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# Roles of C-Terminal Processing, and Involvement in Transacylation Reaction of Human Group IVC Phospholipase A2 (cPLA2γ)

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**The phospholipase A2s (PLA2s) are a diverse group of enzymes that hydrolyze the *sn*-2 fatty acid 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 the 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 the prenylated and non-cleaved form of cPLA2γ using yeast mutants lacking prenyl protein proteases *AFC1* (a-factor–converting enzyme) and *RCE1* (Ras-converting enzyme) revealed decreased expression in the mutant strain compared to that in the 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 it may be involved in fatty acid remodeling of phospholipids and the clearance of toxic lysophospholipids in cells.**

**Key words: CAAX box motif, cPLA2γ, lysophospholipase, phospholipase A2, prenylprotein endoprotease, transacylation.**

Abbreviations: 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 isomers.

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: (i) A phospholipid is cleaved by phospholipase A2 (PLA2) to produce a lysophospholipid and free fatty acid (FFA); (ii) another FFA is activated by acyl-CoA synthetase with the consumption of ATP; and (iii) the fatty acyl moiety of acyl-CoA is transferred to the lysophospholipid by acyl-CoA:lysophospholipid acyltransferases (*1–6*).

Transacylation between phospholipids and (lyso)phospholipids is considered to be important for fatty acid remodeling (*4–6*). Several types of transacylation system are known, including CoA-dependent and independent ones. 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 cofactor (*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, phosphatydylcholine (PC) and GPC being formed (dismutase reaction). Despite the importance of CoA-independent transacylation systems and LPL dismutase in phospholipid remodeling, the mechanism underlying the transacylation systems 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*), although 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

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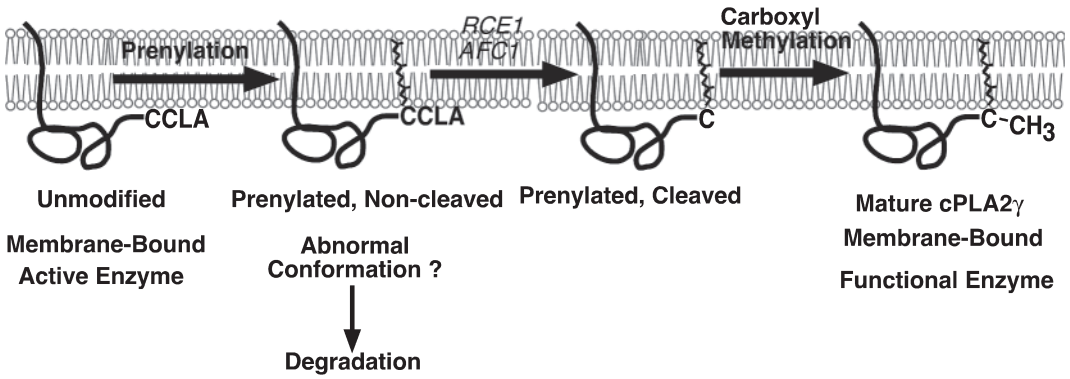



Scheme 1. **Processing of the CAAX motif of cPLA2γ.** cPLA2γ undergoes three steps of post-translational modification. The membrane distribution and activity in each processing step are described. We postulated that cPLA2γ was an integrated membrane protein because it was not solubilized on $Na_2CO_3$-treatment and a membrane-spanning domain in the amino acid sequence of cPLA2γ was predicted by HMMTOP, as described in the text.

be involved in phospholipid remodeling (*21*). cPLA2γ exhibits 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 expressed in insect cells has been 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, although 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 and LPLase A/transacylase activities, suggesting that it may be involved in phospholipid remodeling. We further discuss its 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 Pon–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 Chemicals. 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, 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 sites are underlined. In one construct, the nucleotide sequence coding for the CAAX box tetrapeptide at the C-terminus of cPLA2γ (CCLA) was changed so as to code for SCLA using the same forward primer and an alternative reverse primer: 5′-AAAAAAGCTAGCCTATGCCAAGCAGGAACTTCG-3′. After digestion with *Sal*I and *Nhe*I, the PCR products were subcloned into the *Xho*I–*Nhe*I sites of pESC-URA to yield a plasmid carrying a sequence corresponding to the 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 an N-terminal c-myc epitope were excised from the corresponding plasmid (pESC/cPLA2γ or pESC/

cPLA2γ SCLA) by digestion with *Sma*I and *Nhe*I, and then subcloned into the *Sma*I–*Xba*I sites of the pVL1393 baculovirus transfer vector (Invitrogen) to yield 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 digestion with *Sma*I and *Not*I, and then subcloned into the *Eco*RV–*Not*I sites of the pcDNA4/TO expression vector (Invitrogen) to yield plasmids pcDNA4/TO/cPLA2γ and pcDNA4/TO/cPLA2γ SCLA.

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

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*). The 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 in complete synthetic medium minus uracilplus 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 then 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 at 48 h 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 the 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 ($7 \times 10^6$) were plated onto 150-mm tissue culture plates and then infected with either the control virus or recombinant virus. After 72 h, the infected *Sf9* cells were harvested and subcellular fractions were prepared.

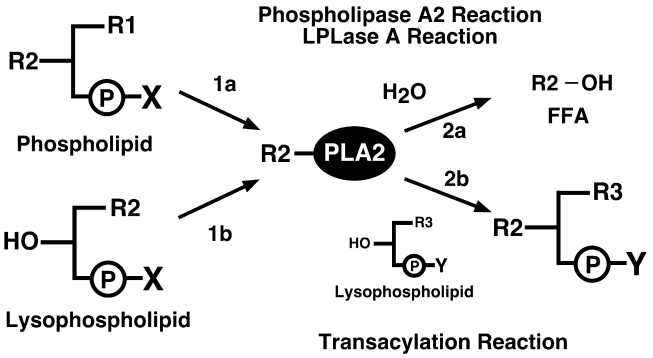
*Preparation of Cytosol and Membrane Fractions from Yeast, HEK293 and Sf9 Cells*—Yeast cells were disrupted using zirconia/silica beads (Biospec, Inc.) in 0.6 M mannitol/15 mM Tris-HCl (pH 7.4)/0.5 mM EDTA/protease inhibitors (1 mM PMSF, 0.1 mM AEBSF, 5 μg/ml aprotinin, 5 μg/ml bestatin, 5 μg/ml E-64, 10 μg/ml leupeptin, and 5 μg/ml pepstatin A). After removing cell debris and nuclear material by centrifugation at 2,000 rpm for 5 min, the lysate was centrifuged at 105,000 × *g* for 1 h to separate the 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 × *g* for 10 min to remove cell debris and nuclear materials. The supernatant was centrifuged at 5,000 × *g* for 10 min, and the resultant pellet was washed with STE buffer, suspended in the same buffer, and used as the heavy mitochondria fraction (HM). The supernatant was centrifuged at 105,000 × *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 fraction was determined by the BCA method using bovine serum albumin as a standard.

*NaCl and $Na_2CO_3$ Treatment of Membrane Fractions*—Light mitochondria and microsome fractions were prepared from *Sf9* cells expressing wild type cPLA2γ or mutant cPLA2γ SCLA as described above. The membrane fractions were treated with 1 M NaCl or 0.1 M $Na_2CO_3$ (mild alkaline), and then centrifuged at 105,000 × *g* for 60 min. The resultant pellets (insoluble fractions) and supernatants (solubilized fractions) were subjected to Western blotting with antibodies against the c-myc epitope and then assayed for LPLase A activity.

*LPLase A Activity*—LPLase A activity was determined by measurement of the [$^{14}$C]fatty acid released from [$^{14}$C]LPC (1-[$^{14}$C]palmitoyl). Aliquots of [$^{14}$C]LPC (110,000 dpm/assay) were dried under a stream of $N_2$ gas and then suspended at 50 μM by brief sonication (Branson bath type sonifier) in 50 mM Tris-HCl (pH 7.5) containing 2 mM EDTA before incubation with the enzyme at 30°C for 2 min. In some cases, the concentration of [$^{14}$C]LPC was varied. The reaction was terminated by the addition of 3 ml of chloroform:methanol (1:2, v/v). After the addition of the 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 with development with petroleum ether:diethyl ether:acetic acid (70:30:1, v/v). The band corresponding to the 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 with 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, lipid extracts were separated by TLC with development with chloroform:methanol:ammonia (65:35:4, v/v), and the radioactivity in PC was measured as described above.




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. The participation of a water molecule in catalysis results in hydrolysis, whereas the participation of a lysophospholipid leads to transacylation.

*Measurement of Transacylation Activity (10, 11)*—The transacylation activity 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 under $N_2$ gas and then 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 the enzyme source at 30°C. The reaction was terminated by the 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 with chloroform:methanol: ammonia (65:35:4, v/v). The band corresponding to the product PC was visualized and its radioactivity was determined as described above.

*Analysis of Transacylation Reaction Products*—After separation of products of the transacylation reaction by TLC, the bands corresponding to diradyl-GPC (1-alkyl-2-acyl-GPC or 1,2-diacyl-GPC) were scraped off from the TLC plate into tubes containing chloroform:methanol: water (1:2:0.8, v/v). Diradyl-GPC was extracted by stirring the mixture, followed by 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 with development 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 then transferred to a nitrocellulose membrane (Protran BA85; Schleicher & Schuell). After blocking the membrane with 5% bovine serum albumin, it was incubated with monoclonal antibodies against the c-myc epitope. Immunoreactive bands were visualized as 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 determine the physiological role of cPLA2γ. We previously investigated the CoA-independent transacylation system that is 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 participant in the transacylation activity.

To determine the biochemical and enzymological properties of cPLA2γ, we expressed the protein in various expression systems, including yeast *Saccharomyces cerevisiae*, *Sf9* insect cells and mammalian HEK293 cells. First, we examined the expression of cPLA2γ in HEK293 cells transfected with a 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. 1A, a 61 kDa band was detected for membrane fractions, such as the heavy mitochondria (lane 1) and microsomal (lane 2) fractions. No signal was detected for 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. 1B) and yeast cells (Fig. 2).

The 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 the C-terminus and thus block prenylation (cPLA2g SCLA). Surprisingly, the SCLA mutant remained localized to membrane 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 the SCLA Mutant Catalyze LPLase A Activity*—We measured LPLase A activity because cPLA2γ has considerable LPLase A activity (*19*, *20*), and because the addition of an N-terminal epitope tag was reported to affect PLA2 activity rather than LPLase A activity (*19*). The LPLase A assay involved [$^{14}$C]LPC (1-[$^{14}$C]palmitoyl-GPC) as the substrate and membranes from HEK293 cells expressing cPLA2γ. Transfection with the cPLA2γ vector markedly increased the LPLase A activity in the membrane fractions; 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 obtained 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

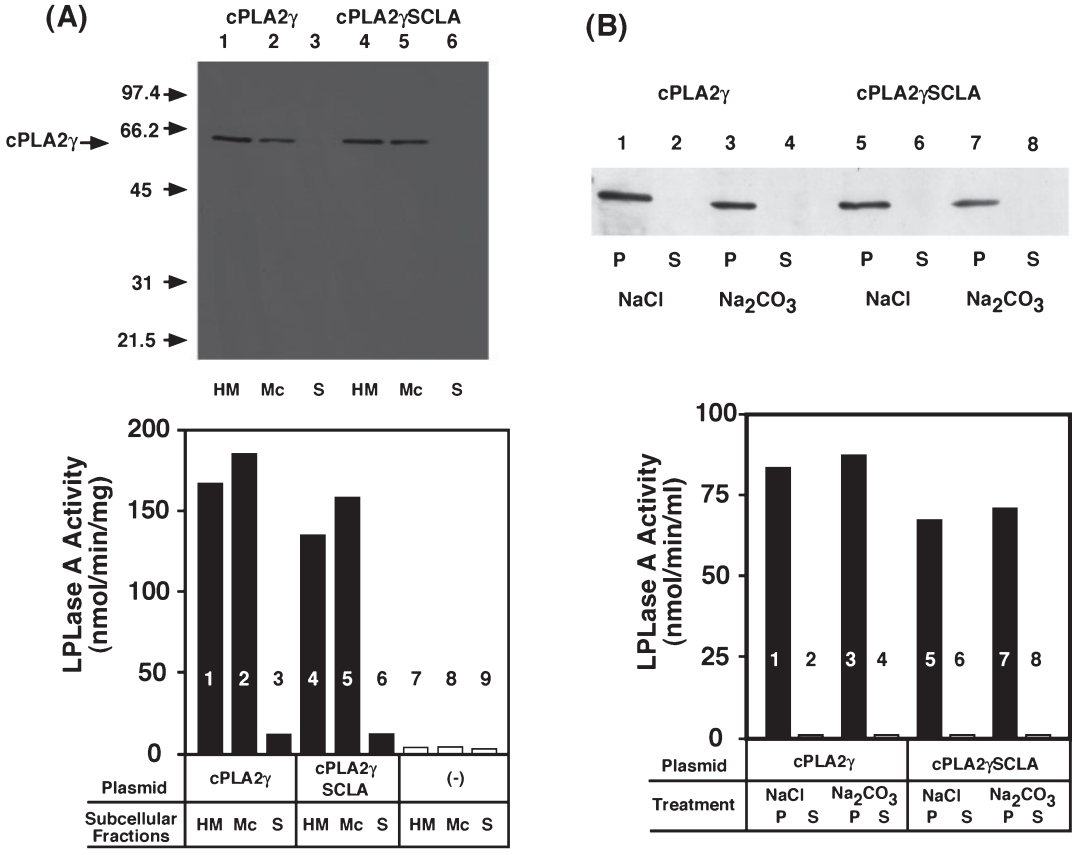



Fig. 1. **Subcellular distribution of cPLA2γ expressed in HEK293 and *Sf9* cells.** A: Distribution of cPLA2γ and cPLA2γSCLA in fractions isolated from HEK293 cells transfected with an expression plasmid harboring the wild type cPLA2γ or mutant cPLA2γ SCLA. Subcellular fractions were prepared as described in the text and then 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 the upper panel; LPLase A activity was also measured in mock-transfected cells (columns 7–9). B: Distribution of cPLA2γ and the cPLA2γ SCLA mutant after treatment with high salt or alkali. *Sf9* cells expressing the wild type cPLA2γ or mutant cPLA2γ SCLA were homogenized and then light mitochondria/microsome membrane fractions were 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 an antibody against the c-myc tag (upper panel), and assaying of LPLase A activity (lower panel). The details are given under "EXPERIMENTAL PROCEDURES."

obtained with the SCLA mutant form of cPLA2γ. Cells expressing the recombinant protein exhibited elevated LPLase A activity in membrane fractions, although the activity of the SCLA mutant was somewhat lower than that of the wild type cPLA2γ (Fig. 1). The results suggest that C-terminal processing is not essential for LPLase A activity.

We analyzed the mode of binding of cPLA2γ to membranes. The membrane fractions 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 as LPLase A activity. Neither the wild type cPLA2γ nor the SCLA-mutant were solubilized on 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 Proteases 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 that in mammals, and mutant strains defective 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 yield the 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 the wild type yeast (Fig. 2A, lanes 1–4). Expression of cPLA2γ in the double-mutant yeast (Δ*afc1*, Δ*rce1*) was even lower, although still detectable (Fig. 2A, lane 4). Recombinant cPLA2γ was not detected in the cytosolic fraction of any of the prenyl protein protease-deficient

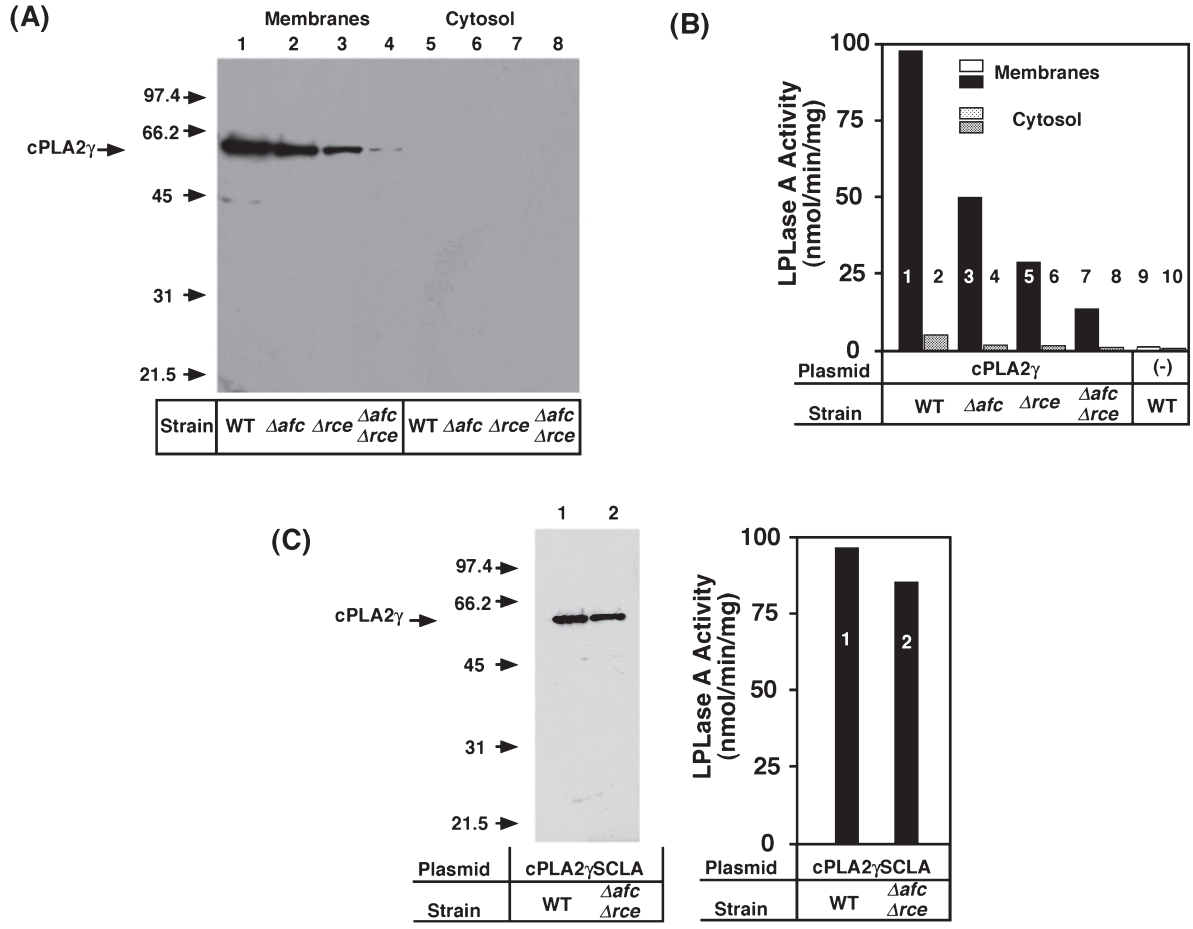



Fig. 2. **Effects of defects in prenyl protein proteases *AFC1* and *RCE1* on expression of recombinant cPLA2γ and the cPLA2γSCLA mutant in yeast.** A: Western blotting of recombinant cPLA2γ produced in the wild type and prenyl protein protease–deficient yeast. Membranes (lanes 1, 2, 3, and 4) or cytosol (lanes 5, 6, 7, and 8) isolated from yeast strains JRY5314 (wild type, lanes 1 and 5), JRY5315 (*afc1*Δ*::HIS3*, lanes 2 and 6), JRY5316 (*rce1*Δ*::TRP1*, lanes 3 and 7), and JRY5317 (*afc1*Δ*::HIS* + *rce1*Δ*::TRP1*, lanes 4 and 8), all harboring cDNA for cPLA2γ, were analyzed by Western blotting with an 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) anamembrane fractions of the wild type (JRY5314) and prenyl protein protease–deficient (JRY5317; *afc1*Δ*::HIS3, rce1*Δ*::TRP1*) yeast. The details are given under "EXPERIMENTAL PROCEDURES."

strains (Fig. 2A, lanes 5–8). The results obtained on Western blotting were confirmed by measurement of LPLase A activity (Fig. 2B). LPLase A activity in the membrane fractions from the Δ*afc1* and Δ*rce1* yeast mutants was lower than that in the 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 at comparable levels in the double-mutant (Δ*afc1*, Δ*rce1*) and wild type yeast, as assessed by Western blotting and as LPLase A activity (Fig. 2C).

Taken together, these results indicate that defects in RCE1p and AFC1p decrease the expression of cPLA2γ in yeast, and that both AFC1p and RCE1p can process the cPLA2γC-terminus. Because the cPLA2γ gene was induced by the same, potent promoter ($P_{GAL1}$) in all the yeast strains, defects in RCE1p and AFC1p probably affect cPLA2γ degradation rather than 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 lysophospholipids (Scheme 2). We therefore examined whether or not cPLA2γ catalyzed CoA-independent transacylation activity with 1-*O*-[$^3$H]hexadecyl-GPC (lyso platelet-activating factor) as a 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 in control membrane fractions (open circles), but membranes from yeast expressing wild type cPLA2γ exhibited markedly higher activity, indicating that the recombinant protein promoted transacylase activity. Membranes from yeast expressing the SCLA mutant also exhibited elevated transacylase activity. The formation of

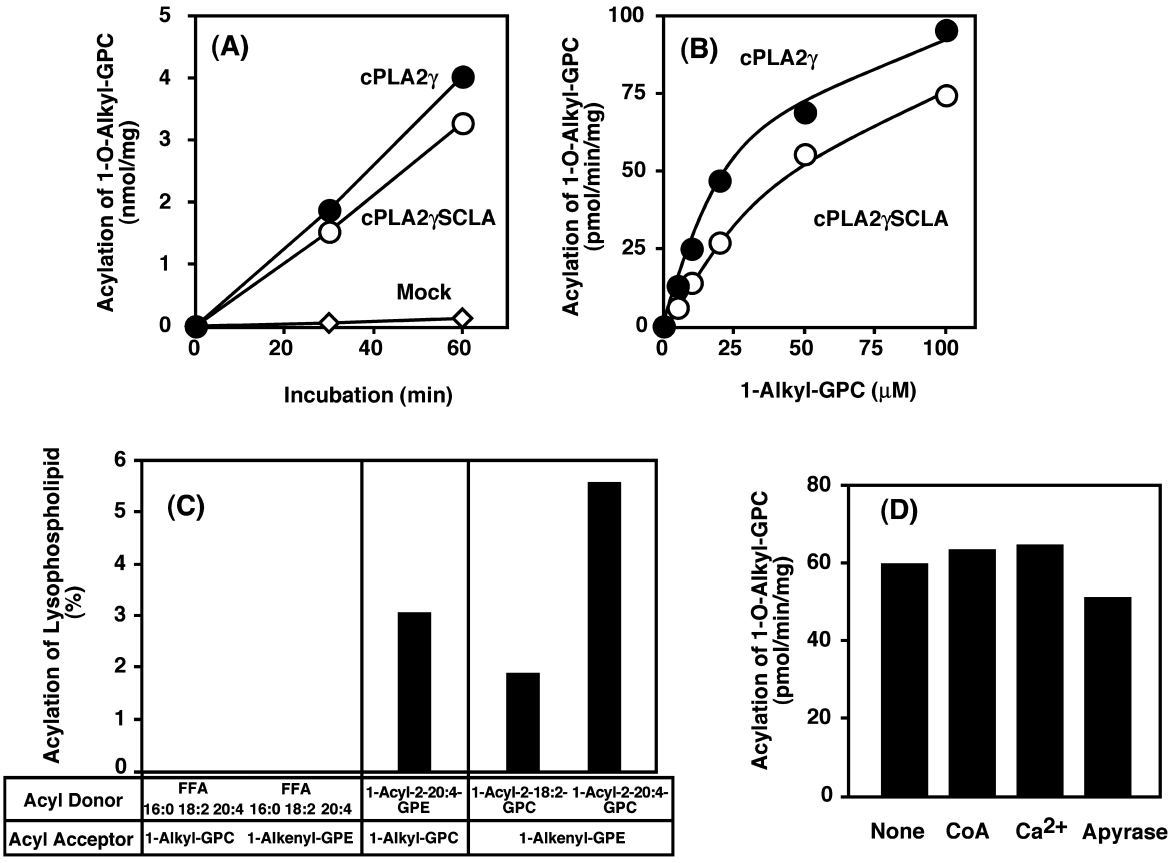



Fig. 3. **Transacylation activities of cPLA2γ and the cPLA2γ SCLA mutant.** A: Time courses of [$^3$H]diradyl-GPC formation by membrane fractions from mock-transfected yeast (open diamonds), yeast expressing cPLA2γ (closed circles), and yeast expressing the cPLA2γ SCLA mutant (open circles) with 50 μM 1-*O*-[$^3$H]hexadecyl-GPC as the 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) and yeast expressing the cPLA2γ SCLA mutant (open circles). The lines represent nonlinear least squares fits to the data points. C: Analysis of the acyl donor and acceptor specificities of the transacylation reaction. Aliquots of membrane fractions from yeast expressing cPLA2γ were incubated with the indicated radiolabeled acyl donor and 1-*O*-alkyl-GPC or 1-alkenyl-GPE as the acyl acceptor. The transfer of fatty acids was quantitated after TLC separation of the 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 the transacylation activity of membranes from yeast expressing cPLA2γ, using 1-*O*-[$^3$H]alkyl-GPC as the acceptor. The details are given under "EXPERIMENTAL PROCEDURES."

[$^3$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 yielded [$^3$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 at the *sn*-2 position of phosphatidylethanolamine was transferred to 1-*O*-hexadecyl-GPC to yield [$^{14}$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 donors in the case of cPLA2γ-catalyzed acylation in assays with 1-*O*-[$^3$H]hexadecyl-GPC.

We next examined the cofactor requirements of the cPLA2γ-catalyzed transacylation activity. Transacylation of 1-*O*-[$^3$H]hexadecyl-GPC occurred with the membrane fraction itself and the 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 investigation of the substrate specificity of the cPLA2γ transacylation activity, we found that the protein could also catalyze LPL dismutase activity (reactions 1b and 2b in Scheme 2 combined). We used [$^{14}$C]LPC (1-[$^{14}$C]palmitoyl-GPC) as the substrate, and the assay was the same as that for LPLase A activity described above. As shown on TLC analysis (Fig. 4A), the products were the [$^{14}$C]free fatty acid (LPLase A activity; reactions 1b and 2a in Scheme 2) and [$^{14}$C]PC (dismutase activity; reactions 1b

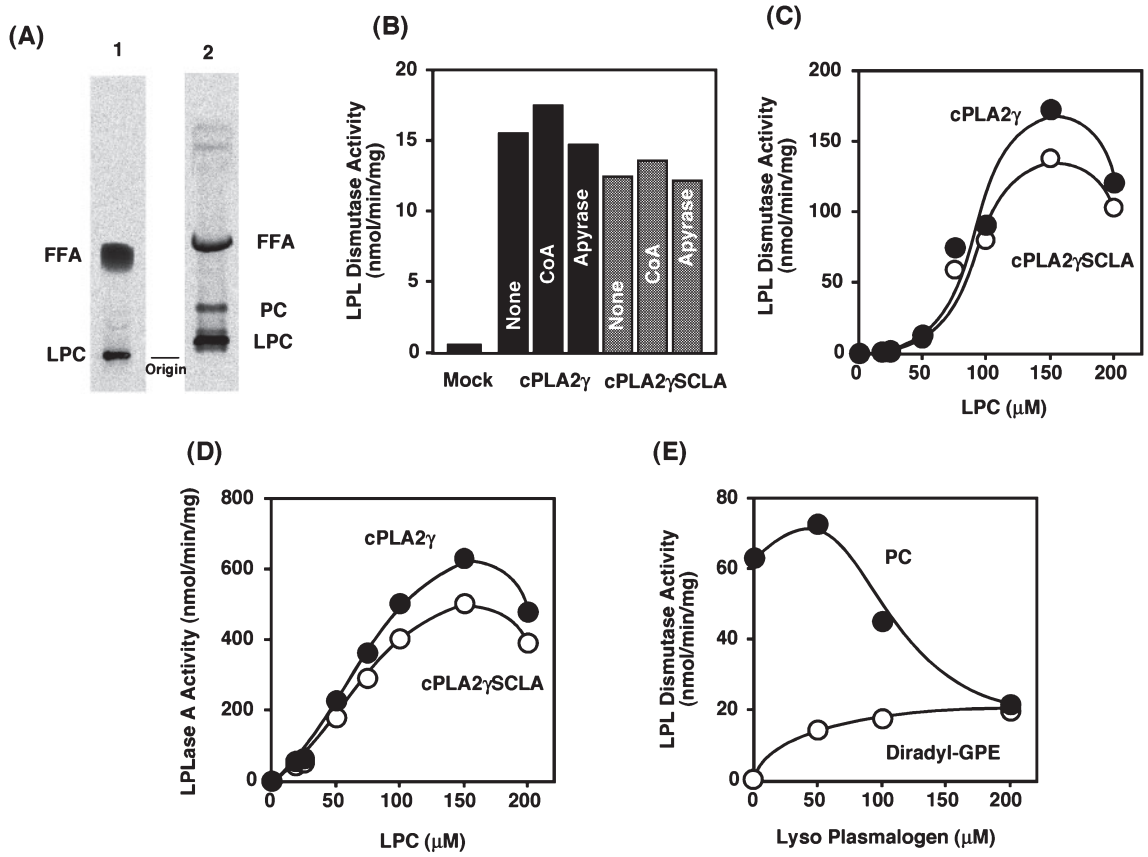



Fig. 4. **LPL dismutase activities of cPLA2γ and the cPLA2γ SCLA mutant.** A: TLC analysis of the reaction products when membranes from HEK293 cells expressing cPLA2γ were incubated with [$^{14}$C]LPC. The developing solvent was petroleum ether:diethylether: acetic acid (70:30:1, v/v, lane 1) or chloroform:methanol:ammonia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

and 2b in Scheme 2). Development 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 the [$^{14}$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 levels of [$^{14}$C]PC formation also occurred with membranes from HEK293 cells expressing the cPLA2γSCLA mutant (Fig. 4B), indicating that C-terminal prenylation was not required for LPL dismutase activity. The LPL dismutase activities of cPLA2γand cPLA2γSCLA were not appreciably affected by the addition of CoA or apyrase (Fig. 4B).

The [$^{14}$C]PC product was analyzed to investigate the mechanism of the dismutase reaction. When [$^{14}$C]PC was treated with snake venom PLA2 and analyzed by two-dimensional TLC, radioactivity was found in both the 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, there being indications of positive cooperativity and substrate inhibition (Fig. 4C). LPLase A activity also exhibited 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 were formed (Fig. 4F); digestion of the product [$^{14}$C]diradyl-GPE with snake venom PLA2 released [$^{14}$C]FFA (data not shown). This 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 decreased upon the addition of 1-*O*-alkenyl-GPE. In any case, the results suggest that cPLA2γ does not discriminate 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 exhibit 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 a deacylation-reacylation cycle was involved in the remodeling system (*1*, *2*). The Lands cycle consists of phospholipid cleavage by PLA to produce a lysophospholipid, ATP-driven activation of another FFA by acyl-CoA synthetase, and transfer of the 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 cofactor (*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 ethanolamine glycerophospholipids as acyl donors and acceptors. The transacylation activity is located in membrane fractions and occurs in a $Ca^{2+}$-independent manner, so it seemed likely a membrane-bound and $Ca^{2+}$-independent PLA2 enzyme such as cPLA2γ was involved. The present results show that cPLA2γ can indeed catalyze the fatty acid transfer from phospholipids to lysophospholipids in a CoA-independent manner (Fig. 3, A, C and D). cPLA2γ-catalyzed transacylation activity has a preference for arachidonic acid over linoleic acid in the transfer 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: (i) Formation of a fatty acyl-enzyme intermediate through the PLA2 half reaction (step 1a in Scheme 2); and (ii) fatty acid transfer from the intermediate to a 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 the 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 LPL dismutase activities is the acyl donor: the former uses phospholipids such as PC and PE, whereas the latter uses LPC. The dismutase activity of cPLA2γ is considerably greater than its PLA2 and CoA-independent transacylation activities. The ratio of the activities of LPLase A and LPL dismutase is approximately 3.5:1. The ratio of the activities of LPL dismutase and CoA-independent transacylation reaction is 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 is 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*) predicted 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 on sequence alignment of cPLA2γ with cPLA2α ($GLS^{228}GS$), (*36*, *37*), but this does not rule out an influence of the membrane on active site exposure to the aqueous environment. In addition, there is some difference in fatty acid preference between the transacylation (Fig. 3C) and PLA2 activities of cPLA2γ; PLA2 activity has been shown to exhibit no fatty acid selectivity (*19*, *21*), while transacylation prefers arachidonic acid over linoleic acid (Fig. 3C). The difference may be due to the dimensions of the catalytic center that is occupied by a different fatty acid, because the transacylation needs space for attack on 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 motif 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 of 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 mem-

branes 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 the hydrolysis of plasmalogen under hypoxic conditions, such as in ischemia, and it is thought that such increased PLA2 activity leads to the accumulation of lysophospholipids and injury to 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 the LPLase A and LPL dismutase reactions (Figs. 1, 2 and 4). In addition, cPLA2γ exhibits 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 enzyme 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 a defect in cPLA2γ function and heart failure would be important issue to investigate in near future.

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