Lipids and Lipoproteins:

Identification of Novel Cytosolic Phospholipase A 2s, Murine cPLA2δ, ?, and ζ, Which Form a Gene Cluster with cPLA2 β

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Identification of Novel Cytosolic Phospholipase A_2s, Murine cPLA$_2\delta$, ϵ, and ζ, Which Form a Gene Cluster with cPLA$_2\beta$*[S]

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Phospholipase A_2 hydrolyzes the *sn*-2 ester bond of glycerophospholipids that produce free fatty acids and lysophospholipids. Cytosolic phospholipase A_2s (cPLA$_2$, group IV) are a subgroup of enzymes that act on the intracellular phospholipid membrane. The best investigated cPLA$_2\alpha$ (group IVA) is a key enzyme for lipid mediator production *in vivo*. Here we report cloning and characterization of novel murine cPLA$_2$s: cPLA$_2\delta$ (group IVD), cPLA$_2\epsilon$ (group IVE), and cPLA$_2\zeta$ (group IVF), that form a gene cluster with cPLA$_2\beta$ (group IVB). The deduced amino acid sequences of cPLA$_2\delta$, ϵ, and ζ demonstrated a conserved domain structure of cPLA$_2$, *i.e.* one C2 domain and one lipase domain. The potential catalytic dyad, Ser and Asp, was conserved for these newly cloned cPLA$_2$s along with relatively high conservation for the surrounding residues. Transcripts of murine cPLA$_2\delta$, ϵ, and ζ appeared to be enriched in certain organs rather than ubiquitous distribution. Major Northern signals for cPLA$_2\delta$ were detected in placenta, cPLA$_2\epsilon$ in thyroid, heart, and skeletal muscle, and cPLA$_2\zeta$ in thyroid. Recombinant proteins expressed in human embryonic kidney 293 cells demonstrated molecular sizes of about 100 kDa by Western blotting and exhibited Ca^{2+}-dependent PLA$_2$ activities on 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylcholine substrate. In contrast to cPLA$_2\alpha$, cPLA$_2\zeta$ preferred phosphatidylethanolamine to phosphatidylcholine. Intracellular localization was visualized by green fluorescent-tagged proteins. Each molecule showed specific localization, and cPLA$_2\delta$ translocated from the cytosol to the perinuclear region by calcium-ionophore stimulation. We thus discovered these functional novel cPLA$_2$ genes, which cluster on murine chromosome 2E5.

Phospholipase A_2 (PLA$_2$, EC 3.1.1.4)[1] hydrolyzes the *sn*-2 ester bond of glycerophospholipids. Mammalian PLA$_2$ can be classified into four major categories: cytosolic PLA$_2$ (cPLA$_2$), Ca^{2+}-independent PLA$_2$ (iPLA$_2$), secretory PLA$_2$ (sPLA$_2$), and platelet-activating factor acetylhydrolase (PAF-AH) (1, 2). So far, 20 gene loci for human PLA$_2$ are known: 4 cPLA$_2$s, 2 iPLA$_2$s, 10 sPLA$_2$s, and 4 PAF-AHs.

We focus on the cPLA$_2$ group with regard to lipid mediator productions and membrane remodeling. cPLA$_2\alpha$ was first identified by purification of the protein (3–5), and its PLA$_2$ activity is characterized by Ca^{2+} dependence and substrate preference for arachidonoyl phospholipids (6–9). A catalytically important dyad structure (Ser-228 and Asp-549) for human cPLA$_2\alpha$ was identified by site-directed mutagenesis (10, 11) and x-ray crystallography (12). The association of cPLA$_2\alpha$ with its substrate is regulated by Ca^{2+}, and interaction with the C2 domain induces translocation from cytosol to the endoplasmic reticulum/Golgi apparatus (ER/Golgi) and nuclear envelope (13–16). Enzyme activity is also regulated by phosphorylation with various protein kinases (17–21). Furthermore, phosphoinositides were reported to activate human cPLA$_2\alpha$ (22), and ceramide 1-phosphate was reported as an activator of human cPLA$_2\alpha$ interacting with the C2 domain (23). Gene-disrupted mice of cPLA$_2\alpha$ demonstrated marked reduction in lipid mediator production, reproductive abnormality, attenuation of symptoms in acute lung injury, and other various inflammatory disease models (24–27). Thus, cPLA$_2\alpha$ is one of the critical enzymes for production of lipid mediators such as prostaglandins, leukotrienes, and PAF under both physiological and pathological conditions (28, 29). Human cPLA$_2\beta$ and γ were identified from the expressed sequence tag (EST) data base (30–32). cPLA$_2\beta$ has Ca^{2+}-dependent PLA$_2$ activity *in vitro*, but its biological function is still unknown. cPLA$_2\gamma$, lacking the C2 domain, has Ca^{2+}-independent PLA$_2$ activity. A possible function for cPLA$_2\gamma$ is remodeling of membrane phospholipids (33, 34). Recently, human cPLA$_2\delta$ was identified as one of psoriasis-related genes (35). Whereas extensive studies have been carried out for secretory PLA$_2$ (36, 37), only limited information is available for the cPLA$_2$ family. Therefore, we started searching for new cPLA$_2$ molecular species. Considering the difficulty of enzymological detection or cDNA library screening for gene products with low levels and/or limited patterns of tissue ex-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables I and II.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB195276 for cPLA$_2\delta$, AB195277 for cPLA$_2\epsilon$, and AB195278 for cPLA$_2\zeta$.

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[1] The abbreviations used are: PLA$_2$, phospholipase A_2; cPLA$_2$, cytosolic phospholipase A_2; iPLA$_2$, calcium-independent phospholipase A_2; sPLA$_2$, secretory phospholipase A_2; PAF-AH, platelet-activating factor acetylhydrolase; ER, endoplasmic reticulum; EST, expressed sequence tag; BLAST, basic local alignment search tool; RT, reverse transcription; RACE, rapid amplification of cDNA ends; d.p.c., day postcoitum; HEK293, human embryonic kidney 293 cell line; CHO-K1, Chinese hamster ovary cell line; BSA, bovine serum albumin; GFP, green fluorescent protein; ORF, open reading frame; PA-PC, 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylcholine; PL-PC, 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine; PA-PE, 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylethanolamine; PL-PE, 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ER, endoplasmic reticulum; EGFP, enhanced GFP.

pression, we adopted a different approach from previous cPLA$_2$ cloning. A comprehensive homology search was carried out against murine genome and EST data bases using conserved exons within cPLA$_2\alpha$, β, and γ as queries. We discovered three cPLA$_2$ genes, termed cPLA$_2\delta$ (group IVD), cPLA$_2\epsilon$ (group IVE), and cPLA$_2\zeta$ (group IVF): the first may correspond to a murine orthologue of human cPLA$_2\delta$. We describe herein the structure, tissue distribution, catalytic activity, and intracellular localization of these newly cloned gene products.

EXPERIMENTAL PROCEDURES

Materials—Thirteen to fifteen-week-old male C57BL/6J mice (CLEA Japan, Tokyo, Japan) were used as RNA sources. [^{32}P]dCTP (~110 TBq/mmol) was purchased from Amersham Biosciences. Complete™ protease inhibitor mixture (EDTA free) was from Roche Diagnostics. 1-Palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylcholine (1.8 GBq/mmol), 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine (2.1 GBq/mmol), 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylethanolamine (1.8 GBq/mmol), and 1-[^{14}C]palmitoyl-2-lyso-phosphatidylcholine (2.0 GBq/mmol) were from PerkinElmer Life Sciences. 1-Palmitoyl-2-[^{14}C]linoleoyl-phosphatidylethanolamine (2.0 GBq/mmol) was from Amersham Biosciences. Triton X-100 and bovine serum albumin (BSA, fatty acid free) were from Sigma. Ionomycin was obtained from Calbiochem (La Jolla, CA), BODIPY-brefeldin A, LysoTracker Red DND-99, and MitoTracker Red CMXRos were from Molecular Probes (Eugene, OR). 1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine (PAF C-16) was obtained from Cayman Chemicals (Ann Arbor, MI).

Gene Identification—Candidates for novel members of the cPLA$_2$ family were compiled from BLAST searches on murine genome and murine EST data bases using cPLA$_2\alpha$, β, and γ as queries. PCR primers for cDNA cloning by RT-PCR were designed based on the search results (see below). For preparation of cDNA templates from murine organs, total RNA extracted by an acid guanidinium-phenol-chloroform method (Isogen, Nippon Gene, Tokyo, Japan) was subjected to μMACS mRNA isolation kit (Miltenyi Biotec, Gladbach, Germany) and to oligo(dT)-primed reverse transcription by Superscript II enzyme (Invitrogen, Carlsbad, CA). The organs investigated were whole brain, lung, liver, spleen, heart, kidney, intestine, thyroid, stomach, placenta (15.5-day postcoitum, d.p.c.), and E15 embryo. Fragments of cPLA$_2$ cDNA were amplified with KOD-plus DNA polymerase (Toyobo, Tokyo, Japan) and a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). 5′ and 3′ Rapid amplification of cDNA ends (RACE) was carried out with SMART-RACE cDNA amplification kit (Clontech, Palo Alto, CA). Placenta (14.5 d.p.c.) was used for cPLA$_2\delta$ RACE, heart and brain for cPLA$_2\epsilon$, and thyroid for cPLA$_2\zeta$. Amplified cDNA fragments were subcloned into cloning vectors, pGEM-T Easy (Promega, Madison, WI) or pCR Blunt II TOPO (Invitrogen), and their nucleic acid sequences were determined with a PRISM 3100 Genetic Analyzer (Applied Biosystems).

Northern Analyses—Murine multiple tissue Northern blots, MTN Blots Mouse (Clontech), MTN Blots Mouse II (Clontech), and Mouse Adult Tissue Blot (Seegene, Seoul, Korea) were hybridized with ^{32}P-labeled DNA probe (Rediprime II DNA labeling system, Amersham) for the entire open reading frames (ORFs) of each cPLA$_2$ following the manufacturer's instructions. Radioactive signals were visualized with a BAS-2000 imaging analyzer (Fujifilm, Tokyo, Japan).

Construction of Expression Vectors—Mammalian expression vectors were constructed using pcDNA4/HisMax A (Invitrogen) and pEGFP-C1 (Clontech) plasmid vectors. The PCR-amplified ORF of each cPLA$_2$ was ligated into expression vectors with the minimal flanking regions to fit in-frame with the N-terminal-tagged design with cloning sites on the opposite ends (supplementary data Table II). An expression vector for mRFP (a red fluorescent protein, cDNA provided by R. Y. Tsien (38))-tagged human cPLA$_2\alpha$ was designed by replacing the EGFP coding region with a pEGFP-C1-human cPLA$_2\alpha$ vector. The integrity of all constructs was confirmed by DNA sequencing.

Cell Culture—Human embryonic kidney (HEK) 293 cells were maintained under 5% CO_2 in air at 37 °C in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 50 units/ml penicillin, and 50 μg/ml streptomycin (Roche). A CHO-K1 cell line, W11A, which stably expresses guinea pig platelet-activating factor receptor (39) was maintained in Nutrient Mixture Ham's F-12 (Sigma) supplemented with 10% (v/v) fetal bovine serum, and 0.3 mg/ml geneticin (Wako, Osaka, Japan).

Transient Expression and PLA$_2$ Assay—HEK293 cells were seeded onto 10-cm culture dishes at a density of 2×10^6 cells/dish. After 24 h, they were transfected with expression plasmids using Lipofectamine Plus reagents (Invitrogen) according to the manufacturer's protocol. Transfection medium was replaced with fresh medium 3 h after transfection. The cells were harvested with trypsin-EDTA 48 h after transfection, rinsed twice with phosphate-buffered saline, frozen with liquid nitrogen, and stored at −80 °C until use. Thawed cells were suspended in the homogenizing buffer (50 mM Tris-HCl (pH 7.5), 0.32 M sucrose, 4 mM dithiothreitol, 3 mM $MgCl_2$, 5 mM EGTA, and 1× Complete™ protease inhibitor mixture), and then homogenized with a sonicator (OHTAKE WORKS, Yamaguchi, Japan). Centrifugations at 10,000 × *g* for 10 min and 100,000 × *g* for 1 h were carried out for fractionation. Protein concentrations were determined by Bradford's method (Bio-Rad) using BSA as a calibration standard. PLA$_2$ activity was measured using mixed micelles each containing phospholipid and Triton X-100 in a molar ratio of 1:2 as a substrate; the final concentration of substrate was 2 μM (54,000–63,000 dpm/reaction). The assay buffer contained

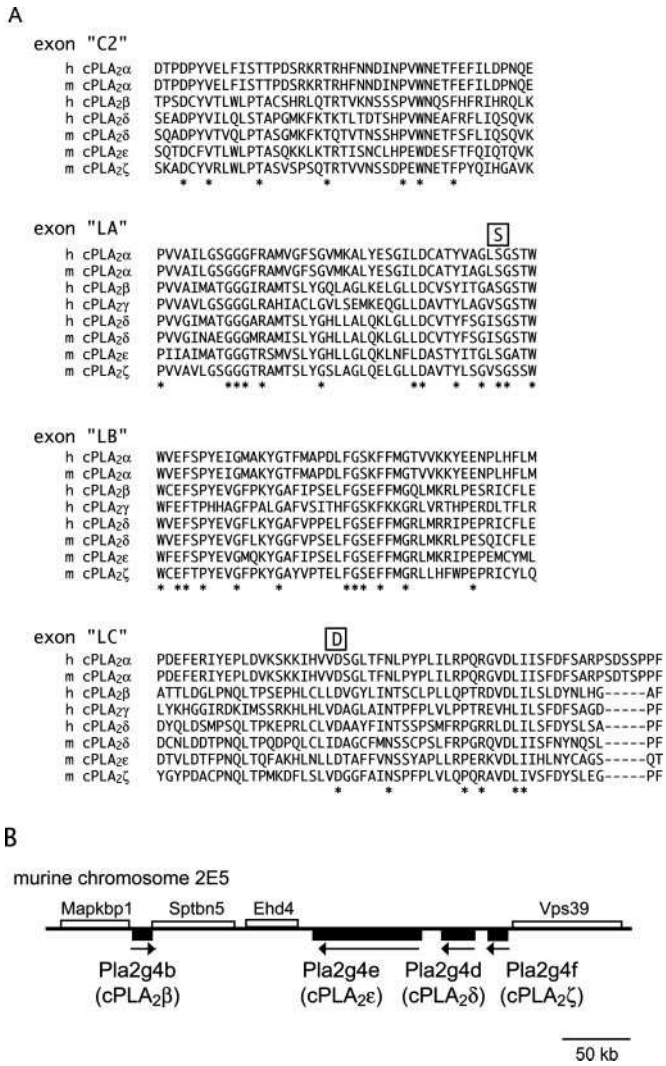

FIG. 1. **Queries for gene search and a cPLA$_2$ gene cluster.** *A*, three relatively well conserved exons were found in the lipase domain among human cPLA$_2\alpha$, β, and γ: exons *LA*, *LB*, and *LC*. A fourth conserved exon was detected in C2 domain of human cPLA$_2\alpha$ and β: exon *C2*. Three novel cPLA$_2$s, cPLA$_2\delta$, ϵ, and ζ, were found by BLAST searches on the human/murine genome using these exon sequences as queries. These conserved exons translated to amino acid sequences were aligned. *h*, human; *m*, mouse; *, conserved residues for all genes; *S*, a consensus residue within a catalytic GX*S*GS motif, *D*, a consensus residue within a catalytic *DX*G motif. *B*, schematic representation of murine cPLA$_2$ gene cluster. Newly discovered cPLA$_2\delta$, ϵ, and ζ form a gene cluster with cPLA$_2\beta$ in murine chromosome 2E5 spanning about 0.3 Mb (ncbi.nlm.nih.gov/genome/seq/MmBlast.html). *Arrows* indicate the direction of translation. *Filled boxes* indicate cPLA$_2$s and *open boxes* indicate other genes. *Mapkbp1*, mitogen-activated protein kinase-binding protein 1; *Sptbn5*, spectrin, beta, non-erythrocytic 5; *Ehd4*, EH-domain containing 4; *Vps39*, vacuolar protein sorting 39.

A

B

	cPLA2α				
cPLA2β	25%			C2 Lipase	
	37%	cPLA2β			
cPLA2δ	28%	***43%***			
	31%	***50%***	cPLA2δ		
cPLA2ε	25%	***35%***	***38%***		
	32%	***45%***	***46%***	cPLA2ε	
cPLA2ζ	27%	***35%***	***33%***	***33%***	
	32%	***45%***	***42%***	***41%***	cPLA2ζ
cPLA2γ	-	-	-	-	-
	30%	28%	32%	31%	33%

C

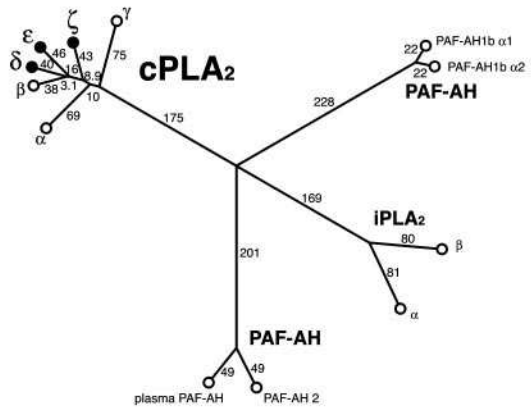

FIG. 2. **Comparison of murine cPLA$_2$δ, ϵ, ζ, with cPLA$_2$α,β, and γ.** *A*, alignment of murine cPLA$_2$δ, ϵ, and ζ. Suggested domains are *boxed*. Conserved residues for all proteins (*) and conserved residues for three proteins (*dots*) are indicated. *Arrows* indicate conserved exons, used for gene search. *B*, percent identity at amino acid levels among murine cPLA$_2$s. Comparisons were made separately for two parts N-terminal region (*C2*) and C-terminal region (*lipase*). The regions (*C2*, *lipase*) examined for comparisons were: cPLA$_2$α (1–185 and 186–748 amino acid residues), cPLA$_2$β (1–291 and 292–782), cPLA$_2$γ (− and 1–597), cPLA$_2$δ (1–328 and 329–825), cPLA$_2$ϵ (1–378 and 379–875), and cPLA$_2$ζ (1–351 and 352–855). *C*, dendrogram of murine cPLA$_2$, iPLA$_2$, and PAF-AH, using the all-against-all matching method (cbrg.inf.ethz.ch/Server/AllAll.html). Values show branch lengths that represent the evolutional distance between each pair of sequences. The sequence divergence is equal to the sum of the value of each branch length. Detailed structural information of murine cPLA$_2$β will be published elsewhere (N. Uozumi and T. Ohto, unpublished data). sPLA$_2$s were not included because they have completely distinct structures from cPLA$_2$, iPLA$_2$, and PAF-AH.

100 mM HEPES-NaOH (pH 7.5), 1 mg/ml BSA, 4.5 mM $CaCl_2$, and 1 mM dithiothreitol. $CaCl_2$ was replaced with EGTA to examine the Ca^{2+} requirement for the enzyme activity. Lysophospholipase activity was measured using monomeric lyso-PC (2 μM, 63,000 dpm/reaction) with a concentration of BSA in assay buffer of 0.5 mg/ml. The reaction was started by the addition of enzyme sources, and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated with Dole's reagent, and silica gel powder was used to recover free fatty acid in an *n*-heptane layer (40, 41). Radioactivity was counted with a liquid scintillation counter LS6500 (Beckman, Fullerton, CA).

Western Blots—SDS-PAGE separated samples were transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA), and probed with mouse monoclonal IgG anti-Xpress antibody (Invitrogen) that recognize the Xpress™ epitope (DLYDDDDK), or BD Living Colors™ full-length rabbit polyclonal antibody (Clontech) to detect N-terminal-tagged proteins. Immunoreactive signals were visualized with anti-mouse or rabbit IgG horseradish peroxidase-conjugated secondary antibody and ECL reagents (Amersham Biosciences).

Confocal Microscopy—CHO-K1 cells (W11A cells) at a density of 7 × 10^5 cells/dish were seeded onto 6-cm dishes 1 day before transfection. Two μg each of pEGFP-murine cPLA$_2$α, pEGFP-murine cPLA$_2$δ, pEGFP-murine cPLA$_2$ϵ, or pEGFP-C1 were transfected using Lipofectamine Plus (Invitrogen). Transfection medium was replaced with

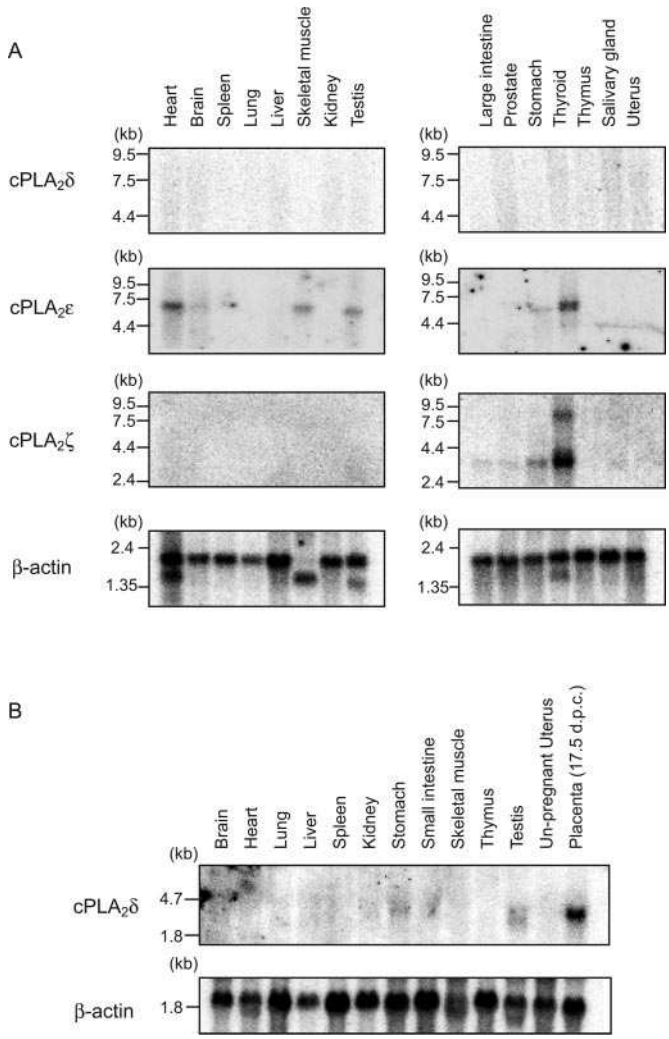

FIG. 3. **Tissue distribution of murine cPLA$_2$δ, ϵ, and ζ mRNAs.** Mouse MTN membrane (Clontech), Mouse MTN membrane II (Clontech) (*A*), loaded with 2 μg of poly(A)$^+$ RNA per lane, and Mouse Adult Tissue Blot (Seegene) (*B*), loaded with 20 μg of total RNA per lane, were sequentially hybridized with ^{32}P-labeled probes. Probes for cPLA$_2$s covered the entire ORF of respective genes. Hybridizations with β-actin probes were carried out as controls. Two sets of MTN membranes were hybridized in this way: cPLA$_2$ζ, cPLA$_2$ϵ, and β-actin for set 1, cPLA$_2$δ and β-actin for set 2. Signals for β-actin were essentially the same for the 2 sets of membranes, and set 2 is shown as a representative. Molecular sizes are indicated on the *left*.

fresh medium at 4 h. After incubation for 24 h, cells were harvested with trypsin/EDTA (Sigma) and seeded onto 35-mm glass-bottomed dishes (IWAKI, Tokyo, Japan) with Nutrient Mixture Ham's F-12 medium containing 10% (v/v) fetal bovine serum. Then, the cells were incubated for 24 h, and serum-starved with medium containing 0.1% BSA for an additional 24 h. The cells were stained with 1 μM BODIPY-brefeldin A or 50 nM LysoTracker Red in Hanks' balanced salt solution containing 10 mM HEPES (pH 7.4) and 0.1% BSA at 37 °C for 30 min. For experiments with murine cPLA$_2$ζ, CHO-K1 cells (W11A cells) were seeded onto 35-mm glass-bottomed dishes (IWAKI) at a density of 2 × 10^5 cells/dish 1 day before transfection. One μg of pEGFP-murine cPLA$_2$ζ and 1 μg of pmRFP-human cPLA$_2$α were co-transfected. Transfection medium was replaced with a medium containing 10% (v/v) fetal bovine serum at 4 h. Then, cells were incubated for 18 h, and serum-starved with medium containing 0.1% BSA for an additional 6 h. Fluorescence images of green fluorescent protein (GFP), BODIPY-brefeldin A, LysoTracker Red, and mRFP were obtained using an LSM510 Laser Scanning Microscope (Carl Zeiss, Germany) with a ×63 water-immersion objective (NA = 1.2) or a ×100 oil-immersion objective (NA = 1.3). GFP fluorescence was monitored by excitation at 488 nm with an argon laser, and by emission with a 505–550-nm band pass filter, or with a 505–530-nm band pass filter when measuring together with BODIPY-brefeldin A to avoid interference. BODIPY-brefeldin A, LysoTracker Red, and mRFP, the excitation was set at 543 nm with a He/Ne laser, and emissions were taken with a 560-nm long path filter for LysoTracker Red, or a 580-nm long path filter for BODIPY-brefeldin A and mRFP.

RESULTS

Gene Identification and cDNA Cloning of Novel Murine cPLA$_2$s—Human cPLA$_2$α, β, and γ share a lipase domain structure with considerable homology. Overall identities at the amino acid levels are between 30 and 34%. When aligned according to the RNA-splicing sites, higher conservations were noticed for three exons within the lipase domain (Fig. 1*A*, exons *LA*, *LB*, and *LC*). Two of these exons include residues of the catalytic dyad, the serine and aspartic acid residues in the G*X*SGS (exon "LA") and *DX*G (exon "LC") motifs, respectively. The functional significance of the exon "LB" in between has not been clarified yet. An extensive BLAST search against the draft human genome sequence using these exon sequences as queries predicted three novel cPLA$_2$ gene loci. Equivalent loci were identified in the murine genome. Furthermore, the fourth conserved exons containing putative C2 domain were detected for each locus (exon *C2*, in Fig. 1*A*). Several EST clones, which are supposed to contain truncated transcripts of these novel genes, were also found using the BLAST search. Based on these DNA sequences, we designed PCR primers; one in the coding region and the other in the putative 3′-untranslated region. We thus obtained truncated cDNA fragments of these novel cPLA$_2$s by RT-PCR from murine tissues (δ from placenta (15.5 d.p.c.), ϵ from whole brain and heart, and ζ from spleen and thyroid). RACE was carried out next to design new sets of PCR primers on 5′- and 3′-untranslated regions (supplementary data Table I). DNA fragments covering putative ORF of three novel cPLA$_2$s were amplified and cloned, and the PCR primers are shown in supplementary data Table II. They were named cPLA$_2$δ (GenBank^{TM} accession number AB195276), cPLA$_2$ϵ (GenBank accession number AB195277), and cPLA$_2$ζ (GenBank accession number AB195278).

The ORF for each clone was assigned so that the coding region should be the longest. The sizes of cloned ORF for murine cPLA$_2$δ, ϵ, and ζ were 2,748, 2,625, and 2,565-bp long corresponding to 825 (molecular mass 93 kDa), 875 (100 kDa), and 855 (96 kDa) amino acid residues, respectively. Recently, human cPLA$_2$δ was discovered in the psoriatic skin cDNA library (35). We figured it to be the orthologue of one of the novel murine cPLA$_2$s based on its chromosomal localization and homology of the primary structure. Human cPLA$_2$δ has the highest homology to murine cPLA$_2$δ: 71% identical at the amino acid level as opposed to 29–49% for other murine cPLA$_2$s. Chromosome localizations of these newly identified cPLA$_2$s, *i.e.* δ, ϵ, and ζ were determined by BLAST search on genome data bases. They were found to form a gene cluster with cPLA$_2$β in murine chromosome 2E5 spanning 0.3 Mb (Fig. 1*B*), and they correspond to human orthologues in chromosome 15q14. Alignment of murine cPLA$_2$δ, ϵ, and ζ revealed that these gene cluster members have considerable homology throughout the molecule, and they seem to have C2 plus lipase domain structures (Fig. 2*A*). Percent identities of amino acid sequences among cPLA$_2$β, δ, ϵ, and ζ are calculated to be 33–43%, and 41–50% for the C2 and lipase domains, respectively (Fig. 2*B*). Lower values were obtained for comparisons with cPLA$_2$α or γ: 25–28% for C2 domain of cPLA$_2$α, and 30–37% for lipase domains of cPLA$_2$α and γ. A dendrogram was drawn by pairwise comparisons of amino acid sequences of murine cPLA$_2$, iPLA$_2$, and PAF-AH (Fig. 2*C*). Six cPLA$_2$s form a branch separately from iPLA$_2$ and PAF-AH, and the gene cluster members, *i.e.* cPLA$_2$β, δ, ϵ, and ζ, come closer together than the remaining cPLA$_2$α and γ.

TABLE I

PLA_2 activity of transiently expressed murine $cPLA_2$s

These data, shown as mean ± S.E., indicate specific activity (pmol/min/mg), pmol of cleaved ^{14}C-labeled arachidonic acid/mg of protein of each of the centrifuge fractions, for 1 min. Centrifugal supernatants of HEK293 cell homogenates transiently transfected with expression vector for $cPLA_2\alpha$, δ, ϵ, or ζ were incubated with 1-palmitoy-2-[^{14}C]arachidonoyl-PC mixed micelles in the presence of 4 mM free Ca^{2+} at 37 °C for 30 min. "*n*" means the number of different transfection batches used for each assay. The protein expression levels are shown in Fig. 4A. In all transfections, the expression patterns are quite similar.

	Mock	Mock (T)[a]	$cPLA_2\alpha$	$cPLA_2\delta$	$cPLA_2\epsilon$ (T)[a]	$cPLA_2\zeta$
10,000 × *g* sup	2.5 ± 0.4	0.4 ± 0.2	190 ± 35	6.1 ± 1.2	7.2 ± 1.9	26 ± 11
100,000 × *g* sup	1.5 ± 0.3	0.6 ± 0.3	291 ± 61	5.1 ± 1.0	7.7 ± 1.5	55 ± 22
n	9	5	5	5	5	5

[a] T, 0.075% (w/v) Triton X-100 included in homogenizing buffer.

Tissue Distribution of $cPLA_2$δ, ϵ, and ζ mRNAs—Expression profiles of $cPLA_2$s were examined at mRNA levels (Fig. 3). The $cPLA_2\delta$ transcript was not clearly detected in MTN blot filters (Clontech, mouse MTN blot and MTN blot II), but was detected in placenta (17.5 d.p.c.) using the Mouse Adult Tissue Blot (Seegene) at about 4.5 kb in size. A transcript of the $cPLA_2\epsilon$ gene (about 5.0 kb in size) was seen predominantly in heart, skeletal muscle, testis, and thyroid, and at low expression levels in brain and stomach. Signals for $cPLA_2\zeta$ were obtained as 2 bands (about 4.0 and 8.0 kb in size). The mRNA was detected strongly in thyroid, moderately in stomach, and very weakly in large intestine and prostate. The combined lengths of RT-PCR and RACE products were 3.3, 4.4, and 3.3 kb (excluding the poly(A) tract) for $cPLA_2\delta$, ϵ and ζ, respectively. We assume that the combined DNA sequence of $cPLA_2\zeta$ corresponds to the shorter Northern signals.

PLA_2 Activity of Transiently Expressed Murine $cPLA_2$s—Mammalian transient-expression experiments were carried out with HEK293 cells and pcDNA4/HisMax expression vectors. Harvested cells were homogenized in the presence of an excess amount of EGTA by sonication, and fractionated by centrifugation at 10,000 × *g* for 10 min and at 100,000 × *g* for 1 h (see "Experimental Procedures").

The enzyme activities were measured with 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylcholine (PA-PC) as a substrate. We performed a series of assays for multiple transfection batches (n = 5–9) to observe consistently elevated PLA_2 activities for both 10,000 × *g* and 100,000 × *g* supernatant fractions of all $cPLA_2$ transfectants, as shown in Table I. To be noted here, preparations for $cPLA_2\epsilon$ were made with homogenizing buffer containing 0.075% (w/v) Triton X-100. When $cPLA_2\epsilon$-expressing cells were processed with the homogenizing buffer without Triton X-100, the enzyme activity was undetectable in the supernatant fractions over mock-transfected cells. We found that addition of Triton X-100 up to 0.1% (w/v) in homogenizing buffer did not inhibit the catalytic activity and improved recovery of PLA_2 activity in the supernatants (data not shown). These effects appeared specific for $cPLA_2\epsilon$ and were not observed for $cPLA_2\alpha$, δ, ζ, or for the intrinsic PLA_2 activity of HEK293 cells (data not shown).

Expression of each $cPLA_2$ protein was confirmed by Western blotting detected with anti-Xpress antibody against the N-terminal tag (Fig. 4). The apparent molecular sizes of the expressed proteins were around 100 kDa (Fig. 4*A*), which are comparable with the estimated sizes from their deduced primary structures with tag sequences (Fig. 2*A*). Cell lysates and centrifugal fractions were prepared in the presence of an excess amount of EGTA in homogenizing buffer (Fig. 4*B*). The majority of expressed proteins were detected in 10,000 × *g* and 100,000 × *g* supernatants for $cPLA_2\alpha$, δ, and ϵ. A portion of expressed signals were detected in the 100,000 × *g* precipitate for $cPLA_2\epsilon$, even in the presence of 0.075% (w/v) Triton X-100. There was no significant difference in the fractionation patterns for $cPLA_2\epsilon$ either with or without 0.075% (w/v) Triton X-100 in homogenizing buffer (data not shown). In contrast to other types of $cPLA_2$s, the $cPLA_2\zeta$ protein was highly recovered in the 10,000 × *g* precipitate (Fig. 4*A*). Nevertheless, supernatants (S2 and S3) contained significant enzyme activity (Table I).

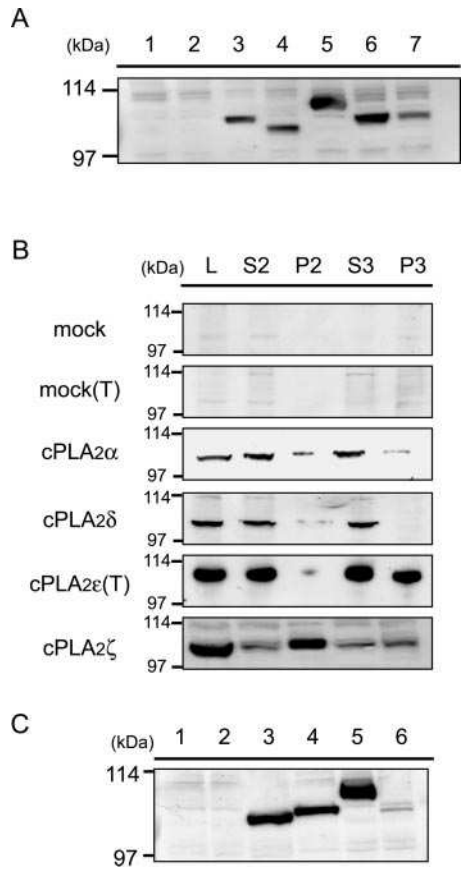

FIG. 4. **Western detection of $cPLA_2$s on a mammalian transient-expression system.** HEK293 cells transiently expressing murine $cPLA_2\alpha$, δ, ϵ, and ζ were sonicated and centrifuged for 10 min at 10,000 × *g* and for 1 h at 100,000 × *g* at 4 °C. Exogenously expressed proteins were detected with N-terminal Xpress tag. Molecular sizes are indicated on the *left* in kDa. *A*, 100,000 × *g* supernatants used in Table I were loaded with 10 μg of total protein/lane. *1*, mock; *2*, mock (T); *3*, $cPLA_2\alpha$; *4*, $cPLA_2\beta$; *5*, $cPLA_2\epsilon$ (T); *6*, $cPLA_2\delta$; and *7*, $cPLA_2\zeta$. These samples were used for the determination of the enzyme activity in Table I. *B*, fractionations of the proteins by centrifugation. *L*, supernatant of 800 × *g*; *S2*, supernatant of 10,000 × *g*; *P2*, precipitate of 10,000 × *g*; *S3*, supernatant of 100,000 × *g*; and *P3*, precipitate of 100,000 × *g*. 10 μg of proteins were loaded on *lane L*, and equivalent parts from fractionation were loaded for the other lanes. *C*, 100,000 × *g* supernatants used in Table III were loaded with 10 μg of total protein/lane. *1*, mock; *2*, mock (T); *3*, $cPLA_2\alpha$; *4*, $cPLA_2\delta$; *5*, $cPLA_2\epsilon$ (T); and *6*, $cPLA_2\zeta$. (*T*) means that 0.075% (w/v) Triton X-100 was added to the homogenizing buffer.

Ca^{2+} Dependence of PLA_2 Activity—The deduced primary structures of murine $cPLA_2\delta$, ϵ, and ζ appear to contain one C2

TABLE II
Ca^{2+} dependence of PLA_2 activity toward PAPC

These data indicate specific activity (pmol/min/mg), picomole of cleaved ^{14}C-labeled free arachidonic acid/mg of protein, for 1 min. Supernatants of 100,000 × *g* centrifugation prepared from HEK293 cells transiently expressing cPLA$_2\alpha$, δ, ϵ, or ζ were incubated with 1-palmitoyl-2-[^{14}C]arachidonoyl-PC mixed micelles in the presence of 4 mM free Ca^{2+} (+) or 4 mM EGTA (−) at 37 °C for 30 min. These results are the mean of triplicates ± S.D.

Ca^{2+}	Mock	Mock (T)[a]	cPLA$_2\alpha$	cPLA$_2\delta$	cPLA$_2\epsilon$ (T)[a]	cPLA$_2\zeta$
+	1.0 ± 0.2	0.5 ± 0.1	100 ± 6.3	3.0 ± 0.5	6.0 ± 0.4	21 ± 17
−	0.6 ± 0.1	0.3 ± 0.1	1.7 ± 0.4	0.8 ± 0.1	1.2 ± 0.2	2.0 ± 0.4

[a] T, 0.075% Triton X-100 included in homogenizing buffer.

TABLE III
Substrate preference of PLA$_2\delta$, ϵ, and ζ

These data indicate specific activity (pmol/min/mg), pmol of cleaved [^{14}C]-labeled fatty acid per mg protein, for one minute. Supernatants of 100,000 × *g* centrifugation prepared from HEK293 cells transiently expressing cPLA$_2\alpha$, δ, ϵ, or ζ were incubated with substrates at 37 °C for 30 min. These results are the mean of triplicates ± S.D., representative of three individual experiments.

Substrates	Mock	Mock (T)[a]	cPLA$_2\alpha$	cPLA$_2\delta$	cPLA$_2\epsilon$ (T)[a]	cPLA$_2\zeta$
PA-PC	5.3 ± 0.5	2.1 ± 0.4	1068 ± 2.0	13.4 ± 0.5	19.2 ± 1.4	162 ± 13
PL-PC	1.5 ± 0.2	0.9 ± 0.1	216 ± 9.4	8.1 ± 3.3	8.4 ± 1.4	92 ± 66
PA-PE	0.6 ± 0.1	0.6 ± 0.1	275 ± 23	14.5 ± 5.0	5.1 ± 1.3	367 ± 27
PL-PE	0.5 ± 0.7	0.4 ± 0.3	42 ± 21	19.9 ± 3.5	4.6 ± 0.8	704 ± 32
Lyso-PC	4.5 ± 2.7	9.5 ± 1.2	65 ± 0.9	16.4 ± 1.4	29.5 ± 4.4	9.8 ± 0.8

[a] T, 0.075% Triton X-100 included in homogenizing buffer.

domain for each molecule on their N termini, as is known for cPLA$_2\alpha$. The C2 domain of cPLA$_2\alpha$ is functionally important in its Ca^{2+}-dependent phospholipid binding properties. To examine the Ca^{2+} dependence of PLA_2 activity of cPLA$_2\delta$, ϵ, and ζ, $CaCl_2$ was replaced with EGTA in the assay buffer. The observed PLA_2 activity for 100,000 × *g* supernatants in the absence of Ca^{2+} was decreased nearly to vector control levels for all cPLA$_2$ transfectants (Table II). Essentially, the same results were obtained for 10,000 × *g* supernatant preparations (data not shown). These results indicate that murine cPLA$_2\delta$, ϵ, and ζ exhibit Ca^{2+}-dependent PLA_2 activity, like cPLA$_2\alpha$ and β.

Substrate Preference of Murine cPLA$_2$s—To estimate substrate specificity for these enzymes, we prepared 100,000 × *g* supernatant fractions of HEK293 cell lysates expressing each cPLA$_2$ molecule (Table III). Expression levels of each enzyme were determined by Western blotting as shown in Fig. 4*C*. A 4–7-fold preference for arachidonyl-PC and -PE over linoleoyl counterparts were observed for cPLA$_2\alpha$, as has been previously documented (42, 43). cPLA$_2\delta$ and ϵ did not appear to have a notable preference among the four substrates tested. Interestingly, cPLA$_2\zeta$ showed higher enzyme activity for PE than PC substrates. When monomeric lyso-PC substrate was used, cPLA$_2\epsilon$ showed relatively high lyso-PLA_1 activity compared with PLA_2 activity. Lyso-PC was not a good substrate for cPLA$_2\zeta$ in the present assay conditions.

Subcellular Localization of cPLA$_2\delta$, ϵ, and ζ—GFP-tagged cPLA$_2$s were transiently expressed in CHO-K1 cells to observe subcellular localization of cPLA$_2$s within living cells. Western blots against N-terminal GFP tags detected expression of GFP-fused cPLA$_2\alpha$, δ, ϵ, and ζ with the expected sizes, and no signals were detected for truncated proteins (data not shown). We detected comparable Ca^{2+}-dependent PLA_2 activity of GFP-fused cPLA$_2\alpha$, δ, ϵ, and ζ (data not shown). Murine cPLA$_2\alpha$ translocated from the cytoplasm to the perinuclear regions within 1 min of 10 μM ionomycin stimulation (Fig. 5*A*), as has been shown for human cPLA$_2\alpha$ (15). Murine cPLA$_2\delta$ is located in the cytoplasm in the resting state and caused translocation to the perinuclear regions by ionomycin stimulation (Fig. 5*B*). Staining of the cells with BODIPY-brefeldin A revealed these perinuclear regions as ER/Golgi. Although translocation targets are similar between cPLA$_2\alpha$ and δ, differences were seen for the latency and ratio of cells with GFP-signal translocation. It took as long as 5 min to show clear accumulation of GFP-cPLA$_2\delta$ in ER/Golgi, in contrast to cPLA$_2\alpha$ that required less than 1 min. Whereas 83% (93 of 112 cells) of GFP-cPLA$_2\alpha$-expressing cells showed translocation by 5 min of ionophore stimulation, 27% (32 of 119 cells) of GFP-cPLA$_2\delta$-expressing cells showed translocation by 10 min of stimulation ($p < 0.0001$, Fisher's exact test). Murine cPLA$_2\epsilon$ showed a dot-like pattern scattered throughout the cells (Fig. 5*C*). Staining with LysoTracker Red partly overlapped with GFP-cPLA$_2\epsilon$ signals (Fig. 5*C*). In contrast, staining with BODIPY-brefeldin A and MitoTracker Red showed distinct patterns from that of GFP-cPLA$_2\epsilon$ (data not shown). Therefore, cPLA$_2\epsilon$ appears to be partly associated with lysosomes, but not with ER/Golgi or mitochondoria. Simultaneous expression of red fluorescent protein-tagged human cPLA$_2\alpha$ and GFP-cPLA$_2\zeta$ showed similar distribution patterns, showing that murine cPLA$_2\zeta$ was localized in the cytoplasm at the resting state (Fig. 5*D*). Stimulation with ionomycin did not cause redistribution of GFP-cPLA$_2\epsilon$ and GFP-cPLA$_2\zeta$ until at least 10 min (data not shown).

DISCUSSION

We performed a genome data base search using the relatively conserved four exons among previously known cPLA$_2$s rather than the entire sequence (Fig. 1*A*). In this top-down strategy, we identified three novel cPLA$_2$s. cPLA$_2\delta$, ϵ, and ζ form a gene cluster with cPLA$_2\beta$ in murine chromosome 2E5 that corresponds to human chromosome 15q14 spanning about 0.3 Mb (Fig. 1*B*). We obtained the cDNA of these cPLA$_2$s along with cPLA$_2\beta$ from murine tissues by RT-PCR. Recently reported human cPLA$_2\delta$ is located within this gene cluster (35). We assigned murine cPLA$_2\delta$ among three newly cloned cPLA$_2$s by homology alignments and comparison of the relative chromosomal location. The remaining two gene products are termed cPLA$_2\epsilon$ and cPLA$_2\zeta$. Their order and direction within the gene cluster are shown in Fig. 1*B*. The deduced amino acid sequence of cPLA$_2\delta$, ϵ, and ζ suggested one C2 domain on the N terminus, and one lipase domain on the remaining part. Counterparts of the catalytic dyad of cPLA$_2\alpha$ (Ser-228 and Asp-549 for human cPLA$_2\alpha$) (10, 11) and a catalytically essential amino acid residue (Arg-200 for human cPLA$_2\alpha$) (10, 11) were conserved for all cPLA$_2$s (Figs. 1*A* and 2*A*). Amino acid residues critical for binding Ca^{2+} in the C2 domain of human cPLA$_2\alpha$ (Asp-43 and Asp-93) (44–46) were also conserved.

Expression profiles of transcripts of murine cPLA$_2\delta$, ϵ, and ζ

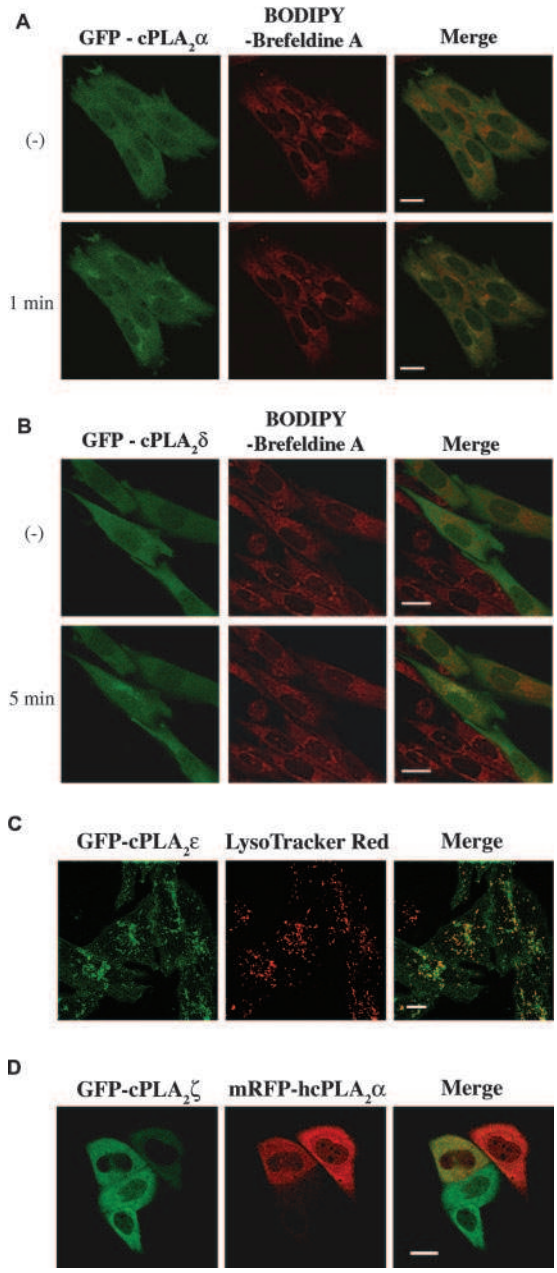

FIG. 5. **Subcellular localization of murine cPLA$_2$α, δ, ϵ, and ζ.** Confocal images of CHO-K1 cells expressing GFP-cPLA$_2$α (*A*) and GFP-cPLA$_2$δ (*B*) show localization of these proteins before (*upper panels*) and after (*lower panels*) 1 (cPLA$_2$α) or 5 min (cPLA$_2$δ) stimulation with 10 μM ionomycin. The cells were stained with 1 μM BODIPY-brefeldin A for 30 min. GFP-cPLA$_2$ϵ (*C*) was expressed in CHO-K1 cells. The cells were stained with 50 nM LysoTracker Red for 30 min. GFP-cPLA$_2$ζ (*D*) is co-expressed with mRFP-human cPLA$_2$α to show similar distribution patterns. The *scale bars* indicate a length of 10 μm.

indicated specific patterns for the organ and molecular species (Fig. 3) in contrast to ubiquitous expression of cPLA$_2$α (32). For cPLA$_2$δ, placenta (17.5 d.p.c.) was the only tissue that gave a significant signal by Northern analyses (Fig. 3). Expression of human cPLA$_2$δ was reported to be restricted to fetal skin, uterine cervix, and prostate under normal conditions (35), but not detected in placenta. Murine cPLA$_2$δ was not observed in prostate (Fig. 3), suggesting different expression patterns between species. Among the organs we used for RT-PCR, placenta showed cPLA$_2$δ-specific amplification. Small intestine and brain also produced weak but specific signals (data not shown). Together with a reported increase in psoriatic skin (35), tissue-specific expression and the inducible nature for cPLA$_2$δ are suggested. The promoter analyses and transcriptional regulation will be the next subject of our research. It is of interest that cPLA$_2$ϵ and ζ have relatively high expression in thyroid. Northern analysis identified two lengths of cPLA$_2$ζ transcripts in thyroid (Fig. 3). RT-PCR designed for ORF together with the untranslated region amplified the single DNA fragment (data not shown). The longer transcript might be a product of immature and/or alternative splicing. We could not obtain further evidence of its presence by PCR-based techniques. An alternative site of transcriptional initiation can also be considered. With regard to PLA$_2$ activities in thyroid, it was reported that thyrotropin receptor stimulating IgG from Graves' disease patients can activate PLA$_2$ activity (47–49). The role of PLA$_2$ activities and its products in the thyroid remain to be clarified.

Initial experiments using PA-PC substrate showed Ca^{2+}-dependent PLA$_2$ activity for murine cPLA$_2$δ, ϵ, and ζ (Tables I and II). Substrate specificity on the *sn*-2 fatty acid moiety and the *sn*-3 polar head group is an important point of investigation. We tested 5 different substrates in the 100,000 × *g* supernatant fractions as the enzyme source (Table III). Murine cPLA$_2$δ did not show a preference for arachidonoyl over linoleoyl phospholipid. This is in contrast to human cPLA$_2$δ, which is reported to have 6-fold preference for PL-PC over PA-PC substrate (35). Because differences in enzyme source preparation (10,000 × *g* supernatant (35) *versus* 100,000 × *g* supernatant in our study) and assay conditions could have affected the results, it is still inconclusive whether human and murine cPLA$_2$δ have different substrate specificity. Murine cPLA$_2$ϵ appeared to act better on monomeric lyso-PC substrates. Considering the unique properties of this enzyme, *i.e.* requirement of Triton X-100 in the homogenizing buffer for effective extraction of enzyme activity and association with lysosomes even in the resting state, murine cPLA$_2$ϵ is speculated to have a higher binding affinity to lipid membrane. If this is the case, cPLA$_2$ϵ could have a lower rate of access to the new substrate molecule in the micelle and show apparently low PLA$_2$ activity. Murine cPLA$_2$ζ showed a preference for PE over PC substrates. Effective binding of human cPLA$_2$α with the PC molecule was shown to be mediated by Phe-35, Leu-39, Tyr-96, and Val-97 (50). These amino acid residues other than Leu-39 are not conserved in murine cPLA$_2$ζ, as well as cPLA$_2$δ, and ϵ. This might be the reason why preference for the PC substrate was not clearly observed for these novel cPLA$_2$s. We have to wait for the final conclusion by the use of purified enzymes.

Intracellular distribution is an important issue for newly identified cPLA$_2$s, because cPLA$_2$ are assumed to require access to membrane phospholipids. Although cPLA$_2$δ was consistently recovered in the cytosol fraction (S3), significant amounts of the cPLA$_2$ϵ and ζ isozymes were observed in precipitate fractions (P2 and P3) as well (Fig. 4*B*). cPLA$_2$ϵ required Triton X-100 for stable recovery and enzyme activity, suggesting strong interaction of this enzyme with membranes. The major part of cPLA$_2$ζ protein expressed in HEK293 cells was recovered in the precipitate fraction at 10,000 × *g* centrifugation (Fig. 4*B*). cPLA$_2$ζ might self-aggregate or associate strongly with cellular organelle. In CHO cells stimulated with Ca^{2+}-ionophore, GFP-cPLA$_2$δ translocated from cytosol to ER/Golgi membranes. The target organelles were similar to those of GFP-cPLA$_2$α (Fig. 5, *A* and *B*). However, GFP-cPLA$_2$δ moved slower (up to 5 min) than GFP-cPLA$_2$α (within 1 min) and the population of cells that show clear translocation is 3-fold smaller in GFP-cPLA$_2$δ (27%, 10 min) compared with GFP-cPLA$_2$α (83%, 5 min). It is possible that maximal cPLA$_2$δ translocation may be accelerated by additional signals or conditions, such as phosphorylation, stabilization, and interaction with phosphoinositides (22, 51, 52) or phosphatidylcholine (50). On

the other hand, a unique dot-like pattern was observed for GFP-cPLA$_2\epsilon$ distribution (Fig. 5*C*). Staining with organelle markers indicated that part of cPLA$_2\epsilon$ is associated with the lysosomes. It is speculated that cPLA$_2\epsilon$ participates in lysosomal functions, *e.g.* metabolism of endocytosed phospholipids, and regulation of fusion events between the endosomes and lysosome. Further study is required to elucidate how cPLA$_2\epsilon$ relates to lysosomal functions. GFP-cPLA$_2\zeta$ seemed to be present in the cytoplasm (Fig. 5*D*) even after stimulation with Ca^{2+} ionophore. We could not observe cPLA$_2\zeta$ translocation in PAF-stimulated W11A cells, or other ionomycin-stimulated cell lines: HEK293 or mouse fibroblast L929, although GFP-tagged human cPLA$_2\alpha$ translocates from cytosol to ER/Golgi membranes in these settings (data not shown). So far, we could not provide a clear explanation for how this enzyme shows Ca^{2+}-dependent PLA$_2$ activity (Table II). Some elements may hinder association of cPLA$_2\zeta$ with membrane phospholipids and/or additional intracellular signals may allow cPLA$_2\zeta$ to gain access to substrates. It is also possible that overexpression of GFP-tagged proteins show artificial distribution, and the accurate localization and translocation study wait the antibody against native enzymes.

In summary, we identified a gene cluster of cPLA$_2$s composed of cPLA$_2\beta$, δ, ϵ, and ζ. Tissue expression of these gene products was confirmed by Northern analysis and RT-PCR. Exogenously expressed proteins have Ca^{2+}-dependent PLA$_2$ activity. Substrate preference was examined with 100,000 $\times$ *g* supernatant fractions to find a specific pattern for each molecule. Further precise analyses are to be carried out by mixed substrate assay with mass spectrometric detection of fatty acids and lysophospholipids. At the cellular level, the key issues are transcriptional and post-translational regulation of enzyme activity by intracellular signaling systems. Further investigations on biochemical and biological functions of these enzymes should advance our understanding of phospholipid metabolism and lipid mediator functions.

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