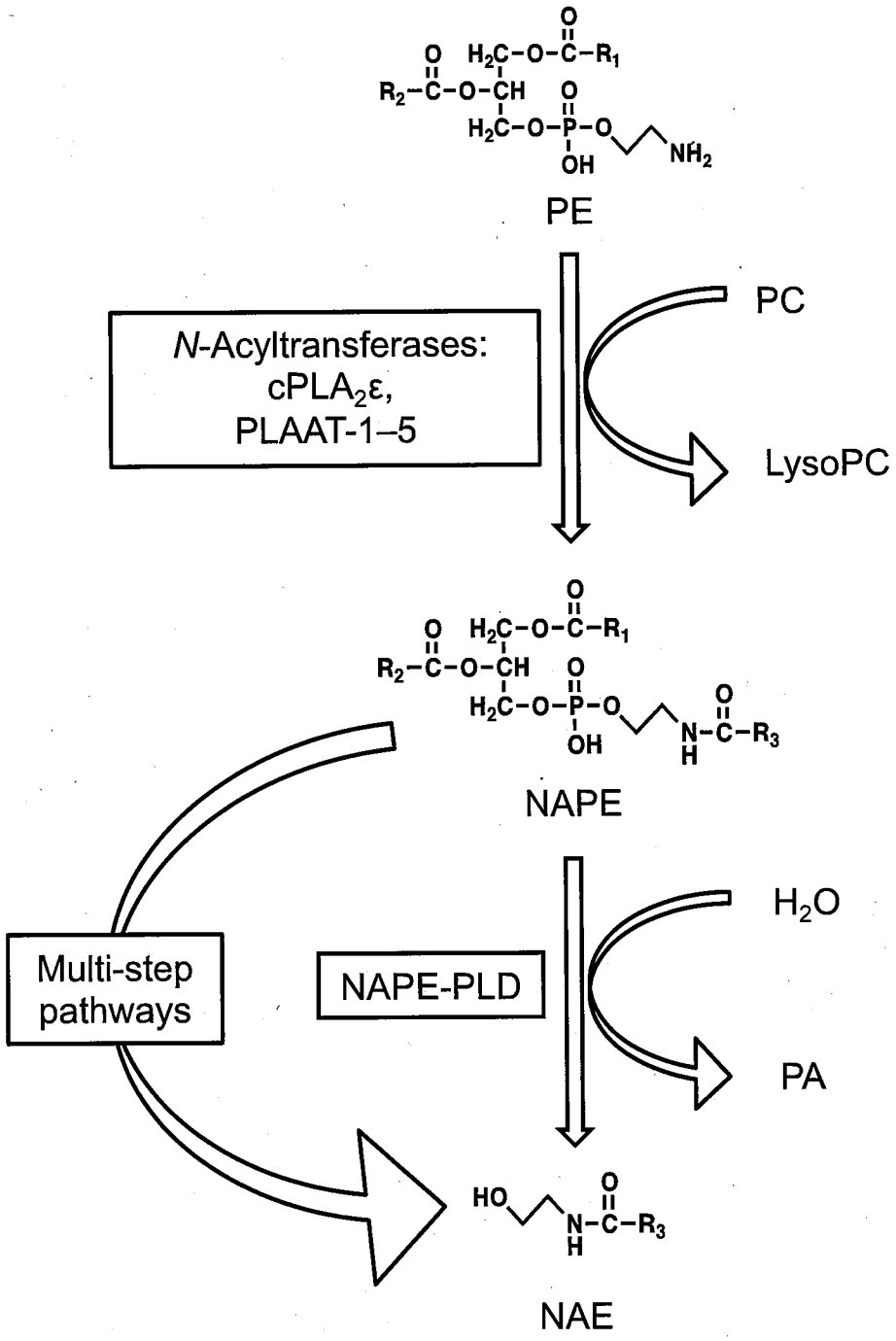

Fig. 1

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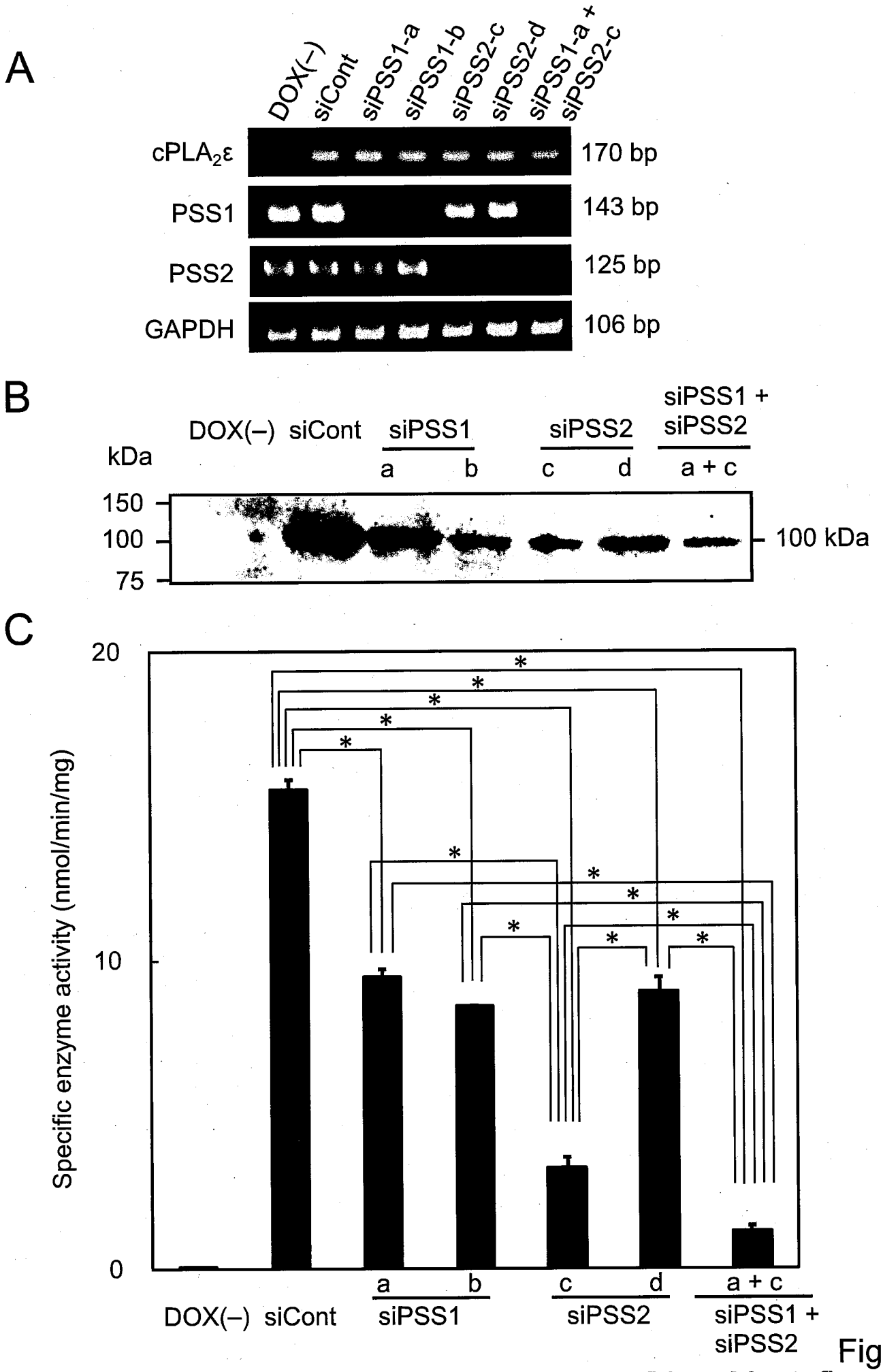

Fig. 2
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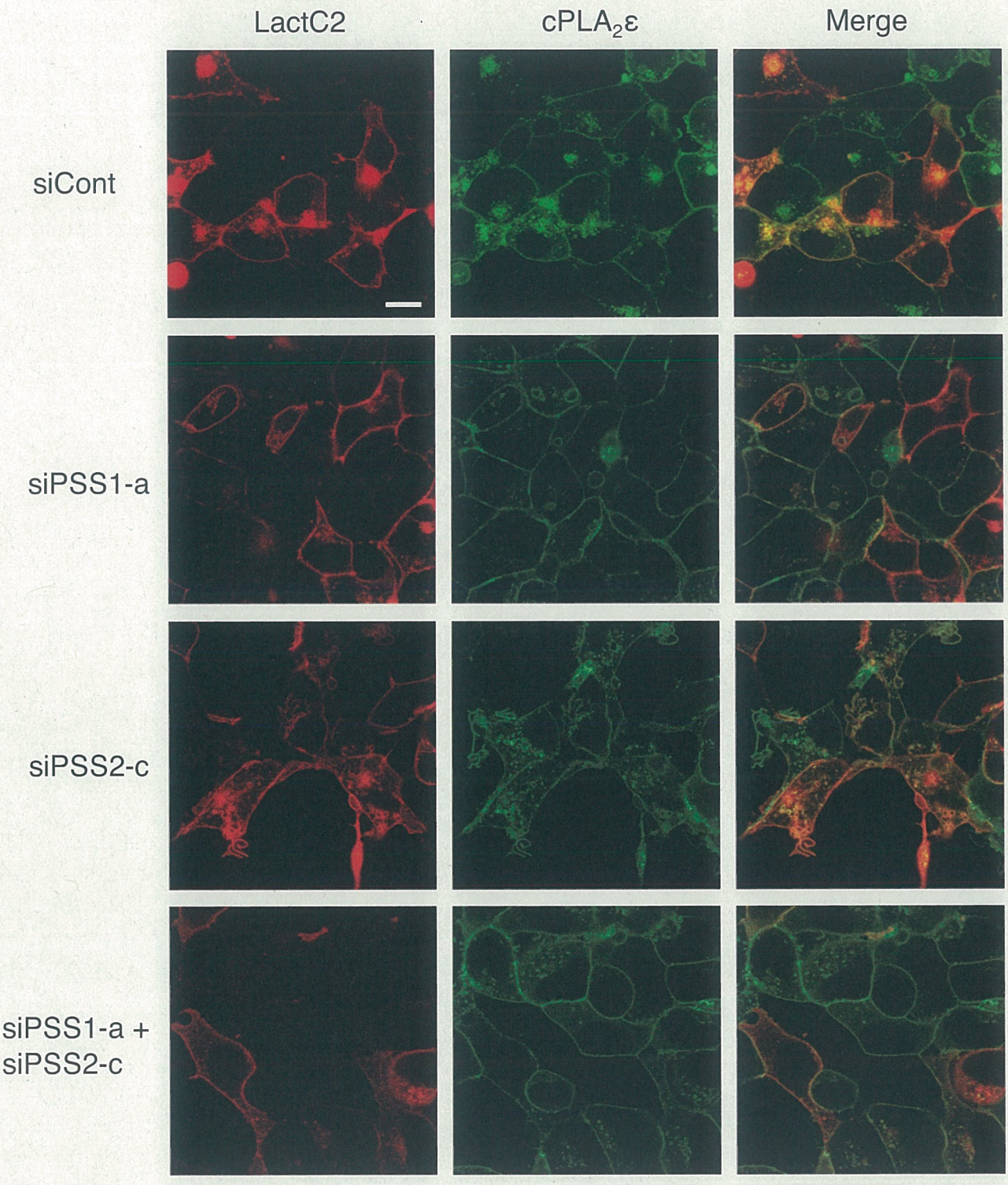

Fig. 3

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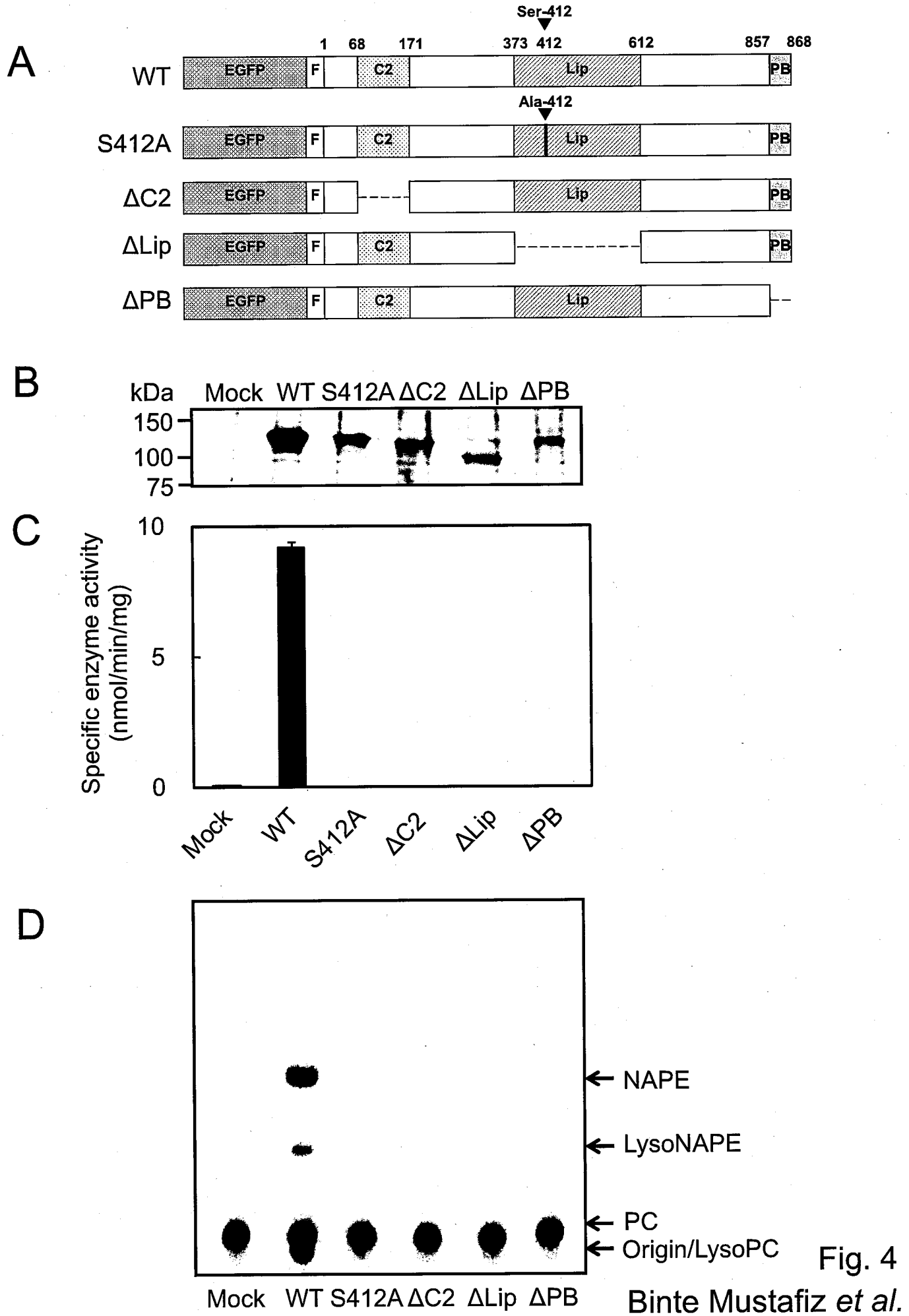

Fig. 4

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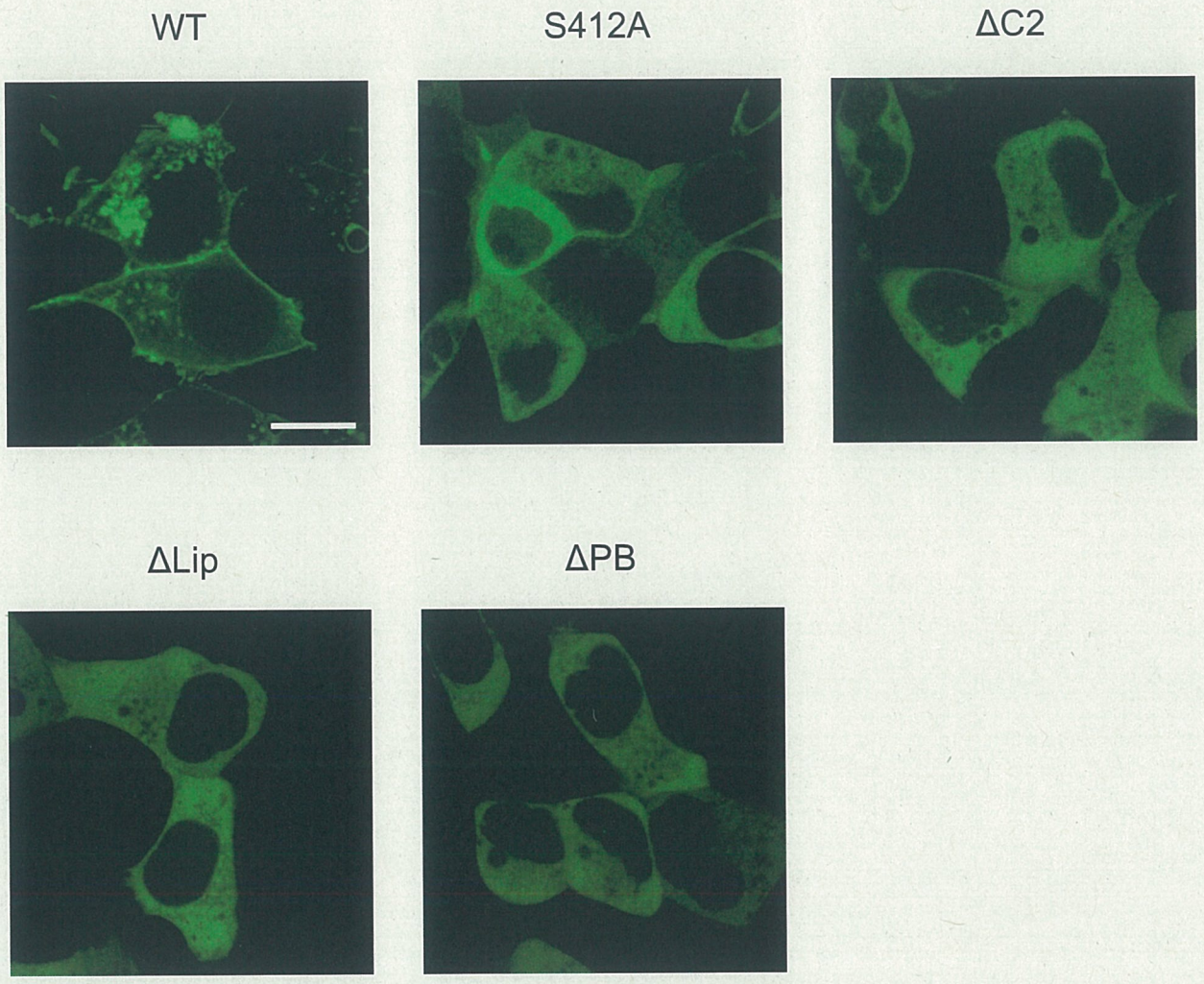

Fig. 5

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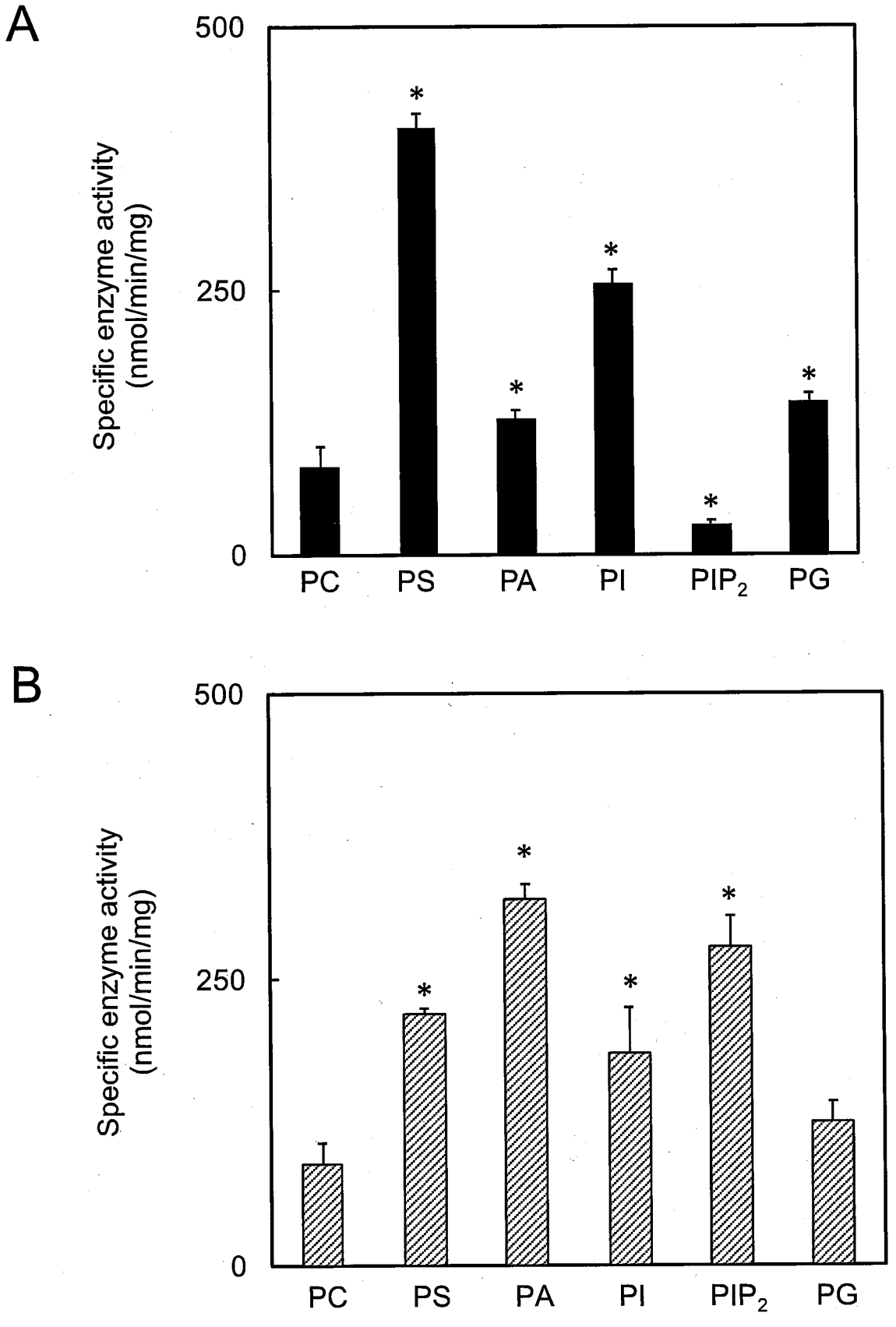

Fig. 6

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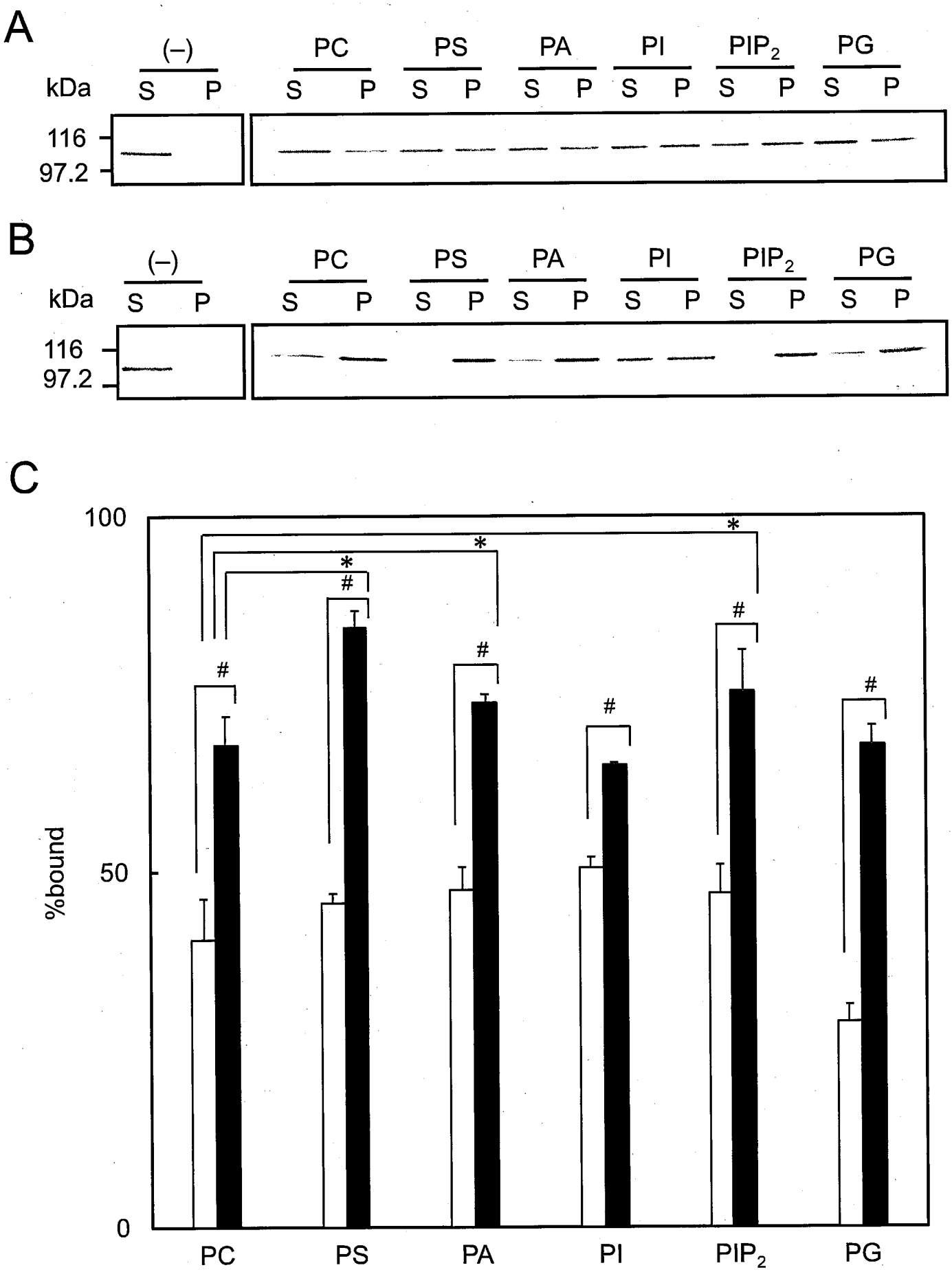

Fig. 7
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