Roles of C-terminal processing, and involvement in transacylation reactions of human group IVC phospholipase A2 (cPLA2γ).

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abbreviations footnote: CoA, coenzyme A; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPL, lysophospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Fatty acids are designated in terms of the number of carbon atoms and double bonds; e.g., 20:4 denotes twenty carbons and four double bonds (arachidonate). Unless otherwise indicated, lysophospholipids are the 1-acyl isomer.

Summary

Phospholipases A2 (PLA2) are a diverse group of enzymes that hydrolyze the *sn*-2 fatty acids from phospholipids and play a role in a wide range of physiological functions. A 61-kDa calcium-independent PLA2, termed cPLA2γ, was identified as an ortholog of cPLA2α with approximately 30% overall sequence identity. cPLA2γ contains a potential prenylation motif at its C terminus and is known to have PLA2 and lysophospholipase activities, but its physiological roles have not been clarified. In the present study, we expressed various forms of recombinant cPLA2γ, including non-prenylated and non-cleaved forms, in order to investigate the effects of C-terminal processing. We examined the expression of wild type and non-prenylated (SCLA) forms of cPLA2γ and found that the SCLA form was expressed normally and retained almost full activity. Expression of prenylated and non-cleaved form of cPLA2γ using yeast mutants lacking the prenyl protein proteases *AFC1* (a-factor converting enzyme) and *RCE1* (Ras converting enzyme) revealed decreased expression in the mutant strain compared to that in wild type yeast, suggesting that complete C-terminal processing is important for the functional expression of cPLA2γ. In addition, cPLA2γ was found to have coenzyme A (CoA)-independent transacylation and lysophospholipid (LPL) dismutase (LPLase/transacylase) activities, suggesting that cPLA2γ may be involved in fatty acid remodeling of phospholipids and clearance of toxic lysophospholipids in the cells.

The fatty acyl moieties of phospholipids are dynamically remodeled by various enzymes, including phospholipases and acyltransferases (1-6). Lands et al. (1, 2) proposed that a deacylation-reacylation cycle (Lands cycle) was involved in the remodeling of phospholipids. The Lands cycle consists of sequential reactions: 1) Phospholipid is cleaved by phospholipase A2 (PLA) to produce lysophospholipid and free fatty acid (FFA); 2) Another FFA is activated by acyl-CoA synthetase with consumption of ATP; and 3) The fatty acyl moiety of acyl-CoA is transferred to lysophospholipid by acyl-CoA:lysophospholipid acyltransferases (1-6).

Transacylation between phospholipid and (lyso)phospholipid is considered important for fatty acid remodeling (4-6). Several types of transacylation system are known, including CoA-dependent and independent systems. We have already proposed a partial mechanism for a CoA-dependent transacylation system that combines the reverse and forward reactions of acyl-CoA:lysophospholipid acyltransferases, based on examination of lysophosphatidic acid acyltransferase and lysophosphatidylinositol acyltransferase (7, 8).

CoA-independent transacylases or transacylation systems catalyze the transfer of fatty acids esterified at the *sn*-2 position of phospholipids to various lysophospholipids in the absence of any cofactors (3-13). Of particular interest is the role of these enzymes in the arachidonic acid mobilization between phospholipids and 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (GPC)(lyso platelet activating factor). Lysophospholipase (lysophospholipase A, LPLase A) catalyzes the hydrolysis of the carboxyl ester bonds of lysophospholipids (14-16). Some isoforms of LPLase A catalyze a transacylation reaction between two lysophosphatidylcholine (LPC) molecules, forming phosphatydylcholine (PC) and GPC (dismutase reaction). Despite the importance of CoA-independent transacylation systems and LPL dismutase in phospholipid remodeling, the mechanism underlying the transacylation system is not fully understood.

A 61-kDa, calcium-independent PLA2, called group IVC PLA2 or cPLA2γ , was identified as an ortholog of cPLA2α (17, 18), though its physiological role has not been established. cPLA2γ is reported to have PLA2 (17, 18) and LPLase A (19, 20) activities, and is postulated to be involved in phospholipid remodeling (21). cPLA2γ shares 29% overall sequence identity with cPLA2α, which is the only PLA2 that selectively releases arachidonic acid, a precursor for eicosanoids (22-24). cPLA2α is regulated by intracellular

calcium via the C2 domain in its N-terminal region. The C2 domain is not conserved in cPLA2γ, suggesting that cPLA2γ is not regulated by calcium.

Interestingly, cPLA2γ contains a potential CAAX-type prenylation motif at its C terminus (17, 18, 20). Upon prenylation, the affected proteins undergo two modifications (25-29) (Scheme 1). First, the last three C-terminal residues are released by a specific endoprotease, such as *Rce1* or *AFC1* (25, 26). Second, the carboxyl group of the newly exposed prenylated cysteine is methylated by a specific methyltransferase, *Icmt*. These modifications render the carboxyl-terminal domains of CAAX proteins more hydrophobic, facilitating interactions with membranes and with protein partners (25-29). The C-terminal farnesylation, proteolytic processing and carboxymethylation of recombinant cPLA2γ expressing in insect cells were confirmed by mass spectrometry analysis (20).

In the present study, we use prenylated and non-prenylated forms of recombinant cPLA2γ expressed in yeast, insect cells and mammalian cells to investigate three important aspects of cPLA2γ: its membrane distribution, the effects of C-terminal processing, and its physiological functions. The results indicate that complete C-terminal processing is important for the functional expression of cPLA2γ, though not for its membrane localization. In particular, prenyl protein-specific endoproteases are essential for the function of cPLA2γ. In addition, cPLA2γ was found to have CoA-independent transacylation activity and LPLase A/transacylase activity, suggesting that cPLA2γ may be involved in phospholipid remodeling. We further discuss the role in protection against cardiovascular pathology through the metabolism of lysophospholipids.

EXPERIMENTAL PROCEDURES

Materials – [1-^{14}C]Palmitic acid (16:0, 2.0 GBq/mmol), [1-^{14}C]linoleic acid (18:2, 2.0 GBq/mmol), [1-^{14}C]arachidonic acid (20:4, 2.0 GBq/mmol), 1-[1-^{14}C]palmitoyl (16:0)-GPC (2.1 GBq/mmol), [^{14}C]PC (1-palmitoyl-2-[^{14}C]linoleoyl, 2.0 GBq/mmol, and 1-palmitoyl-2-[^{14}C]arachidonoyl, 2.0 GBq/mmol) , [^{14}C] phosphatidylethanolamine (PE) (1-stearoyl-2-[^{14}C]arachidonoyl, 2.1 GBq/mmol), and [^{3}H] lyso platelet activating factor (1-O-[^{3}H] hexadecyl-GPC, 185 GBq/mmol) were purchased from Du Pont-New England Nuclear

(Boston, MA). CoA, LPC (1-palmitoyl (16:0)-GPC) was obtained from Sigma (St. Louis, MO). 1-*O*-hexadecyl-GPC was from Cayman Chemical. Lyso plasmalogen (1-alkeny-sn-glycero-3-phosphoenthanolamine (GPE) was from DOOSAN Serdary Research Laboratories. TLC plates precoated with silica gel 60 (Type 5721) were from Merck (Darmstadt, Germany). All other reagents were of reagent grade or better.

Construction of expression plasmids for human cPLA2γ -- Yeast expression constructs of human cPLA2γ were derived from *GAL1*-promoter (P_{GAL1})-driven with a c-myc epitope (EQKLISEEDL) tagging vector, pESC-URA(Stratagene). The cDNA encoding full-length human cPLA2γ was a gift from Drs. N. Uozumi and T. Shimizu, Tokyo University (21). The cDNA was amplified by polymerase chain reaction using *Pfu* DNA polymerase (Promega) and the following primers:
Forward: 5'-AAAAAAGTCGACATGGGAAGCTCTGAAGTTTCC-3'
Reverse: 5'-AAAAAAGCTAGCCTATGCCAAGCAGCAACTTCG-3'
The *Sal I* and *Nhe I* site are underlined. In one construct, the nucleotide sequence coding the CAAX box tetrapeptide at the C-terminus of cPLA2γ (CCLA) was changed to code for SCLA using the same forward primer and an alternate reverse primer: 5'-AAAAAAGCTAGCCTATGCCAAGCAGGAACTTCG-3'. After digestion by *Sal I* and *Nhe I*, the PCR products were subcloned into *Xho I - Nhe I* site of pESC-URA to yield a plasmid carrying the sequences corresponding to N-terminal c-myc-epitope fusion of native cPLA2γ (pESC/cPLA2γ) or mutated cPLA2γ (pESC/cPLA2γ SCLA). The coding regions of the fusion gene were verified by sequencing.

To construct baculovirus system transfer vectors, DNA fragments containing the ORF of cPLA2γ or cPLA2γ SCLA with a N-terminal c-myc epitope were excised from the corresponding plasmids (pESC/cPLA2γ or pESC/cPLA2γ SCLA) by digestion with *Sma I* and *Nhe I*, and were subcloned into *Sma I – Xba I* site of the pVL1393 baculovirus transfer vector (Invitrogen) to yield the transfer vector plasmids pVL1393/ cPLA2γ and pVL1393/ cPLA2γ SCLA.

To construct mammalian expression vectors, DNA fragments containing the ORF of cPLA2γ or cPLA2γ SCLA with the N-terminal c-myc epitope were excised from pVL1393/

cPLA2γ or pVL1393/ cPLA2γ SCLA by the digestion with *Sma I* and *Not I*, and were subcloned into the *EcoRV – Not I* site of the pcDNA4/TO expression vector (Invitrogen) to yield the plasmids pcDNA4/TO / cPLA2γ and pcDNA4/TO / cPLA2γ SCLA.

Yeast Strains and Growth Conditions -- Methods for growth and selection of yeast were reported previously (30, 31). YPD medium consisted of 1% of Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose. In YPG (induction) medium, galactose replaced dextrose as the carbon source. Complete synthetic media (CSM) was described previously (30, 31) and contained the indicated sugar carbon source (dextrose, D or galactose, G). Yeast selection media contained the components of CSM except as noted (e.g., minus uracil (-Ura)). Yeast strains were grown at 30 ° C.

The yeast strains JRY5314 (wild type), JRY5315 (*afc1Δ::HIS3*), JRY5316 (*rce1Δ::TRP1*), and JRY5314 (*afc1Δ::HIS3, rce1Δ::TRP1*) were kind gifts from Prof. Jasper Rine (Unversity of California, Berkley) (28). Transformation of yeast strains JRY5314 (wild type), JRY5315 (*afc1Δ::HIS3*), JRY5316 (*rce1Δ::TRP1*), and JRY5314 (*afc1Δ::HIS3, rce1Δ::TRP1*) with pESC/cPLA2γ or pESC/cPLA2γ SCLA was performed by the lithium acetate protocol (30). Transformants were selected on plates of complete synthetic medium minus uracil. cPLA2γ expression was induced by culturing with complete synthetic medium minus uracil plus 2% galactose.

Expression of cPLA2γ in mammalian cells - HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma), 100 IU/ml penicillin, and 100 μg/ml streptomycin and were transfected with a complex of LipofectAMINE 2000 (Invitrogen) and pcDNA4/TO/cPLA2γ or pcDNA4/TO/cPLA2γ SCLA according to the manufacturer's protocol. Transfected cells were harvested 48 h of post-transfection. Transfection with pcDNA4/TO served as a vector control.

Production of Recombinant Baculovirus and Protein Expression in Sf9 Cells-- *Sf9* cells were routinely grown in suspension at 27 ˚C in TNM-FH medium containing 10% FBS, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B. Recombinant baculoviruses were

generated by co-transfection of *Sf9* cells with the cPLA2γ transfer vector constructs described above (or pVL1393 itself to generate control virus) and linearized baculovirus DNA (BaculoGold, PharMingen) following the manufacturer's instructions. Recombinant viruses were amplified by standard protocols. For expression of recombinant cPLA2γ, *Sf9* cells (7×10^6) were plated into 150-mm tissue culture plates and infected with either control virus or recombinant virus. After 72 h, the infected *Sf9* cells were harvested and subcellular fractions were prepared.

Preparation of cytosol and membrane fractions from yeast, HEK293 and Sf9 cells

Yeast cells were disrupted using zirconia/silica beads (Biospec, Inc.) in 0.6 M mannitol/15 mM Tris-HCl (pH 7.4) /0.5 mM EDTA /protease inhibitors (1 mM PMSF, 0.1 mM AEBSF, 5 μg/ml aprotinin, 5 μg/ml bestatin, 5 μg/ml E-64, 10 μg/ml leupeptin, 5 μg/ml pepstatin A). After removing cell debris and nuclear material by centrifugation at 2,000 rpm for 5 min, the lysates were centrifuged at 105,000 x g for 1 h to separate membrane fractions and cytosol.

HEK293 and *Sf9* insect cells were homogenized by a Potter-Elvehjem glass-Teflon homogenizer in STE buffer (0.25 M sucrose/0.1 M Tris-HCl/1 mM EDTA (pH 7.4) /protease inhibitor cocktail set III (Calbiochem, No 539134)). The homogenate was centrifuged at 700 x g for 10 min to remove cell debris and nuclear materials. The supernatant was centrifuged at 5,000 x g for 10 min, and the resultant pellet was washed with STE buffer and then was suspended with the same buffer and used as the heavy mitochondria fraction (HM). The supernatant was centrifuged at 105,000 x g for 60 min. The pellet was washed with STE buffer and the final pellet was suspended with STE buffer and used as the light mitochondria and microsomal fraction (Mc). The supernatant was used as the cytosol fraction (S). The protein content of each frantions was measured by the BCA method using bovine serum albumin as standard.

NaCl and Na_2CO_3 treatment of membrane fractions -- Light mitochondria and microsome fractions from Sf9 cells expressing wild type cPLA2γ or mutant cPLA2γ SCLA were prepared as described above. The membrane fractions were treated with 1 M NaCl or 0.1 M

Na_2CO_3 (mild alkaline) and then were centrifuged at 105,000 x g for 60 min. The resultant pellets (insoluble fractions) and supernatant (solubilized fractions) were subjected to Western blotting with antibody against the c-myc epitope and assayed for LPLase A activity.

LPLase A activity – LPLase A activity was determined by the measurement of released [^{14}C]fatty acid from [^{14}C]LPC (1-[^{14}C]palmitoyl). Aliquots of [^{14}C]LPC (110,000 dpm/assay) were dried with a stream of N_2 gas and suspended at 50 μM by brief sonication (Branson bath type sonifier) in 50 mM Tris-HCl (pH 7.5) containing 2 mM EDTA before incubating with enzyme at 30 ˚C for 2 min. In some cases, the concentration of [^{14}C]LPC was varied. The reaction was terminated by addition of 3 ml of chloroform:methanol (1:2, v/v). After the addition of unlabeled fatty acid as a carrier, total lipids were extracted according to the method of Bligh and Dyer (32). Total lipids were separated by silica gel TLC developed with petroleum ether:diethyl ether:acetic acid (70:30:1, v/v). The band corresponding to product fatty acid was visualized under UV light (365 nm) after spraying with primuline (0.001 %, in acetone:water, 4:1, v/v), and the radioactivity was determined in an imaging analyzer (Fuji-Bas 1500, Fuji Film) or by scintillation counting (LSC-3500; Aloka, Tokyo, Japan) after scraping the band from the plate into a vial.

For measurement of LPLase A/transacylation activity, the lipid extracts were separated by TLC developed with chloroform:methanol:ammonia (65:35:4, v/v) and the radioactivity in PC was measured as described above.

Measurement of transacylation activity (10, 11)– Transacylation activities with 1-*O*-[^{3}H]hexadecyl-GPC (lyso platelet activating factor) was measured for membrane fractions from cPLA2γ-expressing or mock-transfected yeast strain JRY5314. Aliquots of 1-*O*-[^{3}H]hexadecyl-GPC (440,000 dpm/assay) were dried with N_2 gas and suspended at 50 μM by brief sonication in 50 mM HEPES (pH 7.5) containing 2 mM EGTA, 30% glycerol, 1 mg/ml fatty acid-free bovine serum albumin, and 150 mM NaCl before incubation with enzyme sources at 30 ˚C. The reaction was terminated by addition of 3 ml of chloroform:methanol (1:2, v/v). After the addition of PC as a carrier, total lipids were

extracted as described above and analyzed by TLC in chloroform:methanol:ammonia (65:35:4, v/v). The band corresponding to product PC was visualized and its radioactivity determined as descibed above.

Analysis of transacylation reaction products -- After separation of products of transacylation reaction by TLC, the bands corresponding to diradyl-GPC (1-alkyl-2-acyl-GPC or 1, 2-diacyl-GPC) were scraped off from TLC plate into tubes containing chloroform:methanol:water (1:2:0.8, v/v). Diradyl-GPC was extracted by stirring the mixture and phase separation by method of Bligh and Dyer (32). The extracted diradyl-GPC was treated with snake venom PLA2 (*Naja naja atra*) (8, 33), and the products were then separated by TLC developed with chloroform:methanol:ammonia (65:35:4, v/v). The radioactivity of radyl-GPC and fatty acid was measured by liquid scintillation counting. In some cases, diradyl-GPE was treated with *Naja naja atra*, and the products were analyzed as just described.

Western Blotting – The expression of cPLA2γ in yeast was confirmed by Western blotting using a monoclonal antibody against the c-myc epitope (Clone 9E10; Roche). Briefly, proteins from membrane and cytosol fractions from yeast strains expressing cPLA2γ were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Protran BA85; Schleicher & Schuell). After blocking the membrane with 5 % bovine serum albumin, it was incubated with monoclonal antibody against against the c-myc epitope. Immunoreactive bands were visualized by chemiluminescence using horseradish peroxidase-conjugated anti-mouse IgG (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) and ECL or ECL Plus reagent (Amersham Pharmacia Biotech).

RESULTS

Distribution of recombinant cPLA2γ expressed in *yeast, Sf9 insect cells and HEK293 mammalian cells* –The first aim of this study was to explore the physiological roles of cPLA2γ. We previously investigated the CoA-independent transacylation system that

involved in the fatty acid remodeling of phospholipids (9-13), and suspected that some PLA2 enzymes were involved in CoA-independent transacylation reactions between phospholipids and lysophospholipid (Scheme 2 and Ref. 5). Any enzymes involved in such transacylation reactions are likely to be membrane-bound, and we considered cPLA2γ as a candidate for participation in the transacylation activity.

To characterize the biochemical and enzymological properties of cPLA2γ, we expressed the protein in various expression systems, including the yeast *Saccharomyces cerevisiae*, *Sf9* insect cells and mammalian HEK293 cells. First, we examined the expression of cPLA2γ in HEK293 cells transfected with mammalian expression vector harboring the cDNA coding for the recombinant protein (pcDNA4/TO/cPLA2γ), using a monoclonal antibody against the c-myc epitope tag. As shown in Fig. 1-A, a 61 kDa band was detected in membrane fractions, such as the heavy mitochondria (lane 1) and microsomal fractions (lane 2). No signal was detected in mock-transfected HEK293 cells (data not shown), confirming that the 61 kDa band was recombinant cPLA2γ. This localization of cPLA2γ to membranes was also observed when the recombinant protein was expressed in *Sf9* insect cells (Fig. 1-B) and yeast cells (Fig. 2).

Localization of cPLA2γ to membrane fractions was conceivably due to prenylation of the protein, so we examined the relationship between C-terminal prenylation and the subcellular distribution of cPLA2γ, using recombinant cPLA2γ with a Cys → Ser mutation to disrupt the CAAX box motif at C-terminus and thus block prenylation (cPLA2γ SCLA). Surprisingly, the SCLA mutant remained localized to membranous fractions when expressed in either HEK293 or *Sf9* cells (Fig. 1, panels A and B). These results indicate that C-terminal prenylation is not an indispensable determinant of cPLA2γ membrane binding.

cPLA2γ and SCLA mutant catalyze LPLase A activity –We measured LPLase A activity because cPLA2γ has considerable LPLase A activity (19, 20) and because addition of an N-terminal epitope tag was reported to affect PLA2 activity rather than LPLase A activity (19). The LPLase A assay employed [^{14}C]LPC (1-[^{14}C]palmitoyl-GPC) as substrate and membranes from HEK293 cells expressing cPLA2γ. Transfection with the cPLA2γ vector markedly increased LPLase A activity in membrane fraction; very low activity was found

in membranes from mock-transfected cells (Fig. 1A). Very little LPLase A activity was found in the cytosol. Similar results were observed with *Sf9* and yeast cells expressing recombinant cPLA2γ (Figs. 1B and 2). Thus, membrane-bound, recombinant wild-type cPLA2γ catalyzes LPLase A activity. Similar results were observed with the SCLA mutant form of cPLA2γ. Cells expressing the recombinant protein had elevated LPLase A activity in membrane fractions, though the activity of the SCLA mutant was somewhat lower than with wild type cPLA2γ (Fig. 1). The results suggest that C-terminal processing is not essential for LPLase A activity.

We analyzed the mode of the binding of cPLA2γ to membranes. The membrane fraction from *Sf9* cells expressing wild type cPLA2γ or the SCLA mutant were treated with 1 M NaCl or 0.1 M Na_2CO_3 to solubilize peripheral membrane proteins. The presence of cPLA2γ in the solubilized material was assessed by Western blotting and LPLase A activity. Neither wild type cPLA2γ nor the SCLA-mutant were solubilized by treatment with high concentrations of salt or mild alkali (Fig. 1B), indicating that cPLA2γ remains tightly anchored to membranes even when not prenylated.

Effects of prenyl protein protease AFC1 and RCE1 on expression of cPLA2γ in yeast– The sequential C-terminal processing of CAAX box proteins in yeast is practically the same as in mammals, and mutant strains defect in the processing are available (28). CAAX box proteins undergo three steps of post-translational modification. After isoprenylation, the last three amino acids of the protein (i.e., the AAX of the CAAX sequence) are released by a prenyl protein-specific endoprotease, *RCE1* (for Ras-converting enzyme) or *AFC1* (for **a**-factor-converting enzyme) (28, 29). Finally, the carboxyl group of the newly exposed isoprenylcysteine is methylated (25- 29) to form mature protein. To further assess the effects of C-terminal processing of cPLA2γ, we examined recombinant cPLA2γ and cPLA2γ SCLA expressed in yeast mutants lacking *RCE1* and *AFC1* to establish the effects of C-terminal processing on the enzyme.

Recombinant wild type cPLA2γ was expressed at lower levels in the *Δ afc1* and *Δ rce1* yeast mutants than in wild type yeast (Fig. 2A, lanes 1-4). Expression of cPLA2γ in the double-mutant yeast (*Δ afc1*, *Δ rce1*) was even lower, though still detectable (Fig. 2A,

lane 4). Recombinant cPLA2γ was not detected in cytosolic fractions in any of the prenyl protein protease-deficient strains (Fig. 2A, lanes 5-8). The results obtained by Western blotting were confirmed by measurements of LPLase A activity (Fig. 2B). LPLase A activity in membrane fractions from the *Δ afc1* and *Δ rce1* yeast mutants was lower than that in wild type yeast and only very low LPLase A activity was observed in the double-mutant yeast (*Δ afc1*, *Δ rce1*) mutant (Fig. 2B). These results indicate that complete C-terminal processing is important for the functional expression of cPLA2γ.

In contrast, defects in *RCE1* and *AFC1* in the host yeast had little effect on expression of the SCLA mutant, which was found at comparable levels in the double-mutant (*Δ afc1*, *Δ rce1 1*) and wild type yeast, as assessed by western blotting and LPLase A activity (Fig. 2C).

Taken together, these results indicate that defects in RCE1p or AFC1p decrease expression of cPLA2γ in yeast, and that both AFC1p and RCE1p can process the cPLA2γ C-terminus. Because the cPLA2γ gene was induced by the same, potent promoter (P_{GAL1}) in all the strains of yeast, defects in RCE1p and AFC1p probably impact on cPLA2γ degradation rather than on cPLA2γ synthesis. Given that the non-prenylated SCLA form was expressed in the double-mutant yeast strain, it appears that cPLA2γ becomes unstable and undergoes degradation if prenylated and not cleaved (Scheme 1).

CoA-independent Transacylation Activity of cPLA2γ -- PLA2 enzymes could plausibly be involved in CoA-independent transacylation reactions between phospholipids and lysophospholipid (Scheme 2). We therefore examined whether cPLA2γ catalyzed CoA-independent transacylation activity with 1-*O*-[^{3}H]hexadecyl-GPC (lyso platelet activating factor) as substrate.

Membrane fractions from yeast cells expressing cPLA2γ or mock-transfected cells were incubated with 1-*O*-[^{3}H]hexadecyl-GPC. As shown in Fig.3A, there was little conversion of 1-*O*-[^{3}H]hexadecyl-GPC to [^{3}H]diradyl-GPC by control membrane fractions (open circles), but membranes from yeast expressing wild type cPLA2γ had markedly higher activity, indicating that the recombinant protein promoted transacylase activity. Membranes from yeast expressing the SCLA mutant also had elevated transacylase activity. Formation of

[^{3}H]diradyl-GPC was dependent on the concentration of 1-*O*-[^{3}H]hexadecyl-GPC (Fig. 3B). Fitting the data to the Michaelis-Menten equation indicated Km values of approximately 40 and 70 μM for the wild type and SCLA mutant forms, respectively (Fig. 3B).

Treatment of the product [^{3}H]diradyl-GPC with snake venom PLA2 formed [^{3}H]radyl-GPC, indicating that the transacylase activity transferred the fatty acid to the sn-2 position of 1-*O*-[^{3}H]hexadecyl-GPC.

As shown in Fig. 3C, unesterified [^{14}C]fatty acids, including palmitate (16:0), linoleate (18:2 n-6), and arachodonate (20:4 n-6), were not transferred to 1-*O*-hexadecyl-GPC or 1-*O*-alkenyl-GPE. In contrast, [^{14}C]arachidonate esterified in the sn-2 position of phosphatidylethanolamine was transferred into 1-*O*-hexadecyl-GPC to from [^{14}C]diradyl-GPC (Fig. 3C). A fatty acyl group could also be transferred from the sn-2 position of PC (1-palmitoyl-2-[^{14}C]arachidonoyl-GPC or 1-palmitoyl-2-[^{14}C]linoleoyl-GPC) to 1-*O*-alkenyl-GPE. In this case, arachidonate was more efficiently transferred than linoleate. These results indicate that phospholipids in the membrane preparations are the likely acyl donor in the case of cPLA2γ-catalyzed acylation in assays with 1-*O*-[^{3}H]hexadecyl-GPC.

We next examined the cofactor requirements of cPLA2γ-catalyzed transacylation activity. Transacylation of 1-*O*-[^{3}H]hexadecyl-GPC occurred with the membrane fraction itself and addition of CoA or Ca^{2+} did not significantly affect the activity (Fig. 3D). Treatment of the membrane fraction containing cPLA2γ with apyrase slightly inhibited the acyltransferase activity. These results indicate that recombinant cPLA2γ promotes transacylation independent of CoA, calcium and ATP.

LPL Dismutase Activity of cPLA2γ --During the investigation of substrate specificity of the cPLA2γ transacylation activity, we found that the protein could also catalyze LPL dismutase activity (combining reactions 1 b and 2b in Scheme 2). We used [^{14}C]LPC (1-[^{14}C]palmitoyl-GPC) as substrate and the assay was the same as that for LPLase A activity described above. As shown by the TLC analysis (Fig. 4A), the products were [^{14}C]free fatty acid (LPLase A activity; reactions 1b and 2a in Scheme 2) and [^{14}C]PC (dismutase activity; reactions 1b and 2b in Scheme 2). Developement with chloroform:methanol:ammonia (65:35:5, v/v) separated the substrate ([^{14}C]LPC) and products ([^{14}C] fatty acid and [^{14}C]PC)

(lane 2). Development with petroleum ether: diethylether :acetic acid (70:30:1, v/v) did not separate [^{14}C] fatty acid from [^{14}C]PC (lane 1). [^{14}C]PC formation was due to recombinant cPLA2γ because it did not occur with membranes from control cells (Fig. 4B). Comparable amounts of [^{14}C]PC formation also occurred with membranes from HEK293 cells expressing the cPLA2γ SCLA mutant (Fig. 4B), indicating that C-terminal prenylation was not required for LPL dismutase activity. The LPL dismutase activities of cPLA2γ and cPLA2γ SCLA were not appreciably affected by addition of CoA or apyrase (Fig. 4B).

The [^{14}C]PC product was analyzed to investigate the mechanism of the dismutase reaction. When the [^{14}C]PC was treated with snake venom PLA2 and analyzed by two-dimensional TLC, radioactivity was found in both LPC and fatty acid fractions, indicating that the LPL dismutase reaction had occurred and [^{14}C]palmitic acid from [^{14}C]LPC was transferred to the sn-2 position of [^{14}C]LPC.

The LPL dismutase activity was dependent on the concentration of [^{14}C]LPC, but it was not a simple saturable relationship, with indications of positive cooperativity and substrate inhibition (Fig. 4C). LPLase A activity also had complex kinetics, with an indication of substrate inhibition (Fig. 4D). The ratio of the maximal activities of LPLase A and LPL dismutase was approximately 3.5.

When 1-*O*-alkenyl-GPE was included in the reaction mixtures, both [^{14}C]PC and [^{14}C]diradyl-GPE was formed (Fig. 4F); digestion of the product [^{14}C]diradyl-GPE with snake venom PLA2 released [^{14}C]FFA (data not shown). This result suggests that cPLA2γ catalyzed [^{14}C]fatty acid transfer to the sn-2 position of 1-*O*-alkenyl-GPE. [^{14}C]LPC and 1-*O*-alkenyl-GPE may be competing substrates, because production of [^{14}C]PC was decreased upon addition of 1-*O*-alkenyl-GPE. In any case, the results suggest that cPLA2γ does not discriminate among lysophospholipid head groups.

DISCUSSION

Human group IVC phospholipase A2, also named cPLA2γ, was identified by searching the EST database for orthologs of cPLA2α (17, 18). The physiological roles of cPLA2γ have not been fully elucidated, but cPLA2γ has been reported to have several

activities in vitro, including PLA2, and LPLaseA activities (17-20). We first considered the possible involvement of cPLA2γ in fatty acid remodeling of phospholipids. Lands proposed that deacylation-reacylation cycle was involved in the remodeling system (1, 2). The Lands cycle consists of phospholipid cleavage by PLA to produce lysophospholipid, ATP-driven activation of another FFA by acyl-CoA synthetase, and transfer of fatty acid from the acyl-CoA to the lysophospholipid by acyl-CoA:lysophospholipid acyltransferases (1-6). cPLA2γ may be involved in the first step of the Lands cycle. Asai et al. (21) reported that overexpression of cPLA2γ in HEK293 cells changed the fatty acid composition of phospholipids. This report supports the involvement of cPLA2γ in the remodeling system.

We previously investigated the properties of a CoA-independent transacylation system in rabbit alveolar macrophages that is involved in fatty acid remodeling (10, 11). This transacylation system catalyzes the transfer of fatty acids esterified at the *sn*-2 position of diacyl phospholipids to various lysophospholipids in the absence of any cofactors (3-6, 9-13). The system has a preference for transferring C20 and C22 polyunsaturated fatty acids, including arachidonic acid and docosahexaenoic acid, and for choline or ethenolamine glycerophospholipids as acyl donor and acceptor. The transacylation activity is located in membrane fractions and occurs in Ca^{2+}-independent manner, so it seemed likely to involve a membrane-bound and Ca^{2+}-independent PLA2 enzyme such as cPLA2γ. The present results show that cPLA2γ can indeed catalyze the fatty acid transfer from phospholipid to lysophospholipid in a CoA-independent manner (Figs. 3A, 3C and 3D). cPLA2γ-catalyzed transacylation activity has a preference for arachidonic acid over linoleic acid in transfers from PC to lysoplasmalogen (Fig. 3C).

A possible mechanism for transacylation by cPLA2γ is depicted in Scheme 2. The proposed transacylation process consists of two steps: 1) Formation of a fatty acyl-enzyme intermediate by PLA2 half reaction (steps 1a in Scheme 2); and 2) fatty acid transfer from the intermediate to lysophospholipid (step 2 b in Scheme 2). The fatty acyl-enzyme intermediate can alternatively react with water to complete the PLA2 reaction (step 2a in Scheme 2). At present, the relative contributions of cPLA2γ to the direct transfer of fatty acid (CoA-independent transacylation system) and to the deacylation step of a Lands cycle are unclear. Acyl-CoA synthetase requires ATP, making the Lands cycle more energy-intensive than the CoA-independent transacylation reaction, which has no ATP requirement (Fig. 3D).

The present results indicate that cPLA2γ catalyzes yet another type of transacylation reaction, the fatty acid transfer from LPC to LPC that forms PC and GPC from two molecules of LPC (termed LPL dismutase activity, steps 1b and 2b in Scheme 2). The main difference between the CoA-independent transacylation and the LPL dismutase activities is the identity of the acyl donor: the former uses phospholipids such as PC and PE, whereas the latter uses LPC. The dismutase activity of cPLA2γ-catalyzed is considerably greater than its PLA2 and CoA-independent transacylation activities. The ratio of activities of LPLase A and LPL dismutase was approximately 3.5:1. The ratio of the activities LPL dismutase and CoA-independent transacylation reaction was approximately 13:1.

The ability of cPLA2γ to catalyze both hydrolytic reactions (PLA2 and LPLase A activities) and transacylation reactions (CoA-independent transacylation and LPL dismutase activities) raises the question of what determines the balance between hydrolysis and transacylation. The accessibility of water to the catalytic center is likely an important factor, as its presence promotes hydrolysis and its absence promotes transacylation (steps 2a and 2b in Scheme 2). In this context, it's worth noting that PLA2s from snake and bee-venoms can act as acyltransferases when water is excluded (34). The localization of cPLA2γ to membrane fractions, even in the absence of prenylation (Fig. 1) means that the physical relationship between the cPLA2γ active site and the membrane needs to be considered. Sequence analysis of cPLA2γ by HMMTOP (35) predicts a transmembrane helix near the N-terminus (amino acids 44-63). This putative transmembrane helix does not include the potential active site segments, $GVS^{82}GS$ identified by sequence alignment of cPLA2γ with cPLA2α ($GLS^{228}GS$), (36, 37), but this cannot rule out an influence of the membrane on active site exposure to the aqueous environment. In addition, there is some difference of fatty acid preference between transacylation (Fig. 3C) and PLA2 activities of cPLA2γ; PLA2 activity has been shown to exhibit no fatty acid selectivity (19, 21) while the transacylation preferred arachidonic acid over linoleic acid (Fig. 3C). The difference may be due to the dimensions of the catalytic center that occupied by the different fatty acid, because the transacylation needs the space for attack of another lysophospholipid.

One major structural difference between cPLA2γ and cPLA2α is that cPLA2γ lacks a C2 domain, but instead has a CAAX box motif at its C-terminus (17, 18, 20). In other

proteins, the CAAX box motif is a signal for post-translational modification, including prenylation, protease cleavage, and carboxyl methylation (25-29). Such modifications are thought to be important for the functions and subcellular localization of CAAX-proteins, including ras p21GTPase. The presence of the potential lipidation motifs at the cPLA2γ C-terminus raised the possibility that this region might function in lipid binding and regulating activity.

It was initially expected that blocking the C-terminal processing of cPLA2γ would result in a soluble protein for several reasons. First, the prototype enzyme, cPLA2α, was isolated as a cytosolic protein from platelets (38) and leukocytes (39, 40). Further, most CAAX proteins, including Ras p21GTPase, are soluble until post-translational modifications render them more hydrophobic, facilitating their interaction with membranes (25-29). However, the results shown in Figs. 1 and 2 demonstrate that recombinant cPLA2γ and cPLA2γ SCLA expressed in yeast, insect and mammalian cells were localized to membrane fractions. cPLA2γ binding to membranes was tight, because it was not disrupted by high salt or mild alkali treatment, even for the SCLA mutant (Fig. 1B).

Several calcium-independent phospholipases are known to be present in heart. Hazen *et al.* (41) and McHowat and Creer (42) have identified membrane-bound, calcium-independent PLA2 activity that prefers plasmalogen, a lipid abundant in the myocardium, as a substrate. These enzymes, including iPLA2, increase hydrolysis of plasmalogen under hypoxic conditions, such as in ischemia, and it is thought that such increased PLA2 activity leads to accumulation of lysophospholipids and injury to the heart tissue because of membrane disruption (34). LPC is also known to induce arrhythmia (43, 44). cPLA2γ is abundantly expressed in heart (17, 18), and cPLA2γ can metabolize lysophospholipids through LPLase A and LPL dismutase reactions (Figs. 1, 2 and 4). In addition, cPLA2γ has CoA-independent transacylation activity capable of acylating 1-O-alkyl-GPC and 1-O-alkenyl-GPE (Fig. 3), lysophospholipids that are resistant to LPLase A activity. Thus, cPLA2γ may play an important role in protection against ischemia-induced injury to heart muscle through its conversion of lysophospholipids to less toxic lipids.

RCE1 and AFC1 were identified in yeast as RAS- and a-factor (yeast mating pheromone) converting enzymes, respectively (28, 29). The structure and properties of each

enzymes are quite distinct, but the two enzymes possess the same catalytic activity (28, 29, 45-47). Homologs of RCE1 and AFC1 are widely distributed from yeast to mammals (28, 29, 45-49). The decreased expression of wild type cPLA2γ (but normal expression of cPLA2γ with a disrupted CAAX box) in yeast strains with defects in RCE1 and/or AFC1 (Fig. 2) clearly implicates both proteases in the proper processing of cPLA2γ. RCE1 and cPLA2γ are known to be highly expressed in heart (45, 50), suggesting that RCE1 may be involved in the processing of cPLA2γ in vivo. Mice lacking RCE1 in heart tissue have been established using *Cre/loxP* recombination techniques (51). The heart-specific *Rce1* knockout mice initially appeared healthy but developed dilated cardiomyopathy and started dying at 3-5 months of age; by 10 months of age, 70% of the mice had died. Given the decreased expression of cPLA2γ we observed in yeast defective in RCE1, it seems possible that the Rce1 knockout mice had decreased cPLA2γ levels in cardiac tissues, with a consequent decreased capacity for protective metabolism of lysophospholipids. The connection between defect of cPLA2γ function and heart failure would be important issue to investigate in near future.

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FIGURE AND SCHEME LEGENDS

Scheme 1: Processing of CAAX motif of cPLA2γ .
cPLA2γ undergoes three step of post-translational modification. The membrane distribution and activity in each form of processing step were described. We postulated and illustrated that cPLA2γ was integrated membrane protein because cPLA2γ did not solubilized by Na_2CO_3-treatment and a membrane spanning domain was predicted in the amino acid sequences of cPLA2γ by HMMTOP as described in the text.

Scheme 2: Potential roles for phospholipase in phospholipid transacylation and hydrolysis. R1, R2 and R3 represent fatty acyl moieties. X and Y represent polar head groups. Participation of a water molecule in catalysis results in hydrolysis, whereas participation of lysophospholipid leads to transacylation.

Figure 1: Subcellular distribution of cPLA2γ expressed in HEK293 and *Sf9* cells. A, Distribution of cPLA2γ and cPLA2γSCLA in fractions isolated from HEK293 cells transfected with expression plasmid harboring wild type cPLA2γ or mutant cPLA2γ SCLA. Subcellular fractions were prepared as described in the text and were assayed for the recombinant protein using antibodies against the c-myc epitope (upper panel) or for LPLase A activity (lower panel). Upper panel: heavy mitochondria (HM) fraction, lanes 1 and 4; light mitochondria and microsome (Mc) fraction, lanes 2 and 5; cytosol (S) fraction, lanes 3 and 6. Lower panel: columns 1-6 correspond to lanes 1-6 in upper panel; LPLase A activity was also measured in mock-transfected cells (columns 7-9). B, Distribution of cPLA2γ and cPLA2γ SCLA mutant after treatment with high salt or alkali. *Sf9* cells expressing wild type cPLA2γ or mutant cPLA2γ SCLA were homogenized and the light mitochondria / microsome membrane fraction prepared. The membrane fractions were treated with either 1 M NaCl (lanes 1, 2, 5, 6) or 0.1 M Na_2CO_3 (lanes 3, 4, 7, 8) and then centrifuged to isolate the membrane pellet (P, lanes 1, 3, 5, 7) and the solubilized material (S, lanes 2, 4, 6, and 8) for analysis by Western blotting with antibody against the c-myc tag (upper panel) and assay of LPLase A activity (lower panel). Details are described in EXPERIMENTAL PROCEDURES.

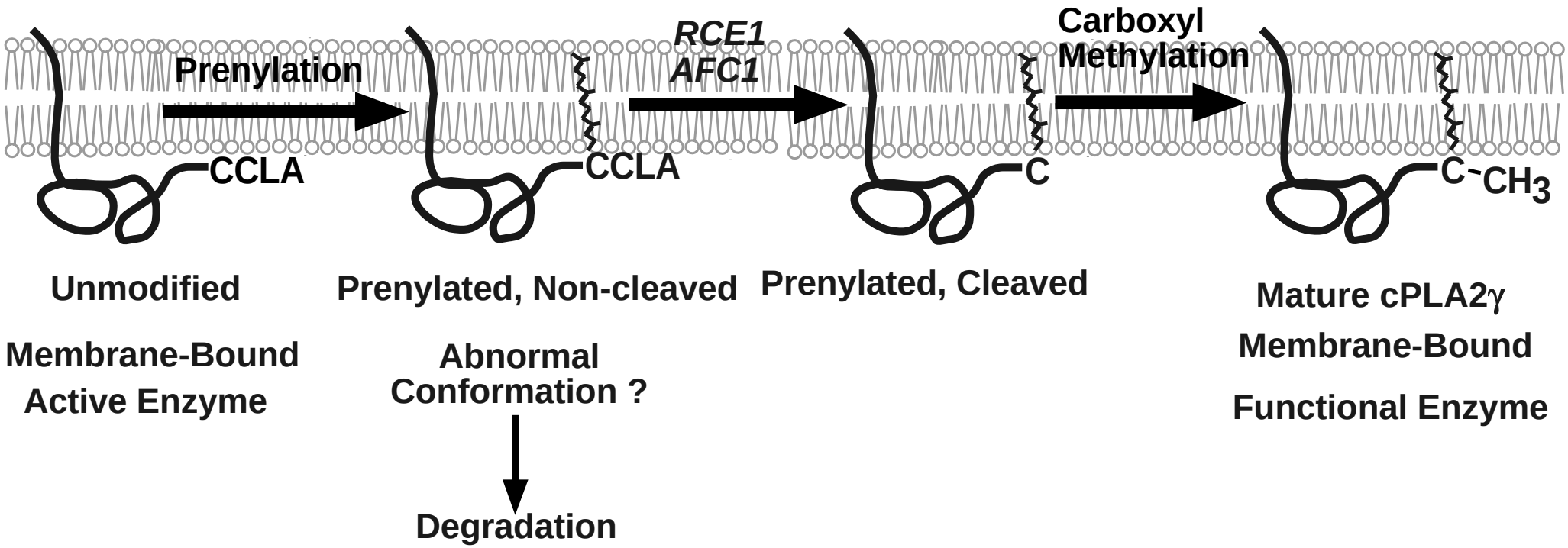
Figure 2: Effects of defects in prenyl protein proteases *AFC1* and *RCE1* on expression of recombinant cPLA2γ and cPLA2γ SCLA mutant in yeast. A, Western blotting of recombinant cPLA2γ produced in wild type and prenyl protein protease-deficient yeast. Membrane (lanes 1, 2, 3, and 4) or cytosol (lanes 5, 6, 7, and 8) isolated from yeast strains JRY5314 (wild type, lanes 1 and 5), JRY5315 (*afc1Δ::HIS3*, lanes 2 and 6), JRY5316 (*rce1Δ::TRP1*, lanes 3 and 7), and JRY5317 (*afc1Δ::HIS + rce1Δ::TRP1*, lanes 4 and 8), all harboring cDNA for cPLA2γ, were analyzed by Western blotting with antibody against the c-myc tag. B, The same fractions (#1-8) were assayed for LPLase A activity, along with membranes (#9) and cytosol (#10) from mock-transfected cells. C, Western blotting (left) and LPLase activity (right) of recombinant cPLA2γ SCLA mutant in membrane fractions of

wild type (JRY5314) and prenyl protein protease-deficient (JRY5317; *afc1Δ::HIS3, rce1Δ::TRP1*) yeast. Details are described in EXPERIMENTAL PROCEDURES.

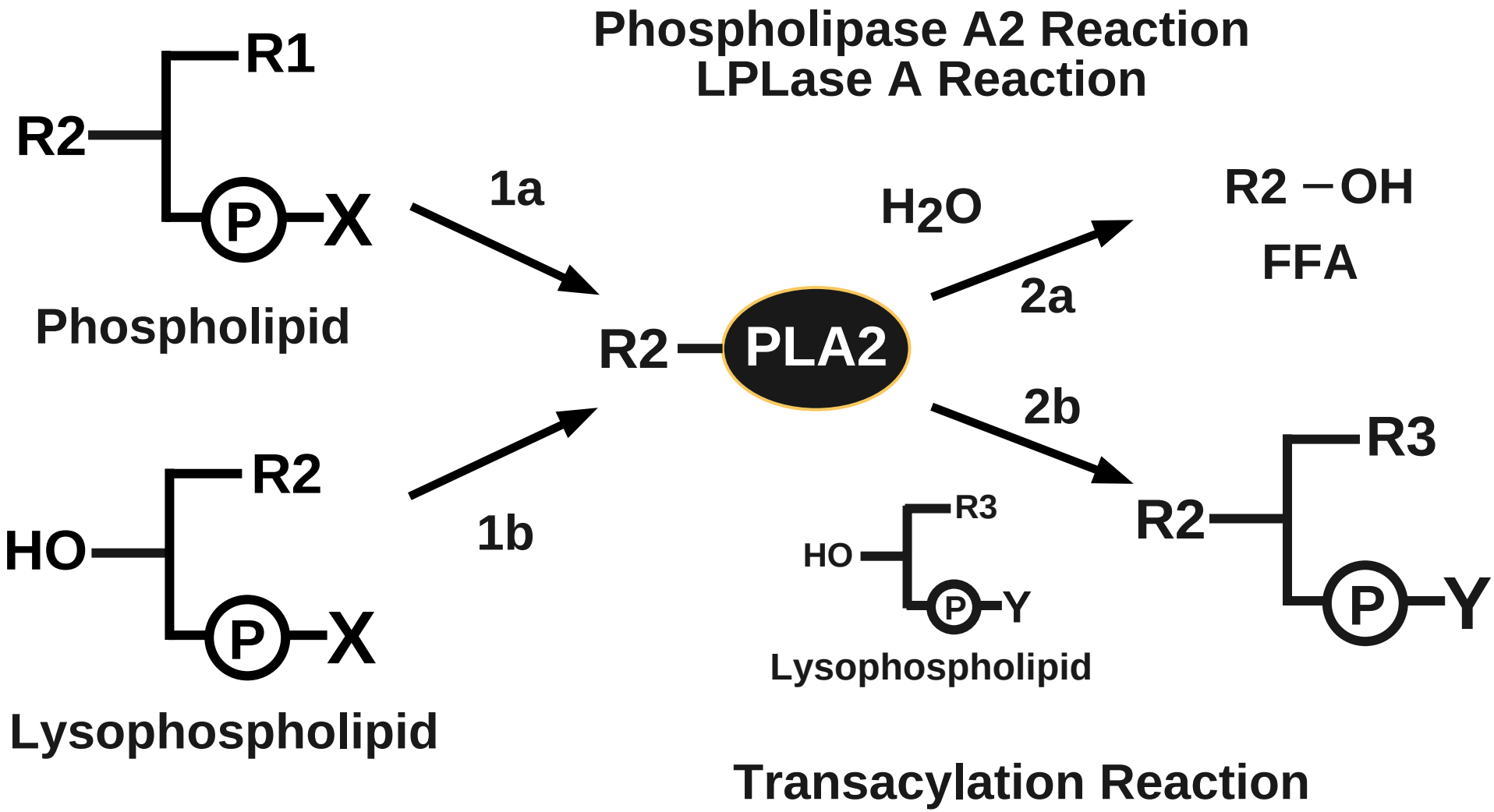
Figure 3: Transacylation activities of cPLA2γ and cPLA2γ SCLA mutant. A, Time courses of [^{3}H]diradyl-GPC formation by membrane fractions from mock-transfected yeast (open diamonds), yeast expressing cPLA2γ (closed circles) or yeast expressing the cPLA2γ SCLA mutant (open circles) with 50 μM 1-*O*-[^{3}H]hexadecyl-GPC as substrate. B, Substrate (1-*O*-[^{3}H]hexadecyl-GPC) concentration dependence of [^{3}H]diradyl-GPC formation by membrane fractions from yeast expressing cPLA2γ (closed circles) or yeast expressing the cPLA2γ SCLA mutant (open circles). The lines represent nonlinear least squares fits to the data points. C, Analysis of acyl donor and acceptor specificities of the transacylation reaction. Aliquots of membrane fraction from yeast expressing cPLA2γ were incubated with the indicated radiolabeled acyl donor and 1-O-alkyl-GPC or 1-alkenyl-GPE as acyl acceptor. Transfer of fatty acid was quantitated after TLC separation of acyl donor and product phospholipids. D, Effect of potential cofactor addition (100 μM CoA or $CaCl_2$) or depletion (pretreatment with 0.5 unit/ml apyrase and 1 mM $MgCl_2$ to degrade ATP) on transacylation activity of membranes from yeast expressing cPLA2γ, using 1-*O*-[^{3}H]alkyl-GPC as acceptor. Details are described in EXPERIMENTAL PROCEDURES.

Figure 4: LPL dismutase activities of cPLA2γ and cPLA2γ SCLA mutant. A, TLC analysis of reaction products when membranes from HEK293 cells expressing cPLA2γ were incubated with [^{14}C]LPC. The developing solvent was petroleum ether:diethylether: acetic acid (70:30:1, v/v, lane 1) or chloroform:methanol:ammonia (65:35:4, v/v, lane 2). B, Effects of potential cofactor addition (100 μM CoA or $CaCl_2$) or depletion (pretreatment with 0.5 unit/ml apyrase and 1 mM $MgCl_2$ to degrade ATP) on LPL dismutase activity of membranes from HEK293 cells expressing cPLA2γ or the cPLA2γ SCLA mutant; [^{14}C]LPC was the substrate. C and D, Substrate ([^{14}C]LPC) concentration dependences of LPL dismutase (C) and LPLase (D) activities of membranes from HEK293 cells expressing cPLA2γ or the cPLA2γ SCLA mutant. E, Effect of lyso plasmalogen concentration on formation of radiolabeled PC (closed circles) or diradyl-GPE (open circles) in incubations

of [^{14}C]LPC with membranes from HEK293 cells expressing cPLA2γ. Details are described in EXPERIMENTAL PROCEDURES.



Scheme. 1 **Yamashita et al**



Scheme. 2 Yamashita et al

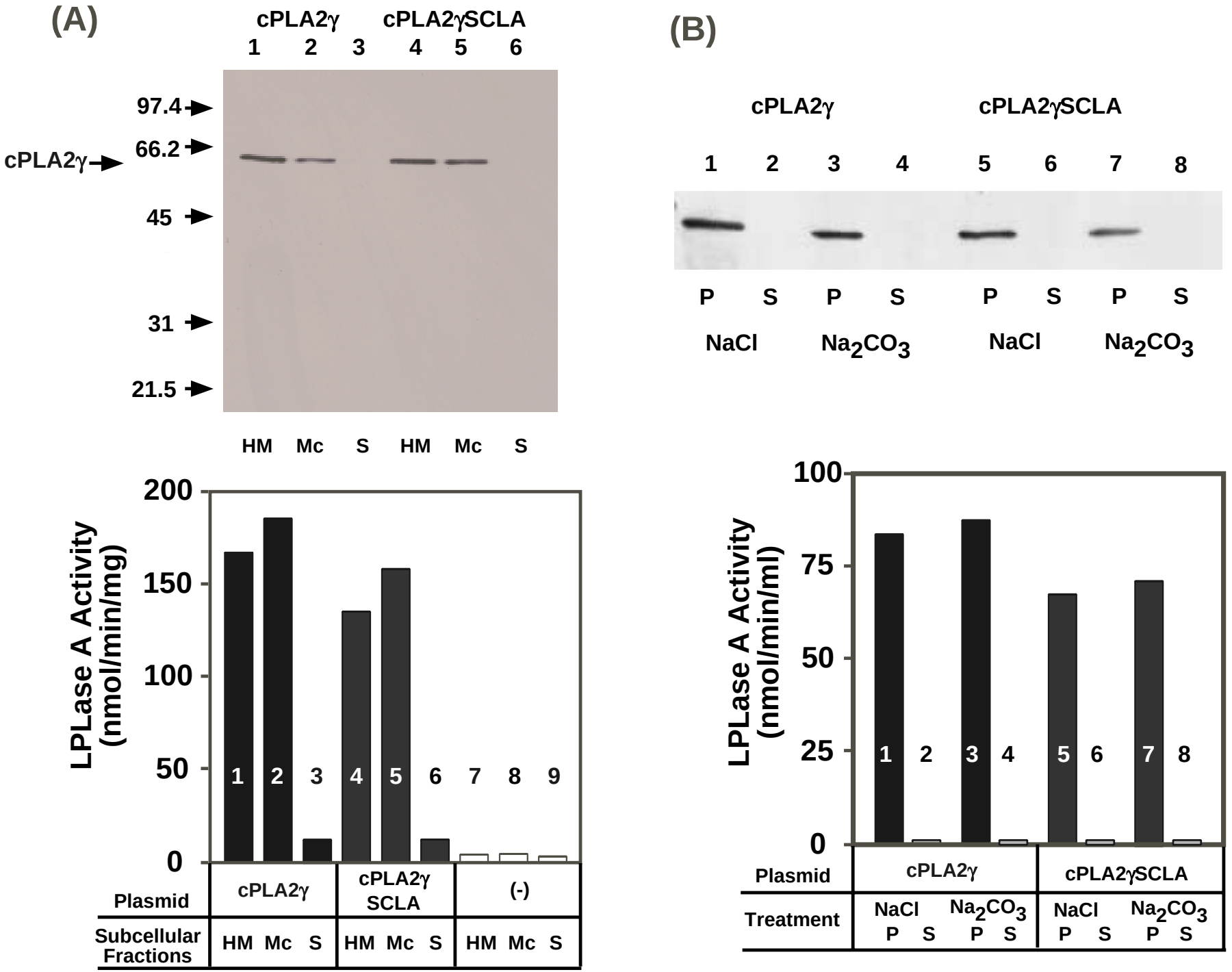


Fig. 1 Yamashita et al

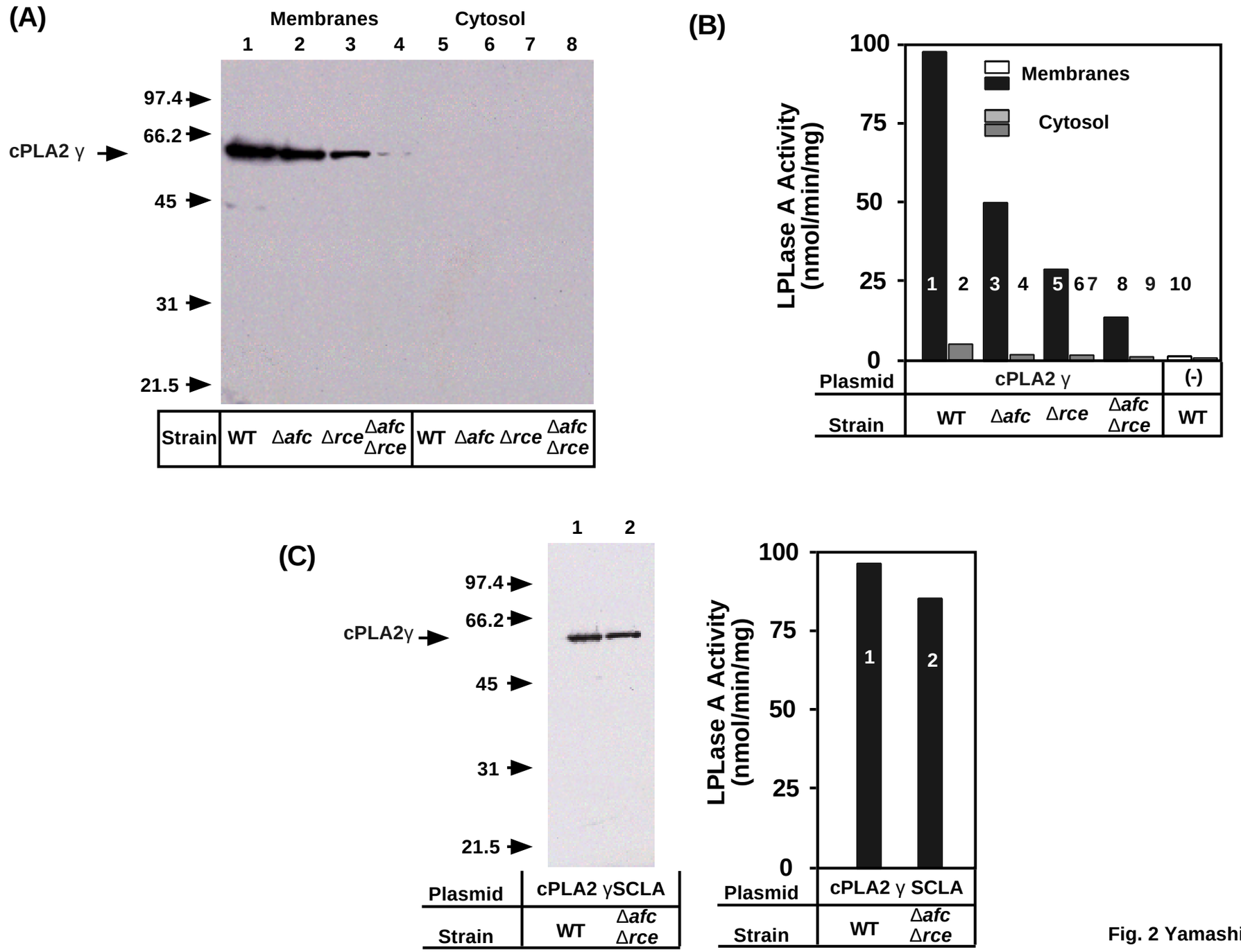
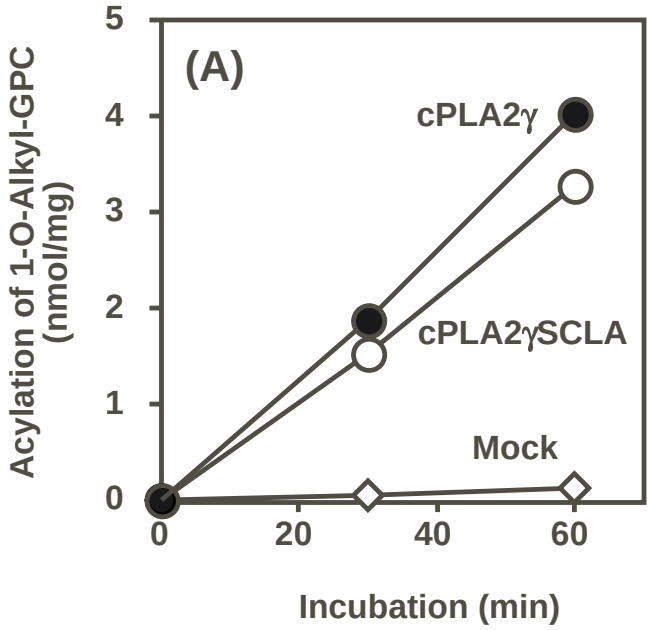
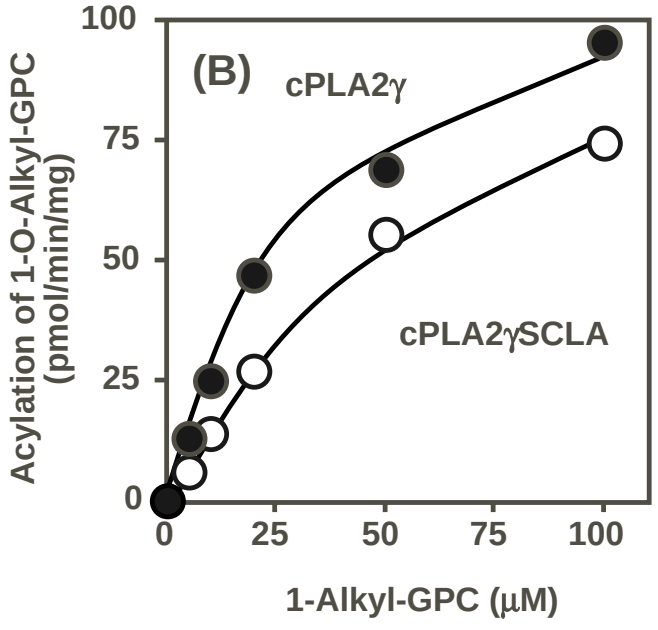
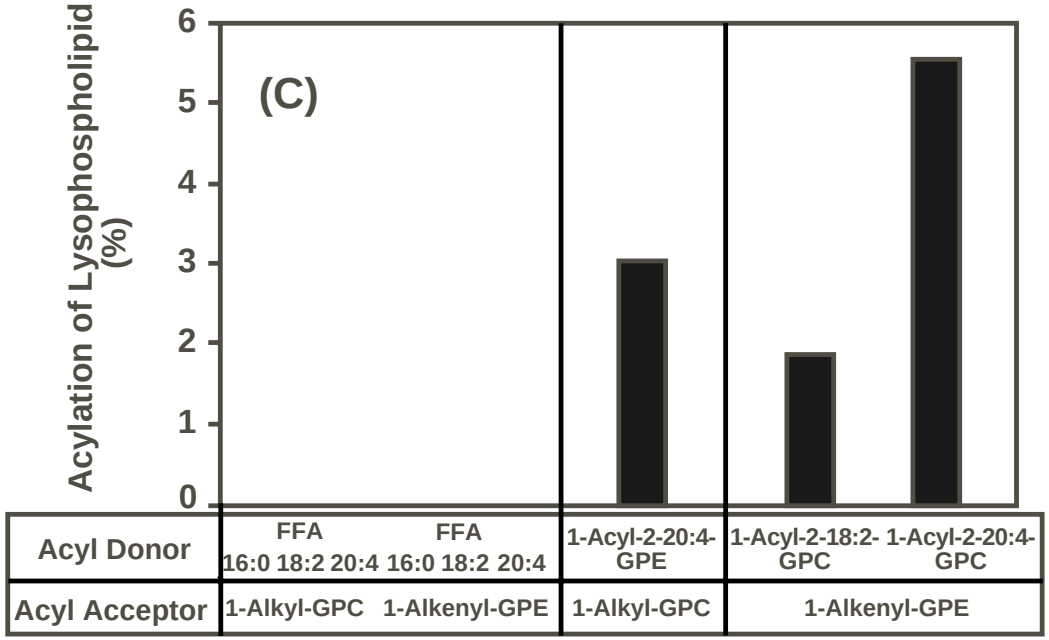


Fig. 2 Yamashita et al







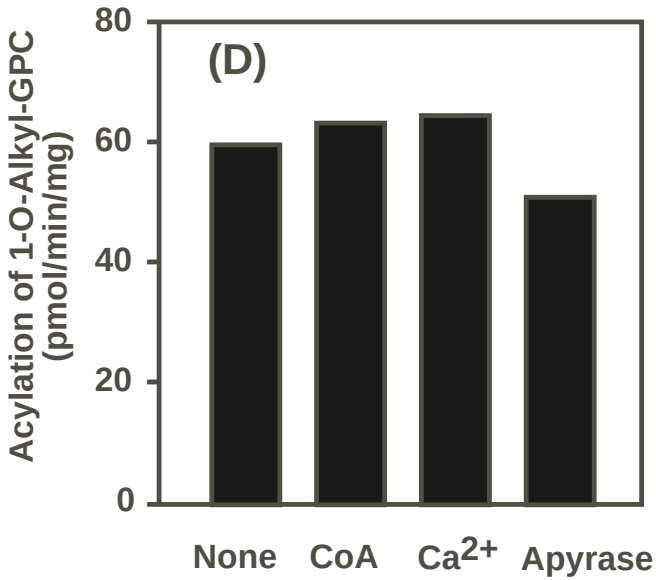


Fig. 3 Yamashita et al

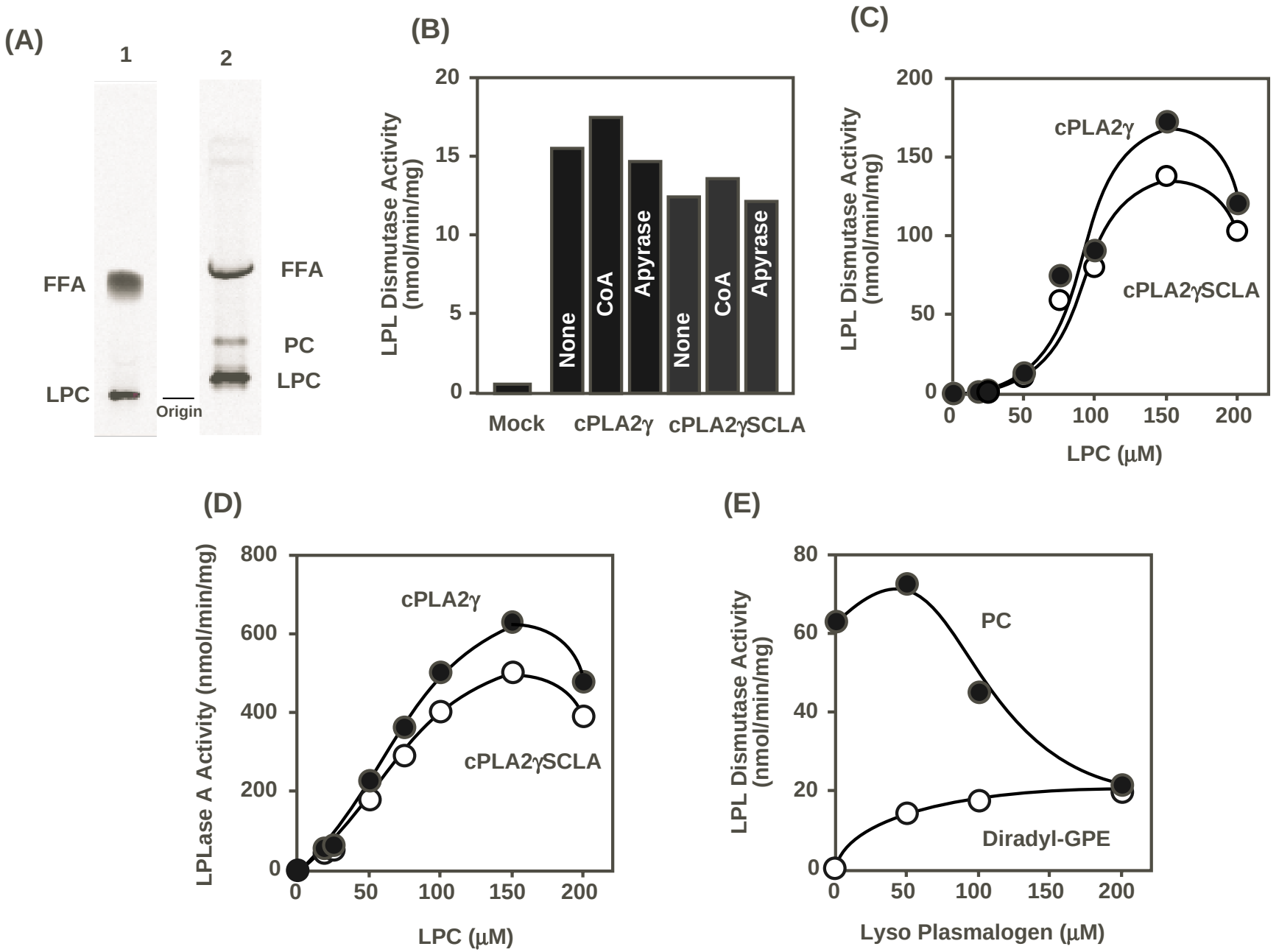


Fig. 4 Yamashita et al