A Novel Calcium-independent Phospholipase A₂, cPLA₂-γ, That Is Prenylated and Contains Homology to cPLA₂*

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Kathryn W. Underwood‡, Chuanzheng Song‡, Ronald W. Kriz, Xiao Jia Chang, John L. Knopf, and Lih-Ling Lin§

From the Small Molecule Drug Discovery Group, Genetics Institute, Cambridge, Massachusetts 02140

We report the cloning and characterization of a novel membrane-bound, calcium-independent PLA₂, named cPLA₂-γ. The sequence encodes a 541-amino acid protein containing a domain with significant homology to the catalytic domain of the 85-kDa cPLA₂ (cPLA₂-α). cPLA₂-γ does not contain the regulatory calcium-dependent lipid binding (CaLB) domain found in cPLA₂-α. However, cPLA₂-γ does contain two consensus motifs for lipid modification, a prenylation motif (–CCLAL) at the C terminus and a myristoylation site at the N terminus. We present evidence that the isoprenoid precursor [3H]mevalonolactone is incorporated into the prenylation motif of cPLA₂-γ. Interestingly, cPLA₂-γ demonstrates a preference for arachidonic acid at the sn-2 position of phosphatidylcholine as compared with palmitic acid. cPLA₂-γ encodes a 3-kilobase message, which is highly expressed in heart and skeletal muscle, suggesting a specific role in these tissues. Identification of cPLA₂-γ reveals a newly defined family of phospholipases A₂ with homology to cPLA₂-α.

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. Of particular interest is the role of these enzymes in the production of factors involved in mediating the inflammatory response. The phospholipases A₂ family is large, and individual members can be classified according to localization (extracellular versus intracellular), sequence homology, and biochemical characteristics. Known PLA₂ members include the secreted PLA₂s and the cytosolic PLA₂s. To date only two cytosolic PLA₂ sequences have been reported: the calcium-dependent PLA₂ (cPLA₂-α) and the calcium-independent PLA₂ (iPLA₂) (2–5). cPLA₂-α has a predicted molecular mass of 85 kDa and contains two domains, a calcium-dependent lipid binding (CaLB) domain and a catalytic domain (2, 6). The catalytic domain contains a lipase consensus sequence and a novel catalytic triad that employs a serine, an aspartate, and an arginine instead of the usual serine, aspartate, and histidine found in many lipases and serine proteases (7–9). cPLA₂-α activity is regulated by the activation of the CaLB domain in response to increased intracellular calcium (6). The activated CaLB domain translocates the enzyme to its substrate in the nuclear envelope and endoplasmic reticulum (10). cPLA₂-α activity is also increased by the phosphorylation of a MAP kinase consensus site, in response to stimulation of cells with cytokines such as tumor necrosis factor and interleukin 1 (11, 12). These same cytokines have also been found to increase the expression of cPLA₂-α (11, 12). Although there have been many studies that suggest the importance of cPLA₂ in the generation of prostaglandins and leukotrienes, the most convincing data have come from studies using mice that are genetically deficient in cPLA₂ (13, 14). Studies demonstrate that cPLA₂-α is essential for both the calcium ionophore, A23187, and lipopolysaccharide-induced prostaglandin E2 and leukotriene B₄ production in peritoneal monocytes (13, 14). The possible importance of cPLA₂ in asthma was also shown (13).

The 85-kDa calcium-independent iPLA₂, purified by two groups, shares no homology with cPLA₂-α except, like other lipases, it contains the critical consensus sequence, GXNXG (4, 5, 15). Interestingly, iPLA₂ contains a domain of eight ankyrin repeats, which may be involved in protein-protein interactions (4). iPLA₂ possesses no clear preference for a fatty acid at the sn-2 position, and it is thought to play a role in the remodeling of phospholipids (16).

Although cPLA₂-α and iPLA₂ are the only intracellular PLA₂s that have been cloned, many other PLA₂ activities, which presently seem to be distinct from cPLA₂-α and iPLA₂, have been reported (17–19). The relationship of the enzymes responsible for these activities to the known PLA₂ enzymes will be clear only upon sequence determination.

Our initial efforts to identify additional PLA₂ enzymes failed using low stringency cross-hybridization techniques with cPLA₂-α sequences.2 A search of the expressed sequence tag (EST) data base was quite successful, and two independent cPLA₂-α related gene fragments were identified. Subsequent sequence analysis of the full-length clones revealed two novel homologs of cPLA₂-α, designated cPLA₂-β and cPLA₂-γ. The characterization of cPLA₂-β will be described elsewhere.3 Here, we report the sequence and characterization of a novel 60.9-kDa calcium-independent, membrane-associated cPLA₂-γ.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—COS cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1 mM glutamine. Cells were incubated in a 37 °C humidified atmosphere with 10% CO₂. Chinese hamster ovary (CHO) cells were maintained in alpha medium (Life Technologies, Inc.) containing 50 units/ml penicillin, 50 μg/ml streptomycin, 1 mM glutamine, 1 mg/ml G418 (Life Technologies,

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‡ Both authors contributed equally to this work.
§ To whom correspondence should be addressed: Genetics Institute, 87 Cambridge Park Dr., Cambridge, MA 02140. Tel.: 617-498-8934; Fax: 617-498-8993.
1 The abbreviations used are: cPLA₂, cytosolic phospholipase A₂; CaLB, calcium-dependent lipid binding; iPLA₂, cytosolic calcium-independent PLA₂; MAP, mitogen-activated protein; EST, expressed sequence tag; PC, phosphatidylcholine; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis.
2 R. Kriz, unpublished results.
3 C. Song, X. J. Chang, K. Bean, M. Proia, J. L. Knopf, and R. W. Kriz, manuscript in preparation.
Inc.), and 10% diazoyed fetal calf serum supplemented with 10 μg each of adenosine, deoxyadenosine, and thymidine per ml for parental cells, 5 μM methotrexate for cPLA₂γ overexpressing cells, and 20 μM methotrexate for cPLA₂α overexpressing cells. Cells were incubated in a 37°C humidified atmosphere with 5% CO₂. Rabbit polyclonal antibodies 42492 and 42493 were used for immunoprecipitation and immunoblotting. Rabbit polyclonal anti-human Ras (Upstate Biotechnology) was used for immunoprecipitation.

Clone Identification—The EST clone 258543 (GenBank accession N56796) was identified by searching the GenBank EST data base using the amino acid sequence of cPLA₂. The 900-base pair EcoRI-NorI fragment from clone 258543 was used to screen 10⁶ recombinates of the library. Two oligonucleotides, 5'-GCCTATTGCAAGCAGCAACTTCGGGCACT-3' and 5'-GGGCCCTATGCCAAGCAGCAACTTCGGGCACT-3', corresponding to the 3' end of cPLA₂γ were used to amplify the 3' end coding region of cPLA₂γ by polymerase chain reaction, using clone 19A DNA as a template. The polymerase chain reaction product was digested with XbaI and EcoRI and ligated with the XbaI-EcoRI fragment of clone 19A and the EcoRI-digested vector pEDΔ. The resulted clone, named pEDΔ-cPLA₂γ-WT, was sequenced to confirm the desired C-terminal coding sequence. The polymerase chain reaction product was digested with XbaI-EcoRI and ligated to a control expression vector pHTOP. pHTOP was modified from pED6 vector (20) essentially by inserting a 289-base pair tetracycline operator sequence (21) at the XhoI site of pED6. The expression vector of transactivator (tTA) was generated similarly as described by Gossen and Bujard (21) using an oligonucleotide that introduces the mutation to the C-terminal coding sequence.

Stable CHO Cell Lines Overexpressing cPLA₂γ and cPLA₂α—The plasmid of wild-type cPLA₂γ was constructed as follows. The restriction fragment (EcoRI-EcoRI) containing the entire coding sequence was isolated from pEDΔ-cPLA₂γ-WT and ligated to a control expression vector pHTOP. pHTOP was modified from pED6 vector (20) essentially by inserting a 289-base pair tetracycline operator sequence (21) at the XhoI site of pED6. The expression vector of transactivator (tTA) was generated similarly as described by Gossen and Bujard (21) using neomycin transferase as selection marker.

Stable CHO cell lines of cPLA₂γ and cPLA₂α were generated by transfecting CHO cells that constitutively express tTA with pHTOP-cPLA₂γ or pHTOP-cPLA₂α. Transfection was performed using Lipofectamine as recommended by the manufacturer (Life Technologies, Inc.). Transfectants were then selected in growth medium containing methotrexate at a concentration of 5–100 μM.

Activity Assay—Cell pellets (either from CHO cells overexpressing cPLA₂γ or cPLA₂α) were resuspended in lysis buffer (10 mM HEPES, pH 7.5, 1 mM EDTA or 1 mM EGTA, 0.1 mM dithiothreitol, 0.34 mM sucrose, and 1 μg/ml leupeptin). Cells were lysed by nitrogen cavitation (750–1000 psi, 10 min) on ice. Approximately 5–15 μg of cell lysate (determined by Bradford assay, Bio-Rad) was used in the assay. 1-[¹⁴C]Palmitoyl-2-[¹³C]arachidonyl-phosphatidylcholine (PC) (57 Ci/mmol), 1-palmitoyl-2-[¹³C]arachidonyl-PC (55–58 Ci/mmol), 1-palmitoyl-2-[¹³C]oleoyl-PC (58 Ci/mmol), 1-palmitoyl-2-[¹³C]linoleoyl-PC (58 Ci/mmol), and 1-O-hexadecyl-2-[¹³C]arachidonyl-PC (200 Ci/mmol) were obtained from DuPont NEN. Unlabeled 1-O-hexadecyl-2-arachidonoyl-PC was obtained from Biomol. The lipids were dried under N₂ and sonicated with vesicle buffer (50 mM HEPES, pH 7.5, 1 mM EDTA or 5 mM EGTA, and 7 mM CaCl₂ for regiospecific assay, 30% glycerol, 1 mg/ml fatty acid-free bovine serum albumin, and 150 mM NaCl) adapted from Ghiomashchi et al. (22). Aliquots of lysate were incubated with substrate (20 μM) at 37°C for the indicated amount of time. Released fatty acid was measured as described (23).

Prenylation Assay—70–80% confluent COS cells (10 cm plate) were transfected with 8 μg of pCMV-RasL61L (obtained as a generous gift from R. Davis) using lipofectamine according to the manufacturer's instructions (Life Technologies, Inc.). Cells were grown for 2 days and then incubated with 20 μM mevistatin (Biomol) for 1 h. The cells were then incubated in 3 ml of growth media containing 40 μM mevistatin and 150 μCi of [³⁷C]mevistatin (29 Ci/mmol) (DuPont NEN) for 14 h (25). Cells were washed twice in phosphate-buffered saline and scraped into 3 ml of phosphate-buffered saline. The cell pellets were lysed in 100 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 137 mM NaCl, 2 mM EDTA, 5 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride) for 15 min on ice. The supernatant was removed and centrifuged, and the pellet was resuspended in 50 μl of 2 x Laemmli’s sample buffer. SDS to 1% was added to the remaining lysate and incubated for 15 min. Lysate buffer was added to the lysate to 1 ml to reduce the SDS to 0.1%, incubated for another 30 min, and then centrifuged 1 h at 100,000 × g, 4°C. The supernatant was immunoprecipitated with 10 μl of antibody (Upstate Biotechnology) for 3 h at 4°C, then incubated with protein A-Sepharose (Amersham Pharmacia Biotech) saturated with 10% bovine serum albumin for 30 min. The beads were washed three times with lysis buffer containing 0.1% SDS and then resuspended in 50 μl of 2 x Laemmli’s sample buffer. Samples were subjected to 4–20% SDS-PAGE (Novex), stained with Coomassie Blue, soaked in densitify (Amersham), and dried and exposed to Biomax MS film (Kodak) for 7 days. To monitor the expression levels of cPLA₂γ, aliquots of the samples were subjected to 4–20% SDS-PAGE. Proteins were transferred to a nitrocellulose filter (Novex), immunoblotted for cPLA₂γ with 44284 antibody, and detected by ECL (Amersham).

RESULTS

cPLA₂γ Is a Novel cPLA₂—The EST data base was searched to identify sequences similar to the human 85-kDa cPLA₂ gene (cPLA₂α). This analysis led to the identification of two related genes, named cPLA₂β and cPLA₂γ. The cPLA₂γ EST clone 258543 was shown to contain a partial cDNA insert with sequence similarity to the C terminus of cPLA₂α. A full-length clone was isolated from a human skeletal muscle cDNA library. This clone contains a 541-aminoc acid open reading frame with predicted molecular mass of 60.9 kDa. Comparison of the amino acid sequences of cPLA₂γ and cPLA₂α (Fig. 1) reveals 28.7% identity. Within this putative catalytic domain there exists two subdomains with greater sequence identity. Interestingly, the spacer region separating these two domains corresponds to an area in cPLA₂α considered to be an exposed hinge region containing many protease-accessible sites, as well as the MAP kinase activation site Ser-505 (6). cPLA₂γ contains a sequence that is similar to the lipase consensus sequence, GLSGS, in cPLA₂α, which has been found to be critical for cPLA₂α activity (7). These sequences are very similar to the lipase consensus sequence, GXGXD, found in many lipases and serine proteases (9, 26). Also conserved in cPLA₂γ are the amino acids that make up the putative catalytic triad of cPLA₂α (8). These amino acids correspond to serine 82, aspartate 385, and arginine 54 in cPLA₂γ.

cPLA₂α contains a CaLB or C-2 domain that has been shown to be important for calcium-dependent binding of the enzyme to membranes (6). cPLA₂γ does not contain a CaLB domain and is therefore likely to be a novel calcium-independent phospholipase. Interestingly, a motif search of cPLA₂γ reveals the presence of a C-terminal –CAAX box (–CCLA). This motif has been identified as a signal for prenylation, where C is the cysteine that becomes modified, A is an aliphatic amino acid, and X is any amino acid (27). The N terminus of cPLA₂γ also contains a sequence that is a potential site for myristoylation (M-G-X-X-X/Small uncharged)-X (Ref. 28 and the Prosite data base). Similar to other lipid-modified proteins, these putative lipid modifications may regulate the localization of cPLA₂γ within the cell.

to determine the tissue distribution of cPLA₂γ, Northern blot analysis was performed. Hybridization of the EST clone with RNA from various human tissues indicates that cPLA₂γ mRNA is approximately a 3-kilobase transcript. Strikingly, cPLA₂γ is most abundant in skeletal muscle and heart, with lower levels in spleen, brain, placenta, and pancreas (Fig. 2).

cPLA₂γ Encodes a Phospholipase A₂—To determine the re-
gioselectivity of cPLA₂-γ, vesicle activity assays were performed using 1-[14C]palmitoyl-2-arachidonyl-PC, 1-palmitoyl-2-[14C]arachidonyl-PC, or 1-O-hexadecyl-2-[3H]arachidonyl-PC. Cell lysates prepared from COS cells transfected with either vector, cPLA₂-γ or cPLA₂-α, were incubated with the various substrates for the indicated times (Fig. 3). Lysate from cPLA₂-γ transfected cells showed at least 4.4-fold more PLA₂ activity than the lysate from vector transfected cells. Importantly, cPLA₂-γ readily liberated arachidonate when 1-O-alkyl phospholipid (1-O-hexadecyl-2-[3H]arachidonyl-PC) was utilized as a substrate, confirming its ability to cleave the sn-2 site. Comparing the activity of cPLA₂-γ in the presence of the substrates using 1-[14C]palmitoyl-2-arachidonyl-PC, 1-palmitoyl-2-[14C]arachidonyl-PC, or 1-O-hexadecyl-2-[3H]arachidonyl-PC, cPLA₂-γ seems to be as proficient at cleaving at the sn-1 site as the sn-2 site. This is dissimilar from cPLA₂-α (Fig. 3B), which under the conditions used has no apparent sn-1 activity. Importantly, the PLA₁ and PLA₂ activity of cPLA₂-γ does not seem to be sequential, as the radiolabeled fatty acid released from 1-palmitoyl-2-[14C]arachidonyl-PC and using 1-[14C]palmitoyl-2-arachidonyl-PC showed similar kinetics.

cPLA₂-γ Is Prenylated—cPLA₂-γ contains the C-terminal sequence -CCLA, which is a motif for prenylation. Protein prenylation is mediated by the addition of either farnesyl (C-15) or geranylgeranyl (C-20) to the cysteine of the CAAX motif (29). The process is initiated by cleavage of the three most C-terminal amino acid residues -AX, followed by methylation of the cysteine carboxyl group (29). The sequence of cPLA₂-γ also resembles the sequence CXX, which is another motif for prenylation (27). This motif signals the addition of geranylgeranyl to the protein and occurs via mechanisms that differ from the CAAX modification. This motif is mostly found on the Rab family of proteins (29).

To investigate the utilization of the prenylation motif on cPLA₂-γ, COS cells were transfected with either cPLA₂-γ or cPLA₂-γ with the C terminus mutated from CCLA to SSLA. A plasmid encoding Ras was transfected as a positive control for prenylation. Approximately 48 h post-transfection, the cells
were incubated for 14 h with the isoprenoid precursor, [3H]mevalonolactone. Cell lysates were prepared as described under “Experimental Procedures” and analyzed by SDS-polyacrylamide gel electrophoresis. Autoradiographic analysis reveals a band at 60 kDa in cells that were transfected with the wild-type cPLA2-γ (Fig. 4A). Whereas cells transfected with plasmid encoding the mutant protein, vector only, or Ras show no bands at 60 kDa. Similar results were obtained when the cell lysate was immunoprecipitated with cPLA2-γ antibody, 44282, confirming that the 60-kDa protein is cPLA2-γ (data not shown). To determine if equal amounts of wild-type and mutant protein were expressed in the COS cells, Western blot analysis was performed on the samples and indicated equal expression of the two proteins (Fig. 4B). These data indicate that cPLA2-γ is prenylated at its C terminus.

cPLA2-γ Is a Membrane-associated Protein—To determine the subcellular localization of cPLA2-γ, CHO cells stably transfected with cPLA2-γ were lysed by nitrogen cavitation and centrifuged for 1 h at 100,000 x g. cPLA2-γ was then detected by Western analysis. As shown in Fig. 5, cPLA2-γ is found to localize to the particulate (pellet) fraction. Treatment of the particulate fraction with 1% Triton X-100 followed by centrifugation at 100,000 x g results in the majority of the enzyme being present in the supernatant. This is unlike cPLA2-α, which is found in the supernatant in the absence of calcium and in the pellet fraction following the addition of calcium (6, 10). Lipid modification may be responsible for the membrane association of cPLA2-γ, as is the case for Ras. However, fractionation of cells transfected with cPLA2-γ mutated at both the N- and C termini, to disrupt possible lipidation sites, revealed that this mutated protein remains in the membrane fraction (data not shown). This result indicates that there is another component that is involved in the association of cPLA2-γ with the membrane.

cPLA2-γ Is Calcium-independent—Unlike the secreted PLA2s, which require millimolar concentrations of calcium for activity, the catalytic domain of cPLA2-α does not require calcium for activity (6). However, cPLA2-α does require micromolar concentrations of calcium for membrane binding through its CaLB domain. We were interested in determining the requirement of calcium for cPLA2-γ activity. Cell lysates prepared from COS cells transfected with cPLA2-γ or cPLA2-α were incubated with 1-palmitoyl-2-[14C]arachidonyl-PC (P*APC) or 1-O-hexadecyl-2[3H]arachidonyl-PC (O-H*APC) vesicles at 37 °C for the indicated times. The amount of released fatty acid was determined. Points are the average of duplicates (± range of the mean), with background activity from vector-transfected cells subtracted from the values. The amount of COS lysate used was approximately 6–7 μg/reaction. Lysates containing cPLA2-α were diluted 1:50 with lysate from vector-transfected cells.

**Fig. 2.** Tissue distribution of cPLA2-γ. Northern blot analysis was performed on various human tissues using a 32P-labeled 900-base pair EcoRI-NcoI fragment of cPLA2-γ. The blot was washed under high stringency conditions. Each lane is composed of approximately 2 μg of poly(A)¹ mRNA.

**Fig. 3.** Regioselectivity of cPLA2-γ in a vesicle assay. Cell lysates prepared from COS cells transfected with cPLA2-γ (A), cPLA2-α (B), or vector were incubated with 1[3H]palmitoyl-2-arachidonoyl-PC (PAPC), 1-palmitoyl-2-[14C]arachidonyl-PC (P*APC), or 1-O-hexadecyl-2[3H]arachidonyl-PC (O-H*APC) vesicles at 37 °C for the indicated times. The amount of released fatty acid was determined. Points are the average of duplicates (± range of the mean), with background activity from vector-transfected cells subtracted from the values. The amount of COS lysate used was approximately 6–7 μg/reaction. Lysates containing cPLA2-α were diluted 1:50 with lysate from vector-transfected cells.

**Table I.** Substrate Specificity of cPLA2-γ at the sn-2 Position—cPLA2-α selectively hydrolyzes arachidonic acid at the sn-2 position in several assay formats, whereas iPLA2-γ selectivity is significantly more assay dependent. To determine if cPLA2-α has a preference for the fatty acid at the sn-2 position, vesicle assays were performed using phosphatidylcholine that contains palmitoyl in the sn-1 position and radiolabeled arachidonyl, oleyl, linoleyl, or palmitoyl in the sn-2 position. The substrates were incubated for 15 min with lysates from CHO cells stably transfected with either cPLA2-γ or cPLA2-α. cPLA2-γ seems to prefer lipids that are unsaturated at the sn-2 position, and it does seem to prefer arachidonic acid approximately 3.5-fold over palmitic acid (Table I).
FIG. 4. Post-translational modification of cPLA2-γ. COS cells were transiently transfected with the mevalonate transporter, pMev, and either wild-type cPLA2-γ (WT), cPLA2-γSSLA mutant (SSLA), vector, or Ras. Approximately 48 h post-transfection, cells were labeled with 150 μCi of [3H]mevalonolactone for 14 h. Cells were processed as stated under “Experimental Procedures.” 1% Triton X-100 pellets were subjected to 4–20% SDS-PAGE, soaked in enhensify and exposed to Biomax MS film for 7 days (A). To examine the expression levels of cPLA2-γ, aliquots of samples were subjected to 4–20% SDS-PAGE followed by Western blot analysis using anti-cPLA2-γ antibody, 44284 (B).

FIG. 5. Localization of cPLA2-γ in CHO cells stably transfected with cPLA2-γ. CHO cells overexpressing cPLA2-γ were lysed by nitrogen cavitation (CL) and spun for 1 h at 100,000 × g at 4 °C. The supernatant was collected (S). The pellet (P) was resuspended in lysis buffer containing 1% Triton X-100 and centrifuged for 1 h at 100,000 × g at 4 °C. The supernatant and pellet fractions were collected. Samples were subjected to 4–20% SDS-PAGE followed by Western blot analysis using anti-cPLA2-γ antibody, 44284.

TABLE I

Fatty acid selectivity of cPLA2-α and cPLA2-α in CHO lysates

| Substrate | cPLA2-α activity pmol/min | cPLA2-α activity |
|-----------|--------------------------|-------------------|
| 1-Palmitoyl-2-[^14C]arachidonoyl-PC | 8.69 ± 0.42 | 7.99 ± 0.83 |
| 1-Palmitoyl-2-[^14C]oleoyl-PC | 4.50 ± 0.73 | 1.14 ± 0.04 |
| 1-Palmitoyl-2-[^14C]linoleoyl-PC | 3.52 ± 1.30 | 2.15 ± 0.14 |
| 1-Palmitoyl-2-[^14C]palmitoyl-PC | 2.51 ± 0.61 | 0.34 ± 0.06 |

DISCUSSION

We have identified a novel 60.9-kDa calcium-independent phospholipase A2, which we termed cPLA2-γ. cPLA2-γ contains 28.7% overall sequence identity with cPLA2-α and was identified by searching the EST data base for related proteins.

A common motif found in many lipases is the consensus sequence, GXSXG, which is essential for enzymatic activity (9, 26). cPLA2-γ contains the sequence GVSXSXG, which is similar but slightly different from the consensus. However, this sequence aligns with the sequence in cPLA2-α, and the change from glycine to serine also occurs in the corresponding region of cPLA2-α, GLSXSXG (7). Mutation of serine-228 and aspartate-549 was shown to abolish cPLA2-α activity, consistent with their role in the putative catalytic triad (7, 8). Catalytic triads of lipases and serine proteases also frequently contain critical histidines; however, mutation of these residues in the catalytic domain of cPLA2-α had no effect on activity (8, 9, 26). Surprisingly, mutation of arginine-200, in what is thought to be the novel catalytic triad of cPLA2-α, abrogated cPLA2-α activity (8). These amino acids may serve as a catalytic triad, providing the active site for hydrolysis, or it is possible that the arginine may...
function in another but unknown critical role, such as in transition state stabilization (8). We have also shown that serine, aspartate, and arginine are conserved in cPLA2-γ, providing further evidence that these amino acids are important and may indeed be part of a novel catalytic triad.

cPLA2-α is regulated by at least two post-translational mechanisms: 1) calcium-induced membrane association through its CaLB domain and 2) phosphorylation of serine-505 by a MAP kinase (6, 10, 30). The phosphorylation site and the CaLB domain of cPLA2-α are not conserved in the sequence of cPLA2-γ, suggesting a different regulatory mechanism. Interestingly, cPLA2-γ contains a potential prenylation motif at its C terminus and a putative signal for myristoylation at its N terminus. Initial studies have failed to indicate that the myristoylation site is utilized, whereas the prenylation site is indeed utilized. The isoprenoid precursor [3H]mevalonolactone is readily incorporated into cPLA2-γ expressed in COS cells. We do not know, however, if the modifying isoprenoid is a farnesyl or a geranylgeranyl. Generally, in the consensus sequence CAAX, when the C-terminal (X) amino acid is a methionine, serine, glutamine, or alanine, this signals that the lipid will be farnesyl (27), whereas a leucine signals that the modifying lipid is a geranylgeranyl. However, there is also a motif XAXXX, CXC, or CCXX found on the Rab family of proteins that modifies the two cysteines with geranylgeranyl (31). Because both of these motifs match the cPLA2-γ sequence (—CCLA), we do not know which of these isoprenoids is modifying the protein.

One of the most striking differences between cPLA2-α and -γ is the lack of a lipid binding CaLB domain in cPLA2-γ. The presence of lipidation motifs suggests that these regions may function as the CaLB domain in cPLA2-α, localizing the enzyme to the membrane and being critical for activity. However, in a preliminary study, cPLA2-γ mutant protein that disrupts the possible N- and C-terminal lipid modification sites did not affect its activity in a phospholipid vesicle assay. Moreover, this mutant protein fails to alter its association with the membrane fraction. However, it remains possible that lipid modification may be important in the subcellular localization of cPLA2-α and/or its ability to associate with other proteins. Interestingly, Ras shows increased affinity toward other proteins when it is prenylated compared with its nonprenylated form, and it has been shown that oncogenic forms of Ras need to be modified to affect its activity in a phospholipid vesicle assay. Therefore, it is possible that lipid modifications may play a role in regulating the activity of cPLA2-γ in the cells.

cPLA2-γ will hydrolyze fatty acids at the sn-1 and sn-2 position of phosphatidylcholine. This suggests that cPLA2-γ contains PLA1 and PLA2 activity. The evidence that cPLA2-γ contains PLA2 activity is also confirmed by its ability to cleave 1-O-hexadecyl-2-arachidonyl-phosphatidylcholine. However, we do not know whether the sn-1 cleavage is PLA1 activity or if it is cleavage of the lyso phospholipid. The kinetics of the reactions suggest that it is PLA1 activity, as the hydrolysis of sn-1 would show a time-dependent lag as compared with sn-2 hydrolysis if sequential cleavage were taking place, unless cleavage of sn-1 from lyso phospholipid occurs rapidly. Taken together, all of these data provide evidence that cPLA2-γ is an enzyme with PLA1 and PLA2 activity and a probable PLA1 activity.

cPLA2-γ prefers arachidonic acid to palmitic acid in the sn-2 position of phosphatidylcholine. However, this preference is modest in comparison to the strong preference that cPLA2-α displays for arachidonic acid, 3.5-fold versus 24.5-fold, respectively. The substrate specificity of cPLA2-γ should be considered cautiously, however, because of the artificial nature of the substrate presentation. The selectivity of the enzyme using a natural membrane as a substrate may be a more relevant method to determine the preferred physiological substrate for this enzyme.

The preferred substrate for an enzyme provides a clue to its physiological role, as can its distribution within tissues. cPLA2-γ is highly expressed in heart and skeletal muscle. The calcium independence of this enzyme may be important for its high expression in muscle, where contractions cause large fluxes in calcium concentrations. Therefore, it may be necessary in this environment to regulate a phospholipase in a calcium-independent manner, such as phosphorylation. As previously stated, cPLA2-α activity is also regulated by MAP kinase phosphorylation of serine-505. This serine is not conserved in the sequence of cPLA2-γ; however, there are several potential protein kinase C phosphorylation sites, which may be utilized to regulate the enzyme.

cPLA2-γ may be highly expressed in these muscles because heart and skeletal muscle encounter physical stress upon increased load. It may be necessary to regulate the remodeling of the phospholipid bilayer when cells undergo stress. This speculation is substantiated by the reports of several calcium-independent phospholipases expressed in heart (17, 19). Hazen et al. (18) and McHowat and Creer (19) have identified membrane-bound, calcium-independent PLA2 activity that prefers the myocardia-abundant lipid, plasmalogens, as a substrate. They have shown increased hydrolysis of plasmalogens under hypoxic conditions, such as in ischemia. It is believed that in ischemia, a PLA2 activity leads to the accumulation of lysophospholipids and subsequent injury to the heart tissue because of disruptions of the membrane. As the PLA2 activity of this myocardial membrane-bound enzyme was shown to increase under hypoxic conditions, it was suggested that this calcium-independent PLA2 is physiologically involved in ischemia (18). Because cPLA2-γ is abundantly expressed in heart, and its properties (including calcium independence and membrane localization) are similar to that reported in heart muscle, it may be that cPLA2-γ is involved in ischemia-induced injury to heart muscle.

In summary, we have described the molecular cloning and initial characterization of a novel 60.9-kDa membrane-associated, calcium-independent PLA2, cPLA2-γ. This enzyme shares identity with cPLA2-α and contains the potential critical amino acids for the catalytic site but is missing the key elements that regulate the activity of cPLA2-α. This suggests that the mechanisms of regulation for cPLA2-γ will be different from that of cPLA2-α and quite possibly employs the use of the lipid modification. Defining the mechanisms of regulation and the physiological substrate of cPLA2-γ should shed some light on the physiological role of this newly identified PLA2.

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