Purification of Recombinant Human cPLA$_2\gamma$ and Identification of C-Terminal Farnesylation, Proteolytic Processing, and Carboxymethylation by MALDI-TOF-TOF Analysis†

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ABSTRACT: Cytosolic phospholipase A$_2\gamma$ (cPLA$_2\gamma$) is a calcium-independent, membrane-associated phospholipase A$_2$ that possesses a C-terminal prenylation motif (-CCLA) whose covalent structure cannot be deduced from the primary sequence alone. Accordingly, we overexpressed human cPLA$_2\gamma$ containing an N-terminal His tag ((His)$_6$cPLA$_2\gamma$) in Sf9 cells and quantitatively solubilized and purified the enzyme by sequential immobilized metal affinity and Mono Q column chromatographies. The final preparation appeared as a single 61 kDa band after SDS−PAGE/silver-staining, possessed high lysophospholipase activity (50 μmol min^{-1} mg^{-1}), and was inhibited by, but did not hydrolyze, palmitoyl-CoA. Radiolabeling of recombinant human cPLA$_2\gamma$ with [^{3}H]-mevalonolactone in the absence of statins and subsequent cleavage of prenyl groups with Raney nickel revealed that the enzyme is only farnesylated and is not geranylgeranylated. Analysis of CNBr-digested cPLA$_2\gamma$ by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI/TOF-TOF) mass spectrometry demonstrated the presence of a farnesyl moiety at Cys-538, cleavage of the Cys^{538}−Cys^{539} bond, and carboxymethylation of the resultant C-terminal prenylated cysteine. Collectively, these results describe the solubilization and purification of recombinant cPLA$_2\gamma$ to homogeneity and identify cPLA$_2\gamma$ as a farnesylated protein that undergoes at least three sequential posttranslational modifications that likely facilitate its targeting and interactions with its membrane substrates.

Acute myocardial ischemia results in the activation of phospholipases A$_2$ (PLA$_2$s), inhibition of lysophospholipases, and the resultant accumulation of lysophospholipids and fatty acids in ischemic zones (*1*−*4*). Both fatty acids (and their oxygenated metabolites) and lysophospholipids have profound effects on sarcolemmal ion channels (*5*−*9*), protein kinase activity (*10*−*12*), and receptor-mediated signaling cascades (*13*−*16*). Initial characterization of intracellular myocardial phospholipase A$_2$ and lysophospholipase activities revealed the presence of multiple cytosolic and membrane-bound enzymes that were predominantly calcium-independent (*17*−*22*). During the course of those studies, two distinct lysophospholipases (a lysophospholipase/acyl-CoA hydrolase and a lysophospholipase-transacylase) and a calcium-independent PLA$_2$ (iPLA$_2$)[1] were purified from the cytosolic fraction of myocardial homogenates. Notably, the two purified cytosolic lysophospholipases are both inhibited by endogenous cardiac amphiphiles (e.g., palmitoyl carnitine and palmitoyl-CoA) that increase during ischemia (*17*−*20*). Conversely, the cytosolic myocardial iPLA$_2$ has been demonstrated to be activated by ATP (*23*), complexed with phosphofructokinase (*24*), and regulated by Ca^{2+}/calmodulin (*25*). In contrast to the successful purification of these soluble enzymes from myocardial cytosol, multiple attempts at purification of the myocardial membrane-associated calcium-independent PLA$_2$ and lysophospholipase activities were unsuccessful.

Molecular biologic approaches have identified the primary structures of at least three myocardial intracellular phospholipases A$_2$ (*26*−*31*), a lysophospholipase/acyl-CoA hydrolase (*32*), and a lysophospholipase-transacylase (*33*). Two of the three intracellular myocardial PLA$_2$s belong to the calcium-independent phospholipase A$_2$ (iPLA$_2$) subfamily (iPLA$_2\beta$ (*26*, *27*) and iPLA$_2\gamma$ (*30*, *31*)) due to their active site sequence similarity to iPLA$_2\alpha$ (patatin) (*34*), while the third is structurally homologous to the cytosolic phospholipase A$_2$ (cPLA$_2$) subfamily and has been designated cPLA$_2\gamma$ (*28*, *29*). Unlike cPLA$_2\alpha$ or the secretory PLA$_2$s, all three of the presently identified heart PLA$_2$ and lysophospholipase iso-

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[1] Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; β-ME, β-mercaptoethanol; cPLA$_2\alpha$, cytosolic phospholipase A$_2\alpha$; cPLA$_2\beta$, cytosolic phospholipase A$_2\beta$; cPLA$_2\gamma$, cytosolic phospholipase A$_2\gamma$; iPLA$_2\alpha$, calcium-independent phospholipase A$_2\alpha$; iPLA$_2\beta$, calcium-independent phospholipase A$_2\beta$; iPLA$_2\gamma$, calcium-independent phospholipase A$_2\gamma$; palm-CoA, palmitoyl-CoA.

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forms do not require a calcium ion for either membrane association or catalysis (*26*−*33*).

In previous work, heterologous expression and characterization of phospholipases A_2 have revealed substantial insights into their roles in lipid second messenger generation and lipid metabolism (*35*−*39*). Recently, recombinant cPLA$_2\gamma$ has been further characterized with respect to inhibitor sensitivity, substrate selectivity, and the ability to release fatty acids from phospholipids in intact cells (*40*). In addition, Stewart et al. demonstrated that cPLA$_2\gamma$ transfected into lung fibroblasts from cPLA$_2\alpha$ deficient mice could be activated by mouse serum, thus suggesting a potential role for cPLA$_2\gamma$ in lipid second messenger generation and cellular signaling (*40*). Following its initial cloning, cPLA$_2\gamma$ was found to lack the C2 Ca^{2+}-dependent lipid binding domain present in cPLA$_2\alpha$ and cPLA$_2\beta$ but possessed a C-terminal prenylation motif (-CCLA) (*28*, *29*). Expression of cPLA$_2\gamma$ in COS cells in the presence of [^{3}H]-mevalonolactone demonstrated that the protein was covalently radiolabeled, but the nature of the isoprenyl group(s) present and additional associated covalent modifications at the C-terminus were not identified (*28*). Although the C-terminal prenylation motif (-CCLA) matches the -CaaX consensus sequence for modification by farnesyltransferase (a = usually an aliphatic amino acid and X = A, M, S, Q, or F) (*41*−*43*), we could not exclude the possibility that it could be alternatively prenylated by geranylgeranyltransferase-I or by geranylgeranyltransferase-II which, in conjunction with Rab escort protein (REP), geranylgeranylates two adjacent cysteines near the C-terminus of Rab5 (consensus sequence -CCXX) (*44*). Given the importance of protein prenylation in protein function (for reviews, see refs *45*−*47*), we sought to identify the isoprenoid group(s) and potential processing of the C-terminus of cPLA$_2\gamma$. Accordingly, we overexpressed (His)$_6$cPLA$_2\gamma$ in Sf9 cells, quantitatively solubilized membrane-associated activity, and purified the resultant solubilized polypeptide to apparent homogeneity by sequential column chromatographies. Subsequent analysis of the purified protein by matrix-assisted laser desorption/ionization (MALDI) time-of-flight/time-of-flight (TOF/TOF) tandem mass spectrometry demonstrated that cPLA$_2\gamma$ is farnesylated (and not geranylgeranylated) at Cys-538 and that after farnesylation the peptide is proteolytically processed (i.e., cleaved at Cys^{538}−Cys^{539}) and subsequently carboxymethylated. Collectively, these results provide the first detailed mass spectral analysis of the covalent chemical modifications at the C-terminus of cPLA$_2\gamma$ that likely influence its interactions with subcellular membrane compartments and modulate its role in lipid second messenger generation.

EXPERIMENTAL PROCEDURES

Materials. Radiolabeled phospholipid substrates 1-palmitoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine, 1-[1-^{14}C]-palmitoyl-*sn*-glycero-3-phosphocholine, and [1-^{14}C]-palmitoyl CoA were purchased from Perkin-Elmer Life Sciences. RS-[5-^{3}H(N)]-mevalonolactone was obtained from American Radiolabeled Chemicals. A peptide corresponding to residues 230−245 (CPERDLTFLRGLWGSA), containing an N-terminal cysteine, was synthesized by the Protein Chemistry Laboratory at Washington University. HPLC-grade organic solvents and channeled LK6D silica gel 60 Å thin-layer chromatography plates (Whatman) were obtained from Fisher Scientific. TALON metal affinity resin was purchased from BD Biosciences. ECL reagents were purchased from Amersham Pharmacia. Sf9 cell culture media and reagents were obtained from GIBCO BRL. Octaethyleneglycol mono-*n*-dodecyl ether ($C_{12}E_8$) was purchased from Anatrace. *N*-Acetyl-*S*-farnesyl-L-cysteine and *N*-acetyl-*S*-geranylgeranyl-L-cysteine were purchased from Calbiochem. Most other reagents were purchased from either Fisher Scientific or Sigma.

Cloning of Human cPLA$_2\gamma$ and Construction of a Baculovirus Vector Encoding (His)$_6$cPLA$_2\gamma$ and Expression in Sf9 Cells. The full-length human cPLA$_2\gamma$ (1.6 kb) was amplified by PCR from a human heart cDNA library (Clontech) utilizing primers that incorporated 5′ *EcoRI* and 3′ *SalI* restriction sites for subcloning into a pFASTBac baculovirus vector (Life Technologies). A sequence encoding a proximal N-terminal (His)$_6$-tag followed by the cPLA$_2\gamma$ coding sequence was then constructed utilizing Excite mutagenesis (Stratagene) to allow affinity purification of cPLA$_2\gamma$ by immobilized metal affinity chromatography while preserving the native prenylation consensus sequence (-CCLA) at the C-terminus of cPLA$_2\gamma$. After sequencing, to ensure the integrity of the construct, a bacmid encoding (His)$_6$cPLA$_2\gamma$ was prepared using the Bac-to-Bac Baculovirus Expression System (Invitrogen Life Technologies) for subsequent Cellfectin-mediated transfection of Sf9 cells to produce infectious recombinant baculovirus. Amplified recombinant baculovirus was then used to infect a 50 mL spinner culture of Sf9 cells for 72 h, and the supernatant was collected as the high titer viral stock. For expression of (His)$_6$cPLA$_2\gamma$, 300 mL cultures of Sf9 cells in spinner flasks were infected at an MOI ≈5 for 48 h at 27 °C.

Solubilization and Purification of (His)$_6$cPLA$_2\gamma$. Sf9 cells containing (His)$_6$cPLA$_2\gamma$ (0.3 L culture grown in a 0.5 L spinner flask) were pelleted by centrifugation (900 rpm × 10 min), resuspended in 50 mL of ice cold phosphate-buffered saline, and repelleted. All subsequent steps were performed at 4 °C. The cell pellet was resuspended in 40 mL of lysis buffer (25 mM $NaHPO_4$ buffer, pH 7.5, containing 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.34 M sucrose, 5 μg/mL aprotinin, and 5 μg/mL leupeptin) and sonicated utilizing a Vibracell sonicator (15 × 1 s pulses at 40% power). Membranes were separated from the cytosol by centrifugation (100 000*g* for 1 h), resuspended utilizing a Teflon homogenizer in 40 mL of lysis buffer, sonicated (15 × 1 s pulses), and were again pelleted by centrifugation (100 000*g* for 1 h). The resultant membrane pellet was resuspended in 40 mL of buffer A (25 mM $NaHPO_4$ buffer, pH 7.5 containing 1 mM β-mercaptoethanol (β-ME) and 20% glycerol) to which 10% $C_{12}E_8$ was added dropwise (while stirring) to a final concentration of 0.5%. After $C_{12}E_8$ addition, the resuspended membrane fraction was stirred for 10 min and was stored overnight on ice in a capped 50 mL conical tube. Insoluble membrane proteins were pelleted by centrifugation (10 000*g* for 10 min). Sodium chloride was added to the supernatant (100 mM final concentration) containing solubilized (His)$_6$cPLA$_2\gamma$ and mixed by inversion with 5 mL of Co^{2+}-TALON affinity resin equilibrated with buffer B (buffer A containing 0.1% $C_{12}E_8$ and 100 mM NaCl) for 4 h. This suspension was then poured into a Bio-Rad Econo-Pac column containing 1 mL of Co^{2+}-TALON affinity

resin equilibrated with buffer A. This column was washed with 60 mL of buffer B containing 200 mM NaCl to reduce nonspecific binding. Next, bound $(His)_6cPLA_2\gamma$ was eluted from the resin in 20 mL of buffer B containing 250 mM imidazole. Following dialysis against buffer B, $(His)_6cPLA_2\gamma$ was applied to a 5 mL Ni^{2+}-charged Hi-Trap chelating column (Amersham) equilibrated with the same buffer utilizing an FPLC system at a flow rate of 0.25 mL/min. The column was then washed with buffer B (10 mL) at a flow rate of 0.5 mL/min followed by a linear gradient of buffer C (buffer B containing 200 mM NaCl and 250 mM imidazole) as follows: 5% buffer C (20 mL), 10% buffer C (10 mL), and 100% buffer C (30 mL). Fractions containing $cPLA_2\gamma$ activity were pooled and dialyzed against buffer D (20 mM imidazole, pH 7.5 containing 0.1% $C_{12}E_8$, 20% glycerol, and 0.5 mM DTT). The dialyzed $(His)_6cPLA_2\gamma$ was directly applied to a Mono Q HR 5/5 FPLC column (Amersham) equilibrated with buffer D at a flow rate of 0.5 mL/min, washed with 8 mL of buffer D, and eluted with a 15 mL linear gradient (0−100%) of buffer D containing 1 M NaCl. Fractions containing purified $(His)_6cPLA_2\gamma$ were pooled, flash-frozen in liquid N_2, and stored at −80 °C. The purified flash-frozen material was stable for at least 3 months.

Cytosolic Phospholipase $A_2\gamma$ Activity Assays. Samples containing $(His)_6cPLA_2\gamma$ (0.02−0.2 μg of protein) were diluted in assay buffer (20 mM HEPES, pH 7.5 containing 150 mM NaCl, 5 mM EGTA, 0.1 mM DTT, 20% glycerol, and 1 mg/mL fatty acid free bovine serum albumin) prior to injection of radiolabeled substrate dissolved in ethanol (for phospholipids) or water (for lysophospholipids) to initiate the reaction. In some experiments, palmitoyl-CoA (palm-CoA), palmitoyl carnitine, CoA, and/or palmitic acid were added simultaneously with the radiolabeled substrate. After gentle brief vortexing, samples were incubated at 37 °C for 2−5 min before extracting the radiolabeled product and remaining substrate into butanol. Samples were spotted on TLC plates, overlaid with a fatty acid standard, dried, and developed in petroleum ether/ethyl ether/acetic acid (70:30:1). The region of the plate corresponding to the fatty acid standard (visualized by iodine staining) was scraped into scintillation vials and quantified by liquid scintillation chromatography.

Generation and Immunoaffinity Purification of Anti-Human $cPLA_2\gamma$ Antibodies. A peptide corresponding to residues 230−245 of human $cPLA_2\gamma$ (G230: CPERDLTFLRGLWGSA) was coupled to maleimide-activated mariculture keyhole limpet hemocyanin according to the instructions of the manufacturer, emulsified with Freund's complete adjuvant, and injected subcutaneously into New Zealand white rabbits. At two week intervals, the rabbits were boosted with the peptide-KLH conjugate emulsified in Freund's incomplete adjuvant until seroconversion occurred. The G230 peptide was coupled to activated thiol-Sepharose 4B for immunoaffinity purification of the anti-G230 peptide antibodies as previously described for the purification of anti-$iPLA_2\beta$ antibodies (*48*). Immunoreactive rabbit antisera were diluted 1:10 with 10 mM Tris-HCl, pH 7.5, prior to application to the G230 peptide affinity column equilibrated with the same buffer. The resin was extensively washed with 10 column volumes of 10 mM Tris-HCl, pH 7.5 containing 500 mM NaCl prior to elution of bound antibodies with 0.1 M glycine-HCl, pH 1.0, into collection tubes containing 1 M Tris-HCl, pH 9.0 (1:3 fraction volume). Antibodies were concentrated, dialyzed against phosphate-buffered saline containing 20% glycerol, and stored at −80 °C prior to use.

Radiolabeling of $(His)_6cPLA_2\gamma$ with [^{3}H]-Mevalonolactone. Twenty mL of a suspension of Sf9 cells (3×10^6 cells/mL) was infected with baculovirus encoding $(His)_6cPLA_2\gamma$ for 30 h. The cells were pelleted (900 rpm for 5 min) and resuspended in 20 mL of fresh media containing 1 mCi of [^{3}H]-mevalonolactone and incubated for an additional 18 h. Cells were pelleted (900 rpm for 5 min), washed once with 10 mL of ice-cold PBS, and resuspended in 7 mL of 25 mM $NaHPO_4$ buffer, pH 7.5 containing 0.34 M sucrose, 1 mM EDTA, 1 mM β-ME, 5 μg/mL leupeptin, and 5 μg/mL aprotinin. Following sonication (20×1 s bursts, 30% power), cell membranes were isolated by centrifugation (100 000*g* for 1 h) and solubilized in 10 mL of 25 mM $NaHPO_4$ buffer, pH 7.5 containing in addition, 20% glycerol, 1 mM β-ME, and 1% $C_{12}E_8$ utilizing a Teflon homogenizer followed by sonication (10×1 s bursts, 30% power). The solubilized membranes were incubated with 3 mL of Co^{2+}-TALON affinity resin (equilibrated with solubilization buffer) for 1 h, washed with 30 mL of 25 mM $NaHPO_4$ buffer, pH 7.5 containing 20% glycerol, 500 mM NaCl, 1 mM β-ME, and 0.1% $C_{12}E_8$. Radiolabeled $(His)_6cPLA_2\gamma$ was eluted with 25 mM $NaHPO_4$ buffer, pH 7.5 containing 20% glycerol, 1 mM β-ME, and 250 mM imidazole.

Raney Nickel Cleavage of [^{3}H]-$(His)_6cPLA_2\gamma$ and Analysis of the Radiolabeled Products by RP-HPLC. Purified [^{3}H]-$(His)_6cPLA_2\gamma$ was precipitated with $CH_3OH/CHCl_3/H_2O$ at 4 °C as described (*49*), pelleted by centrifugation (14 000 rpm for 10 min), and dissolved in 0.5 mL of 8 M guanidine hydrochloride buffered by 0.2 M sodium phosphate, pH 7.0. Raney Ni cleavage and subsequent analysis of the reaction products were performed essentially as described (*50*). Internal standards (100 μg each of *N*-acetyl-*S*-farnesyl-L-cysteine and *N*-acetyl-*S*-geranylgeranyl-L-cysteine) were added to each reaction or separately in control reactions. Samples were extracted twice with pentane (2 mL) to remove the noncovalently bound radiolabel and then overlaid with 2 mL of pentane. Raney nickel (∼50 mg), previously washed with water and ethanol, was added to each reaction that was then sealed under N_2 and incubated in an oil bath (100 °C) for 15 h while mixing with a magnetic stir bar. The pentane layer containing the isoprenoid products was removed, and the aqueous layer was extracted once with fresh pentane that was combined with the original layer. After drying under N_2 (to ≈50 μL), each sample was diluted 5-fold in methanol and injected onto a Vydac RP C18 90 Å pharmaceutical HPLC column equilibrated with deionized water at a flow rate of 0.5 mL/min. The column was then washed with deionized water (2.5 mL), a 0−70% acetonitrile linear gradient (2.5 mL), and a 70−100% acetonitrile linear gradient (30 mL), followed by washing with 100% acetonitrile (15 mL). Isoprenoid products were detected by UV absorbance (210 nm), and 0.5 mL fractions were collected and analyzed by scintillation spectrometry.

Cyanogen Bromide Digestion, RP-HPLC, and Mass Spectral Analysis of $(His)_6cPLA_2\gamma$ Prenylation. For CNBr digests, purified $(His)_6cPLA_2\gamma$ (45 μg) was precipitated as described previously, dissolved in 50 μL of 0.2 M Tris-HCl, pH 8.0, containing 6 M guanidine-HCl and 10 mM DTT,

sealed under N_2, and incubated at 50 °C for 30 min. After allowing the sample to cool to room temperature, a 6 μL aliquot of methanol/4-vinylpyridine (1:1 v/v) was added, and the sample was incubated for 1 h at room temperature in the dark. Reductively alkylated (His)$_6$cPLA$_2\gamma$ was reprecipitated with $CH_3OH/CHCl_3/H_2O$ at 4 °C, dissolved in 50 μL of 70% trifluoroacetic acid, and subsequently incubated with CNBr (100−500 μg) under N_2 for 6 h in the dark at 23 °C. The samples were next dried in a Speed-Vac, dissolved in 20% acetonitrile, absorbed and desorbed from a C18 Zip-Tip, and mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (CHCA, 10 mg/mL in 50% acetonitrile containing 0.1% trifluoroacetic acid). Samples were hand painted onto 192-well plates (ABI) and were allowed to air-dry in preparation for MALDI-TOF-TOF analysis. Mass spectral (MS) and MS/MS analyses were performed utilizing an Applied Biosystems 4700 Proteomics Analyzer (Framingham, MA), which possesses a 200 Hz Nd:YAG laser that operates at 355 nm. Mass accuracy of the instrument was externally calibrated to the 4700 Proteomics Analyzer Calibration Mixture of peptides. For MALDI-MS and MS/MS analyses, spectra were obtained by the accumulation of 1000 and 5000 consecutive laser shots, respectively, at a collision energy of 1 kV with air serving as the collision gas. Calculations of predicted peptide and peptide fragment masses were performed using the programs developed at the UCSF Mass Spectrometry Facility (http://prospector.ucsf.edu).

Other Procedures. For SDS−PAGE, proteins were separated according to the method of Laemmli (*51*) and visualized by silver staining (*52*). For Western analyses, the separated proteins in SDS−PAGE gels were transferred to poly(vinylidene difluoride) membranes and subsequently probed with antibodies directed against the G230 peptide in conjunction with a protein A−horseradish peroxidase conjugate. Protein concentrations were determined by the Coomassie Plus Protein Assay Reagent (Pierce) using BSA as standard.

RESULTS

Cloning, Expression, and Solubilization of (His)$_6$cPLA$_2\gamma$. In the initial reports of the human cPLA$_2\gamma$ sequence, Northern analyses of various human tissues demonstrated that cPLA$_2\gamma$ is highly expressed in both heart and skeletal muscle (*28*, *29*). Accordingly, we cloned cPLA$_2\gamma$ from a human heart cDNA library and engineered an N-terminal (His)$_6$-tag proximal to the coding sequence to facilitate affinity purification while preserving the C-terminal -CaaX prenylation consensus sequence. Upon sequencing, the cPLA$_2\gamma$ cDNA was found to be identical to that reported previously (*28*). The human (His)$_6$cPLA$_2\gamma$ cDNA was then subcloned to create a recombinant bacmid from which baculoviruses were generated for expression of (His)$_6$cPLA$_2\gamma$ in Sf9 cells. Western analyses of membrane and cytosolic fractions from Sf9 cells infected with either wild-type baculovirus (pFB) or baculovirus encoding human (His)$_6$cPLA$_2\gamma$ revealed that the overwhelming majority of the expressed protein is present in the membrane fraction (Figure 1A). Since the majority of the (His)$_6$cPLA$_2\gamma$ heterologously expressed in Sf9 cells is membrane-bound, attempts were made to solubilize the membrane-associated (His)$_6$cPLA$_2\gamma$ form with various detergents. Of those tested, polyoxyethelene(8)dodecyl ether ($C_{12}E_8$) was found to be effective at

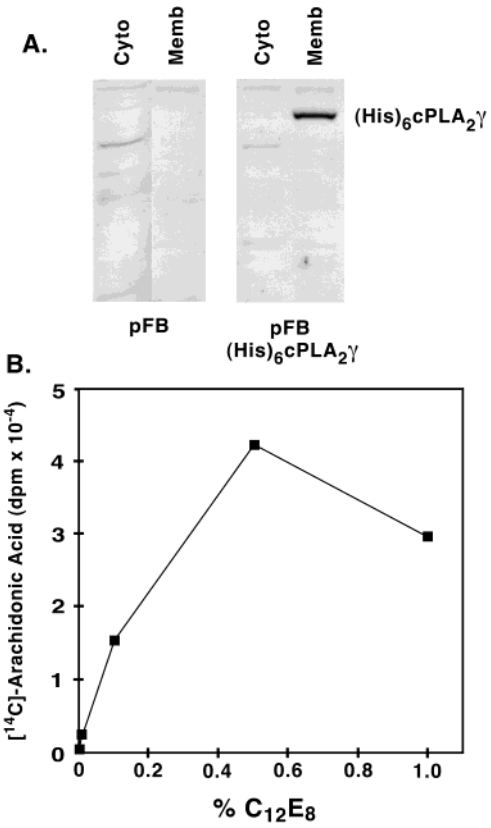

FIGURE 1: Western analysis of the subcellular localization of (His)$_6$cPLA$_2\gamma$ in Sf9 cells and activation of (His)$_6$cPLA2γ by $C_{12}E_8$. Membrane (Memb) and cytosolic (Cyto) fractions from wild-type (pFB) and (His)$_6$cPLA$_2\gamma$ baculovirus-infected Sf9 cells were prepared as described in Experimental Procedures. In panel A, each fraction (10 μg of protein) was subjected to SDS−PAGE and analyzed by ECL Western blotting with an antibody directed against residues 230−245 of the human cPLA$_2\gamma$. In panel B, the membrane fraction containing (His)$_6$cPLA$_2\gamma$ was first solubilized with the indicated concentration of $C_{12}E_8$ and centrifuged at 100 000*g* for 1 h, and the supernatant (containing solubilized (His)$_6$cPLA$_2\gamma$) was incubated with 5 μM 1-palmitoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine for 3 min at 37 °C in the presence of 50 mM HEPES, pH 7.5 containing 5 mM EGTA, 1 mg/mL fatty-acid free bovine serum albumin, and 150 mM NaCl. Reaction products were extracted into butanol and separated by thin-layer chromatography, and regions corresponding to the fatty acid product were analyzed by liquid scintillation spectrometry. The activity of resuspended Sf9 membranes containing (His)$_6$cPLA$_2\gamma$ before the addition of detergent was 0.78×10^4 dpm [^{14}C]-arachidonic acid in the above assay.

solubilizing and apparently activating (up to 5−10-fold) the enzyme relative to the resuspended membrane fraction before the addition of detergent and ultracentrifugation (Figure 1B). This increase in catalytic activity may be caused by a number of factors, such as removal or dilution of the endogenous phospholipid substrate, improved access of enzyme to substrate, altered substrate presentation (i.e., micellar vs bilayer), or disruption of cPLA$_2\gamma$-inhibitor complexes. In contrast, solubilization of cPLA$_2\gamma$-containing membranes with Triton X-100 did not result in a similar activation of enzyme activity (data not shown).

Purification of (His)$_6$cPLA$_2\gamma$ to Homogeneity. The purification strategy undertaken exploited the specificity inherent in immobilized metal affinity chromatography, which represents a powerful method to purify His-tagged proteins (*53*, *54*). Accordingly, Sf9 cell membranes containing (His)$_6$cPLA$_2\gamma$

Table 1: Purification of Human $(His)_6cPLA_2\gamma$ from Sf9 Cell Membranes[a]

fraction	protein (mg)	total activity[b]	specific activity[c]	% yield
solubilized membranes	33	330	10	
Co^{2+} TALON	4.1	160	40	48
Ni^{2+} Hi-Trap	0.59	150	250	45
Mono Q	0.31	80	260	24

[a] Solubilized Sf9 cell membranes containing $(His)_6cPLA_2\gamma$ and eluates from Co^{2+} TALON, Ni^{2+} High-Trap, and Mono Q FPLC columns were incubated with 5 μM 1-palmitoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine in the presence of 5 mM EGTA. Released arachidonic acid was extracted into butanol, separated by thin-layer chromatography, and quantified by scintillation spectrometry as described under Experimental Procedures. [b] nmol arachidonic acid min^{-1}. [c] nmol arachidonic acid min^{-1} mg of $protein^{-1}$.

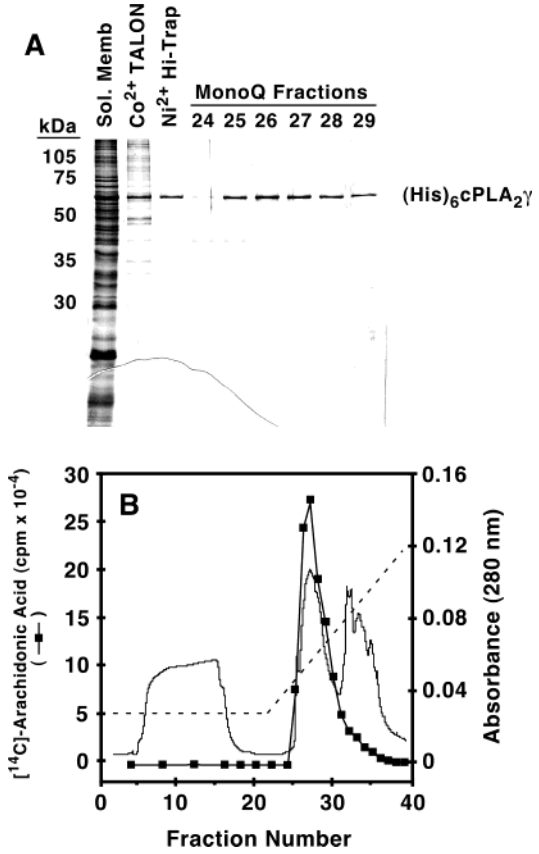

FIGURE 2: Purification of $cPLA_2\gamma(His)_6$. Panel A: samples from $C_{12}E_8$-solubilized Sf9 cell membranes containing $(His)_6cPLA_2\gamma$ (lane 1), Co^{2+}-TALON affinity resin eluate (lane 2), Ni^{2+}-High Trap affinity column eluate (lane 3), and fractions of purified $(His)_6cPLA_2\gamma$ following MonoQ FPLC (lanes 4−9) were boiled in SDS−PAGE loading buffer, separated by SDS−PAGE, fixed, and stained with silver. Panel B: dialyzed fractions from the chelating Ni^{2+} Hi-Trap column containing $(His)_6cPLA_2\gamma$ were applied to a Mono Q FPLC column equilibrated with buffer C. After extensive washing with buffer C, $(His)_6cPLA_2\gamma$ was eluted with a 0−600 mM NaCl gradient (dashed line). Aliquots of column eluates were assayed by quantifying radiolabeled fatty acid released from palmitoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine as described in Experimental Procedures. —, absorbance at 280 nm.

were solubilized with 0.5% $C_{12}E_8$ and directly applied to a Co^{2+}-charged TALON affinity resin utilizing a batch absorption procedure as described in Experimental Procedures. This step resulted in an ≈4-fold increase in specific activity (Table 1). Next, a high-trap Ni^{2+} chelating resin employing an imidazole gradient was utilized, leading to a near homogeneous preparation (Figure 2A). The final step utilized a Mono Q FPLC column employing a NaCl gradient (Figure 2B) resulting in a single intense 61 kDa band after silver-staining of SDS−PAGE gels (Figure 2A). A typical purification of $cPLA_2\gamma(His)_6$ is summarized in Table 1 (representative of five preparations). The specific activity of the purified enzyme utilizing 5 μM 1-palmitoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine as substrate was ≈260 nmol min^{-1} mg^{-1}. Purification of His-tagged proteins to homogeneity from eukaryotic cells can be challenging due to the presence of numerous endogenous proteins that can bind tightly to immobilized metal affinity columns (*55*). The use of tandem immobilized metal affinity columns with different chelated metal ions in conjunction with Mono Q chromatography successfully circumvented visible contamination in the purified fractions.

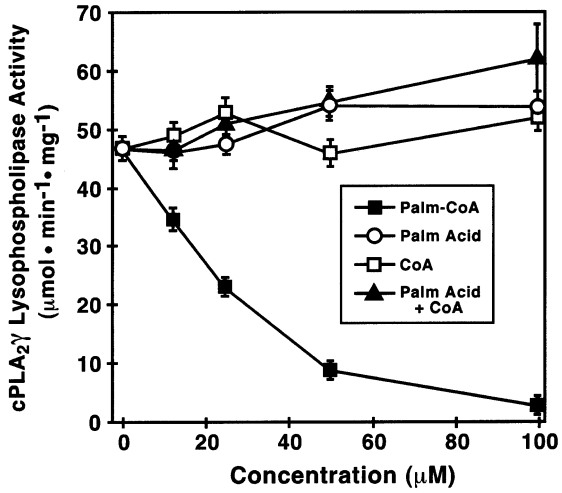

FIGURE 3: Inhibition of $(His)_6cPLA_2\gamma$ lysophospholipase activity by palmitoyl-CoA. Purified $(His)_6cPLA_2\gamma$ (25 ng) in 20 mM HEPES, pH 7.5 containing 5 mM EGTA, 150 mM NaCl, 0.5 mM DTT, 1 mg/mL BSA, and 20% glycerol was incubated for 2 min with 100 μM 1-[1-^{14}C]-palmitoyl-*sn*-glycero-3-phosphocholine in the presence of the indicated concentrations of palmitoyl-CoA (solid squares), palmitic acid (open circles), CoA (open squares), or palmitate and CoA combined (solid triangles). Reactions were terminated by the extraction of remaining substrate and reaction products into butanol, separation by TLC, and quantification of released radiolabeled fatty acid by scintillation spectrometry as described under Experimental Procedures. Points and error bars represent the averages and standard errors, respectively, of four separate determinations.

Palmitoyl-CoA (Palm-CoA) Inhibition of $(His)_6cPLA_2\gamma$ Lysophospholipase Activity. Recombinant $cPLA_2\gamma$ has previously been reported to have severalfold higher lysophospholipase activity relative to phospholipase A_2 activity (*40*). Incubation of purified $(His)_6cPLA_2\gamma$ with 100 μM 1-[1-^{14}C]-palmitoyl-*sn*-glycero-3-phosphocholine demonstrated that the enzyme possessed lysophospholipase activity of approximately 50 μmol min^{-1} mg^{-1} (Figure 3), which is comparable to that previously reported (*40*). Previous work has demonstrated that myocardial microsomes contain robust lysophospholipase activity that is inhibited by palmitoyl-CoA (*17*). Since $cPLA_2\gamma$ mRNA is highly expressed in heart (*28*, *29*) and the recombinant enzyme is a potent membrane-bound lysophospholipase, we examined the possibility that $cPLA_2\gamma$ was inhibited by palm-CoA. As shown in Figure 3, incubation of $(His)_6cPLA_2\gamma$ with increasing amounts of palm-CoA inhibited $cPLA_2\gamma$ lysophospholipase activity with an IC_{50}

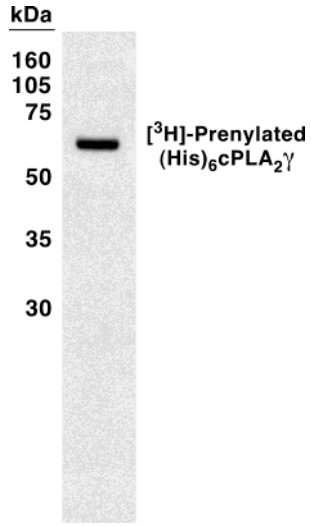

FIGURE 4: Radiolabeling of (His)$_6$cPLA$_2\gamma$ in the presence of [^{3}H]-mevalonolactone. Sf9 cells (20 mL at 3×10^6 cells/mL) were infected with baculovirus encoding (His)$_6$cPLA$_2\gamma$ for 30 h, pelleted by centrifugation, and resuspended in media containing 1 mCi [^{3}H]-mevalonolactone. After incubation for 18 h, cell membranes were prepared and solubilized in buffer containing 1% $C_{12}E_8$ as described in Experimental Procedures. Solubilized (His)$_6$cPLA$_2\gamma$ was bound to TALON Co^{2+}-affinity resin, washed extensively with buffer, and eluted with buffer containing 200 mM imidazole. [^{3}H]-(His)$_6$-cPLA$_2\gamma$ (~10 000 dpm) in the eluate fractions was precipitated with methanol/chloroform/water, dried in a Speed-Vac, dissolved in SDS−PAGE loading buffer, and resolved by SDS−PAGE. After drying the gel, [^{3}H]-(His)$_6$cPLA$_2\gamma$ was visualized by autoradiography.

of approximately 25 μM. In contrast, similar concentrations of palmitic acid, CoA, and the combination of palmitic acid and CoA together do not appreciably inhibit cPLA$_2\gamma$ lysophospholipase activity (Figure 3). Palmitoyl-CoA was not measurably hydrolyzed by (His)$_6$cPLA$_2\gamma$ despite utilizing up to 100-fold more enzyme as was present in the lysophosphatidylcholine hydrolase assays (data not shown). Moreover, purified (His)$_6$cPLA$_2\gamma$ bound to palm-CoA agarose in the presence of supramicellar concentrations of $C_{12}E_8$ detergent (0.1% = 1.85 mM, cmc = 0.11 mM) was largely retained in the presence of up to 400 mM NaCl and could be eluted with 0.5 mM palm-CoA (data not shown). In contrast to the myocardial cytosolic lysophospholipases, cPLA$_2\gamma$ lysophospholipase activity was not significantly inhibited by up to 100 μM palmitoyl carnitine (data not shown).

Identification of the Covalent Nature of the Isoprenoid Moiety in (His)$_6$cPLA$_2\gamma$. Human cPLA$_2\gamma$ has been previously demonstrated to be prenylated when heterologously expressed in COS cells (*28*). The C-terminus of cPLA$_2\gamma$ contains a -CCLA prenylation consensus motif that, from analyses of similar motifs (-CC) in the Rab family of proteins and the -CaaX motifs of other prenylated proteins, can either be farnesylated or be geranylgeranylated. Since the precise covalent nature of the C-terminal prenylation and processing of cPLA$_2\gamma$ was not known, we sought to determine whether either or both cysteines were farnesylated, geranylgeranylated, or further processed utilizing the (His)$_6$cPLA$_2\gamma$ purified from Sf9 cells. Prior characterization of heterotrimeric G protein γ subunits (*56*, *57*) and rab proteins (*58*) in insect cells has demonstrated that Sf9 cells are capable of both farnesylating and geranylgeranylating heterologously expressed proteins. Thus, we first sought to determine if (His)$_6$cPLA$_2\gamma$ was farnesylated or geranylgeranylated. As anticipated, (His)$_6$cPLA$_2\gamma$ is covalently radiolabeled in Sf9 cells cultured in the presence of [^{3}H]-mevalonolactone (Figure 4). To determine if the isoprenoid radiolabel is present as either farnesyl or geranylgeranyl moieties (or both), we subjected purified radiolabeled cPLA$_2\gamma$ to treatment with Raney Ni under denaturing conditions prior to extracting the released isoprenoids into pentane. Reverse-phase HPLC analysis of the [^{3}H]-labeled pentane-extracted isoprenoids in the presence of farnesyl (all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene) and geranylgeranyl (all-*trans*-2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene) internal standards revealed that cPLA$_2\gamma$ is almost exclusively farnesylated with almost no radioactivity detected in the peak corresponding to all-*trans*-2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene (Figure 5).

Identification of the Covalent Structure of the C-Terminus of (His)$_6$cPLA$_2\gamma$ by MALDI-TOF-TOF. Since cPLA$_2\gamma$ contains two potential sites of prenylation within the C-terminal

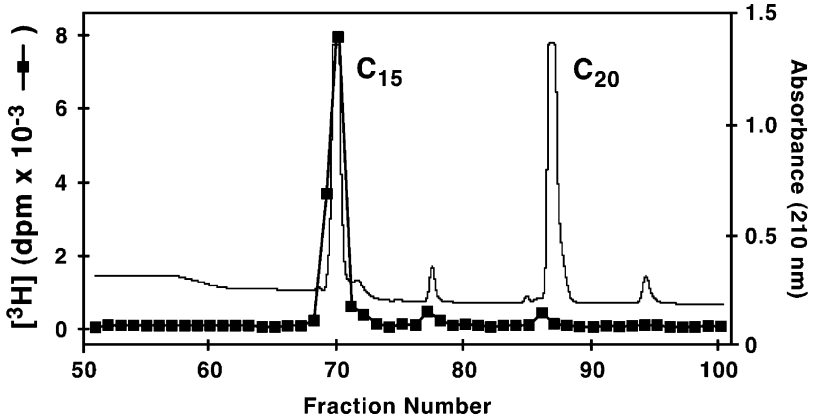

FIGURE 5: Reverse-phase HPLC of pentane extracted products of the Raney nickel cleavage of [^{3}H]-(His)$_6$cPLA$_2\gamma$. Precipitated [^{3}H]-(His)$_6$cPLA$_2\gamma$ (prepared as described in Figure 6) was dissolved in 0.5 mL of 0.2 M sodium phosphate buffer, pH 7.0 containing 8 M guanidine-HCl and 100 μg *N*-acetyl-*S*-farnesyl-L-cysteine and *N*-acetyl-*S*-geranylgeranyl-L-cysteine as standards. Raney Ni (≈50 mg) was added to the sample, overlayed with 2 mL of pentane, sealed, and stirred vigorously for 15 h at 100 °C. Reaction products extracted into the pentane layer were concentrated by evaporation under N_2, applied to a Vydac RP C18 90 Å pharmaceutical HPLC column at a flow rate of 0.5 mL/min, and eluted with an acetonitrile gradient as described in Experimental Procedures. UV-absorbing peaks at 210 nm (solid line) corresponding to the internal standard products: all-*trans*-2,6,10-trimethyl-2,6,10-dodecatriene (C_{15}) and all-*trans*-2,6,10,14-tetramethyl-2,6,10,14-hexadecatetraene (C_{20}) are as indicated. Radioactivity in each fraction was quantified by scintillation spectrometry.

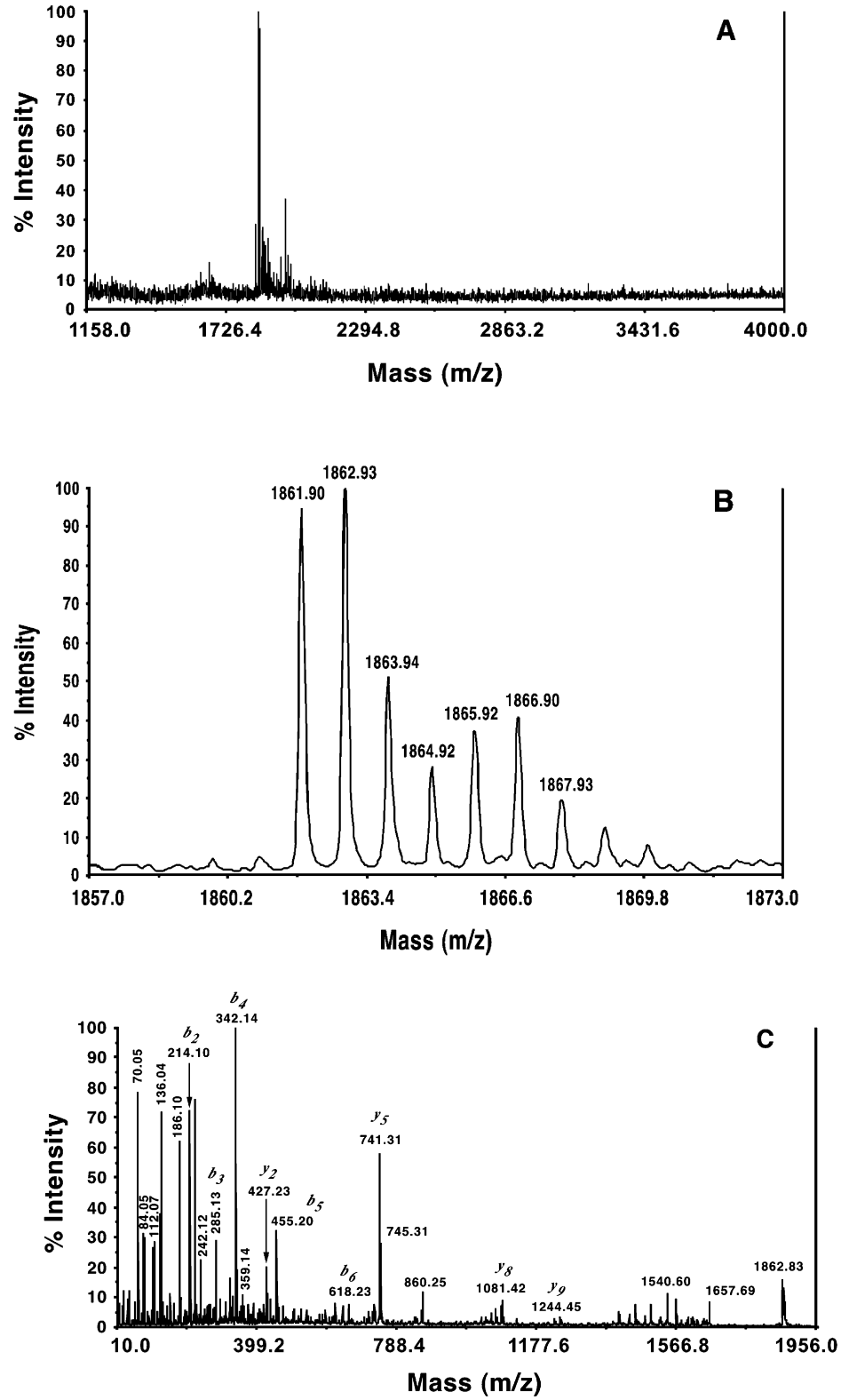

FIGURE 6: MALDI-MS and MALDI-TOF-TOF analysis of a CNBr-cleaved C-terminal peptide of $(His)_6cPLA_2\gamma$. Purified $(His)_6cPLA_2\gamma$ was precipitated, reductively alkylated with 4-vinylpyridine, reprecipitated, dissolved in 70% trichloroacetic acid, and incubated with CNBr for 8 h under N_2 as described in Experimental Procedures. Samples were evaporated to dryness in a Speed-Vac, redisolved in 20% acetonitrile containing 0.1% TFA, and processed utilizing a Zip-Tip. After mixing with CHCA matrix, samples were hand painted on a 192-well ABI plate for mass spectral analysis. Spectra were obtained utilizing an Applied Biosystems 4700 Proteomics Analyzer. Panels A and B: MALDI mass spectra. For MS/MS analyses of the 1861.90 monoisotopic peak (panel C), spectra were obtained by the accumulation of 5000 consecutive laser shots at a collision energy of 1 kV with air serving as the collision gas.

-CCLA motif, we were interested to investigate whether Cys-538 or Cys-539 (or both) were farnesylated. Accordingly, $(His)_6cPLA_2\gamma$ was digested with CNBr to produce a C-terminal peptide (resulting from the cleavage at Met-523), which produced a predicted fragment with a *m*/*z* conducive to mass spectral analysis. Initial MALDI analysis of the

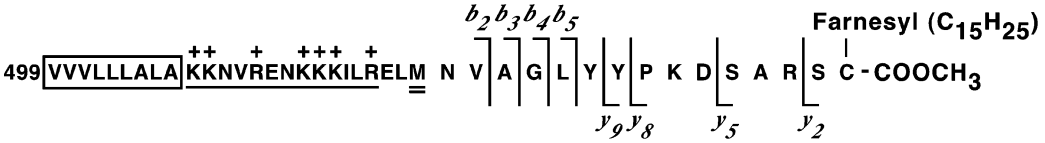

FIGURE 7: Posttranslational modifications at the C-terminus of recombinant (His)$_6$cPLA$_2\gamma$. Fragment ions (*b* and *y*) obtained from MALDI-TOF-TOF analysis (panel C) of the 1861.90 *m*/*z* monoisotopic peak (panel B) are as indicated and identify the presence of a farnesyl group and carboxymethyl group on Cys-538 at the C-terminus of the peptide generated by CNBr cleavage at Met-523 (double underlined) of cPLA$_2\gamma$. The hydrophobic (boxed) and polybasic (underlined) sequences are as indicated.

CNBr digest of (His)$_6$cPLA$_2\gamma$ revealed a series of peaks near 1862 amu (Figure 6A) which, as anticipated, consisted of several peaks separated by one mass unit resulting from ^{13}C isotope effects (Figure 6B). The mass of the major monoisotopic peak ($m/z = 1861.90$) is consistent (within 43 ppm) with the calculated molecular weight of the predicted peptide from the CNBr cleavage reaction with the following modifications: addition of a single farnesyl group, removal of the three C-terminal amino acids, and carboxymethylation of the prenylated C-terminal cysteine (calculated $m/z = 1861.98$). Importantly, no significant peaks (above the background) were observed near *m*/*z* 1930 (corresponding to the geranylgeranyl peptide) or for higher *m*/*z* ratios for the doubly prenylated peptides. To further confirm the identity of the 1861.90 *m*/*z* peak, tandem mass spectrometry was performed employing a floating collision cell. MS/MS spectra demonstrated that the presence of several diagnostic *b* and *y* ions formed upon fragmentation (Figure 6C) of which all of the observed *y* ions ($m/z = 427.23$, 741.31, 1081.42, and 1244.45) were consistent with the presence of a farnesyl moiety, proteolytic processing of the C-terminus, and methylation (Figure 7). In addition, immonium ions representing proline ($m/z = 70$), arginine ($m/z = 112$), lysine ($m/z = 84$), and tyrosine ($m/z = 136$) were also observed substantiating the identity of this peptide as the C-terminus of cPLA$_2\gamma$ (Figure 7).

DISCUSSION

To our knowledge, cPLA$_2\gamma$ represents the only phospholipase A$_2$ yet discovered that contains a prenylation motif. By analogy to other prenylated proteins (e.g., Ras), posttranslational covalent modifications that increase the hydrophobicity of the C-terminus are likely important for the functional interaction of cPLA$_2\gamma$ with membrane bilayers and may enhance productive associations with other proteins or lipids through lateral diffusion in the plane of the membrane surface (*45*−*47*). An intriguing function for the farnesyl group of cPLA$_2\gamma$ is that it may serve to direct cPLA$_2\gamma$ to specialized membrane domains or to specific membrane-associated proteins. Membrane-bound (His)$_6$cPLA$_2\gamma$ was easily removed from Sf9 cell membranes with an actual apparent increase in activity in the presence of $C_{12}E_8$ detergent. This contrasts with the myriad of membrane-bound enzymes that are difficult or impossible to extract with a full retention of activity. The apparent activation manifest during solubilization with detergent could result from many effects, including the following: (1) conformational alterations in the active site or at the C-terminus; (2) detergent-enhanced interactions with substrate; or (3) disassociation of enzyme−inhibitor complexes. One reason potentially underlying the high yield of enzyme activity after detergent extraction is that the native bilayer environment does not have to be replicated but rather that the micelle serves as a nidus that facilitates productive interactions between enzyme (perhaps at the C-terminus promoted by the farnesyl group) and substrate.

The present results unambiguously demonstrate that the C-terminus of cPLA$_2\gamma$ is farnesylated and carboxymethylated when expressed in Sf9 cells and thus is anticipated to be firmly anchored to membrane bilayers in intact cells. Calculated water−octanol partition coefficients (*P* values) for proteins or peptides containing isoprenyl moieties indicate that prenylation and carboxymethylation impart significant hydrophobicity to the C-terminus (*59*, *60*). For the unmodified -CCLA sequence of cPLA$_2\gamma$, the calculated log(*P*) value (i.e., the logarithm of the partition coefficient) is 0.611. Farnesylation and subsequent cleavage of the CLA tripeptide increases the partition coefficient by approximately 4 orders of magnitude (log(*P*) = 4.684). Methylation of the C-terminal cysteine carboxyl group further increases the *P* value by over 2 orders of magnitude (log(*P*) = 7.027), indicating significant nonpolarity. In addition, hydropathy plots identified a hydrophobic region near the C-terminus (residues 499−507) that in conjunction with farnesylation at Cys-538 make it extremely likely that the C-terminus of cPLA$_2\gamma$ is in contact with and/or anchored to the membrane bilayer (Figure 7). Furthermore, cPLA$_2\gamma$ possesses a polybasic region (residues 508−520) adjacent to the hydrophobic region that may interact with anionic lipids (e.g., phosphatidylserine or palm-CoA), as has been demonstrated in prior binding studies of prenylated peptides that bind negatively charged phospholipid vesicles (*61*).

Prior studies of mammalian myocardial subcellular fractions have demonstrated the presence of robust calcium-independent lysophospholipase activity in both microsomal and cytosolic fractions (*17*−*20*). While the cytosolic lysophospholipase activity can largely be assigned to the 23 kDa lysophospholipase/acyl-CoA hydrolase and the 63 kDa lysophospholipase/transacylase, the molecular identity of the enzyme(s) contributing to the myocardial microsomal lysophospholipase activity are unknown. As reported by Stewart and co-workers, we have also found that cPLA$_2\gamma$, like cPLA$_2\alpha$ (*62*, *63*), possesses higher lysophospholipase activity in the presence of high concentrations of lysophosphatidylcholine relative to phospholipase A$_2$ activity (~20−50 μmol min^{-1} mg^{-1} vs 1−5 μmol min^{-1} g^{-1}, respectively). Interestingly, cPLA$_2\gamma$ does not exhibit significant PLA$_1$ activity but is able to sequentially hydrolyze fatty acid from the *sn*-2 position followed by hydrolysis of the fatty acyl chain at the *sn*-1 position (*40*). For reasons that may be due to the amphipathic effects and/or membrane-perturbing properties of lysolipids, all intracellular PLA$_2$s of which we are aware have higher lysophospholipase than phospholipase activities that may protect cells from the accumulation of lysolipids and membrane disruption. Previous work has demonstrated that rabbit myocardial microsomes contain a robust lyso-

phospholipase activity that, like cPLA$_2\gamma$, is inhibited by palmitoyl-CoA (*17*). Inhibition of the lysophospholipase activity of cPLA$_2\gamma$ by palmitoyl-CoA may contribute to the increase in lysophospholipids induced by PLA$_2$ activation in ischemic myocardium.

Identification of the covalent linkages present at the C-terminus of cPLA$_2\gamma$ by mass spectrometry is of particular importance in light of its atypical -CaaX prenylation motif that contains two potential sites (Cys^{538} and Cys^{539}) for prenylation. Posttranslational processing of the C-terminus of cPLA$_2\gamma$ could result in a variety of possible metabolic fates including the following: (1) prenylation of one or both cysteine residues with either farnesyl or geranylgeranyl moieties (*43*); (2) presence or lack of proteolytic processing; and (3) presence or lack of carboxymethylation at the C-terminal residue. Since Sf9 cells have the catalytic machinery to either farnesylate or geranylgeranylate -CaaX box containing proteins, the exclusive presence of the farnesyl moiety is significant. The present results unambiguously identify the farnesylated Cys^{538} carboxymethylated cPLA$_2\gamma$ as the predominant species produced in Sf9 cells. Studies of synthetic prenylated peptides have shown that methylation of the C-terminal cysteinyl carboxyl group results in enhanced association of the peptides with lipid bilayers when the isoprenyl group is farnesyl but not geranylgeranyl (*61*). Methylation may also promote protein−protein interactions, such as has been demonstrated with the association of the *a*-factor with its transporter, Ste6 in yeast (*64*), and the preferential binding of the prenylated and methylated delta subunit of rod phosphodiesterase PDE6 to its catalytic subunits (*65*). We point out that differential processing of cPLA$_2\gamma$ may occur, which would lead to their elution in the leading or trailing edges of the activity peak that may have been selected against by pooling fractions based on the activity assay. However, since the recombinant cPLA$_2\gamma$ protein was purified utilizing a histidine tag at the N-terminus, it seems unlikely that substantial amounts of cPLA$_2\gamma$ mass is present as other C-terminally modified variants when expressed in Sf9 cells. We believe it likely that other posttranslational modifications of cPLA$_2\gamma$ may be utilized in mammalian skeletal or cardiac muscle, which may underlie its specific intracellular distribution and/or physiologic roles. To this end, we are currently preparing transgenic mice that overexpress human cPLA$_2\gamma$ to address this question utilizing the purification and mass spectrometry techniques described herein.

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