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Functional Characterization of Mutations in Inherited Human cPLA$_2$ Deficiency†

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ABSTRACT: Group IVA cytosolic phospholipase A$_2$ (cPLA$_2$α) catalyzes the first step in the arachidonic acid cascade leading to the synthesis of important lipid mediators, the prostaglandins and leukotrienes. We previously described a patient deficient in cPLA$_2$α activity, which was associated with mutations in both alleles encoding the enzyme. In this paper, we describe the biochemical characterization of each of these mutations. Using saturating concentrations of calcium, we showed that the R485H mutant was nearly devoid of any catalytic activity, that the S111P mutation did not affect the enzyme activity, and that the known K651R polymorphism was associated with activity slightly higher than that of the wild type. Using MDCK cells, we showed that translocation to the Golgi in response to serum activation was impaired for the S111P mutant but not for the other mutants. Using immortalized mouse lung fibroblasts lacking endogenous cPLA$_2$α activity, we showed that both mutations S111P and R485H/K651R caused a profound defect in the enzyme catalytic activity in response to cell stimulation with serum. Taken together, our results show that the S111P mutation hampers calcium binding and membrane translocation without affecting the catalytic activity, and that the mutation R485H does not affect membrane translocation but blocks catalytic activity that leads to inactivation of the enzyme. Interestingly, our results show that the common K651R polymorphism confers slightly higher activity to the enzyme, suggesting a role of this residue in favoring a catalytically active conformation of cPLA$_2$α. Our results define how the mutations negatively influence cPLA$_2$α function and explain the inability of the proband to release arachidonic acid for eicosanoid production.

Group IVA cytosolic phospholipase A$_2$ (cPLA$_2$α)[1] is an enzyme widely expressed in mammalian cells that catalyzes the hydrolysis of phospholipids in the *sn*-2 position to release arachidonic acid (AA). It is stimulated in response to diverse factors and represents the first step of metabolic cascades leading to the generation of important lipid mediators such as prostaglandins and leukotrienes. These eicosanoids mediate physiological and pathophysiological processes, making it important to understand the factors regulating cPLA$_2$α activity. Two knockout mouse models provided important information concerning the physiological function of cPLA$_2$α (*1*−*3*).

Recently, we described a patient with globally decreased eicosanoid production, intestinal ulcers, and platelet dysfunction (*4*). We showed that these conditions were associated with loss-of-function mutations in both alleles encoding cPLA$_2$α. Release of thromboxane (TxA$_2$) and 12-hydroxyeicosatetraenoic acid (12-HETE) by the patient's platelets was inhibited by more than 95% compared to control individuals, suggesting that cPLA$_2$α is the main phospholipase responsible for platelet release of eicosanoids.

Sequencing of cDNA revealed that the patient was a compound heterozygote for variants in the *PLA2G4A* gene. An allelic variant, S111P, was inherited from his mother, and the paternal allele contained the variant R485H as well as the known K651R single-nucleotide polymorphism (SNP). In this work, we describe the biochemical characterization of these mutations.

cPLA$_2$α has two functional domains: an N-terminal C2 domain that binds calcium and a C-terminal catalytic domain (*5*, *6*). Calcium binding to the C2 domain initiates translocation of the enzyme from the cytosol to the membranes of the endoplasmic reticulum, the Golgi, and the nuclear envelope for cPLA$_2$α to access the phospholipid substrate (*3*). The cPLA$_2$α C2 domain is an antiparallel β-sandwich composed of two, four-strand sheets with interconnecting loops (*7*, *8*). Two calcium ions bind to residues in calcium-binding loops (CBL) 1−3 at the membrane-binding end of the C2 domain. The S111P variant is located as the first amino acid in the loop at the opposite end of the β-strand from CBL3, a region that provides the basis for a hypothesis that this allelic variant alters the calcium binding function of the C2 domain. The catalytic domain contains a large, positively charged

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[1]Abbreviations: ^{14}C-PAPC, 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine; 12-HETE, 12-hydroxyeicosatetraenoic acid; AA, arachidonic acid; BSA, bovine serum albumin; CBL, calcium-binding loops; cPLA$_2$α, group IVA cytosolic phospholipase A$_2$; DMEM, Dulbecco's modified Eagle's medium; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FBS, fetal bovine serum; IMLF, SV40 immortalized MLF; MLF, mouse lung fibroblasts; MDCK, Madin-Darby canine kidney; PI-(4,5)-P$_2$, phosphatidylinositol 4,5-bisphosphate; *Sf*9, *Spodoptera frugiperda*; TxA$_2$, thromboxane A$_2$.

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region that is present in the membrane-facing region of cPLA$_2\alpha$ (*9*), and the patient's R485H variant is located in this region. Residue R485 is in the proximity of the cluster of lysine residues (K488, K541, K543, and K544) that is the site of interaction with phosphatidylinositol 4,5-bisphosphate [PI-(4,5)-P$_2$], which increases catalytic activity in vitro (*10*−*12*).

This patch of basic residues is required for cPLA$_2\alpha$-mediated AA release in cells but does not regulate calcium-dependent translocation in cells or membrane binding in vitro (*10*, *12*). We hypothesized that the R485H variant could result in altered catalytic activity. The K651R variant is a recognized SNP located in the C-terminal region of the catalytic domain. Although there is no information about this region that engenders a specific functional hypothesis, K651 is a highly conserved residue across species, suggesting the possibility of a functional consequence of this variant.

To improve our understanding of the effect of each mutation on cPLA$_2\alpha$ function, we compared their interfacial binding and activity in vitro using purified enzymes, and their ability to translocate to membrane and release AA in response to calcium increases when expressed in mammalian cells.

MATERIALS AND METHODS

Materials. [5,6,8,9,11,12,14,15-^{3}H]AA (100 Ci/mmol) was from Amersham Biosciences. 1-Palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (^{14}C-PAPC) was from PerkinElmer Life Sciences. Dulbecco's modified Eagle's medium (DMEM) was from BioWhittaker. Penicillin, streptomycin, and L-glutamine were from Invitrogen. Fetal bovine serum (FBS) was from Irvine Scientific. Protease inhibitor tablets were from Roche Applied Science. The plasmid isolation kit was from Qiagen, and the QuikChange II Site Directed Mutagenesis kit was from Stratagene. Mouse serum was from Atlanta Biologicals. Sodium phosphate, potassium phosphate, sodium chloride, ethylenediaminetetraacetic acid, imidazole, Hepes, dithiothreitol, Tris-HCl, and bovine serum albumin (BSA) were purchased from Sigma. Vectors pCR 2.1 and pcDNA 3 were from Invitrogen as were the Nu-PAGE 10% Bis-Tris gels and the Ni-NTA agarose. Restriction enzymes and T4 DNA ligase were from New England Biolabs. The Qiagen kit for polymerase chain reaction (PCR) was from Qiagen. Baculovirus vector pAcHLT-B and the Baculogold Baculovirus Expression System were purchased from BD Biosciences. Glycerol was from Fisher Scientific. Slide-a-lyzer dialysis cassettes were from Pierce.

Preparation of a Baculovirus Vector for cPLA$_2\alpha$. The cDNA for the double mutant cPLA$_2\alpha$ R485H/K651R was purified from the patient's lymphoblasts (*4*) and cloned in pcDNA 3 according to the manufacturer's instructions. This construct was used as a template for PCR cloning into baculovirus transfer plasmid pAcHLT-B. The upper primer including the Xho restriction site and in frame with the baculovirus vector was GCT CGA GAA ATG TCA TTT ATA GAT CCT TAC CAG CAC A. The lower primer containing the stop codon and the KpnI restriction site was CGG TAC CCT ATG CTT TGG GTT TAC TTA GAA ACT CCT T. The PCR product was cut with XhoI and KpnI restriction enzymes, purified with the Qiagen kit for PCR, and ligated into pCR2.1 using T4 DNA ligase. The ligation was transformed into DH5-Alpha competent cells, utilizing blue-white screening, cultured, sequenced, and purified for ligation into the purified baculovirus vector, pAcHLT-B. The construct was confirmed by sequencing. Mutants S111P, R485H, and K651R and the wild type were prepared by site-directed mutagenesis using the QuikChange II kit following the manufacturer's instructions. The mutations were confirmed by sequencing.

Expression and Purification of cPLA$_2\alpha$. *Spodoptera frugiperda* (*Sf*9) insect cells were a generous gift from L. Marnett (Vanderbilt University). Cells were maintained in SF 900 II SFM medium in Erlenmeyer flasks at 27 °C in a rotating incubator set at 150 rpm. Baculovirus vector pAcHLT-B containing each cPLA$_2\alpha$ insert and the Baculogold linearized baculovirus DNA were cotransfected into *Sf*9 insect cells according to the Baculovirus Expression system protocol.

To amplify, insect cells were infected with recombinant baculovirus and purified according to a modification of the method of Gelb (*13*). Supernatant-containing virus from each mutant and the wild type was used to infect *Sf*9 cells, which grew in culture for 68 h. After cells had been harvested, all steps were conducted at 4 °C. Briefly, infected cells were centrifuged at 3000 rpm for 5 min. The pellet was resuspended in buffer composed of 25 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, and protease inhibitors, placed on ice, and homogenized in a Potter dounce. After 20 min on ice, cell lysates were centrifuged for 45 min at 100000*g*.

Supernatant was loaded without dilution onto a Ni-NTA agarose column previously prepared by washing with the Tris buffer described above containing 700 mM NaCl. The column was then washed with 50 mM NaH_2PO_4 (pH 6.0), 750 mM NaCl, and 20 mM imidazole and then with 20 mM imidazole in NaH_2PO_4 (pH 8.0), followed by 100 mM imidazole in the same. All wash and imidazole fractions were collected and electrophoresed on a 10% polyacrylamide gel to check for purity. Fractions containing cPLA$_2\alpha$ were pooled and dialyzed against 50 mM Hepes (pH 7.5), 1 mM DTT, and 10% glycerol in a cassette overnight at 4 °C.

Postdialysis material was electrophoresed on a 10% gel and stained with Coomassie Blue to determine purity. The protein concentration of affinity-purified wild-type and mutant cPLA$_2\alpha$ preparations was determined by the Bradford dye binding assay using BSA as a standard and by measuring OD_{280} using 0.87 mL mg^{-1} cm^{-1} (calculated from the amino acid sequence of cPLA$_2\alpha$), which gave comparable results.

Enzymatic Activity and Interfacial Binding Assays of cPLA$_2\alpha$ Mutants. Vesicle hydrolysis studies were conducted as described previously (*12*) using extruded vesicles of ^{14}C-PAPC (2.7 Ci/mol) (200 μM phospholipid) and 200 ng of enzyme (based on the Bradford assay) in 10 mM MOPS (pH 7.2), 0.5 mM EGTA, 100 mM KCl, 0.5 mg/mL fatty acid-free BSA, and various amounts of $CaCl_2$ (as a Ca^{2+} buffer) to give the indicated concentration of free Ca^{2+}. Reaction mixtures were incubated at 37 °C for 5 min, and the amount of free fatty acid released was determined as described previously (*12*). Values were corrected for the small amount of radioactive fatty acid in the reaction mixture that lacked enzyme.

Vesicle binding studies were conducted as described previously (*12*) using PAPC vesicles. In the case of cPLA$_2\alpha$ R485H, we used 200 ng of enzyme instead of 50 ng because the activity of this mutant is low. Also for this mutant, we estimated the amount of enzyme remaining in the supernatant by Western blot analysis. In this case, 0.3 mL of supernatant above the pelleted vesicles was mixed with 1 mL of cold acetone and left at 4 °C for 1 h. The sample was centrifuged at 16000*g* for 20 min at 4 °C, the supernatant removed, and the pellet treated with Laemmli sample buffer and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The Western blot was probed

with anti-cPLA$_2$α antiserum (Santa Cruz catalog no. sc-438), and the blot was visualized with ECL.

Production of cDNA Constructs and Recombinant Adenovirus. DNAs encoding monomeric (A206K) wild-type enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP) cPLA$_2$α were inserted into the pVQAd5CMVK-NpA shuttle plasmid (ViraQuest, Inc.). The EYFP-cPLA$_2$α construct was used to generate by site-directed mutagenesis the following mutants: S111P, R485H, K651R, and R485H/K651R. Constructs were confirmed by sequencing. Adenoviruses were generated by ViraQuest, Inc.

Cell Culture and AA Release Assay. MDCK cells were cultured as previously described (*14*). Mouse lung fibroblasts (MLF) were isolated from wild-type (MLF$^{+/+}$) and cPLA$_2$α knockout (MLF$^{-/-}$) mice, and SV40 immortalized MLF (IMLF) were generated as previously described (*15*). IMLF$^{-/-}$ were plated at a density of 1.25×10^4 cells/cm^2 in 250 μL of DMEM containing 10% FBS, 0.1% nonessential amino acids, 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.29 mg/mL glutamine (growth medium) in 48-well plates. After 18 h in 5% CO_2 at 37 °C, cells were washed with, and incubated in, serum-free and antibiotic-free DMEM containing 0.1% BSA (stimulation medium). Cells were infected with adenoviruses (100 μL/well). After 1.5 h, 150 μL of stimulation medium containing 0.2 μCi/mL [^{3}H]AA was added to each well. After 26 h, cells were washed twice and fresh stimulation medium was added. Culture medium was collected after stimulation and centrifuged for 10 min at 15000 rpm, and radioactivity was determined by scintillation counting. Cells were scraped into 50 μL of 0.1% Triton X-100 containing protease inhibitors, and the lysates were used to determine the total cellular radioactivity and to determine expression levels of wild-type and mutant cPLA$_2$α via immunoblotting as previously described (*12*). The amount of AA released into the culture medium was calculated as a percentage of the total radioactivity (cells plus media) in each well.

Microscopy. MDCK cells and IMLF$^{-/-}$ were plated at a density of 1.25×10^4 cells/cm^2 in 250 μL of growth medium in glass-bottomed MatTek plates and infected with adenoviruses for expression of wild-type and mutant cPLA$_2$α as described above. Microscopy was conducted on an inverted Zeiss 200 M microscope driven by Intelligent Imaging Innovations Inc. (3I) software (Slidebook 4.1). We calculated fluorescence values after subtracting background fluorescence and correcting for differential bleaching at each wavelength.

Immunoblotting. For Western blotting, cell lysates were prepared in ice-cold buffer containing 50 mM Hepes (pH 7.4), 150 mM sodium chloride, 1.5 mM magnesium chloride, 10% glycerol, 1% Triton X-100, 1 mM EGTA, and protease inhibitors. Lysates were centrifuged at 15000*g* for 10 min at 4 °C, and protein concentrations were determined using the bicinchoninic acid reagent. Lysates were diluted in Laemmli buffer and boiled for 5 min at 100 °C. Proteins were separated on 10% SDS−polyacrylamide gels, transferred to nitrocellulose, and blocked for 1 h in Tris-buffered saline containing 0.25% Tween 20 and 5% nonfat dry milk. Nitrocellulose membranes were incubated overnight with a 1:5000 dilution of anti-cPLA$_2$α antibodies diluted in blocking buffer. Immunoreactive protein was detected using the Amersham Biosciences anti-rabbit secondary antibody and ECL system.

RESULTS

Effect of the Mutations on cPLA$_2$α Catalytic Activity and Interfacial Binding. To study the effect of the mutations on cPLA$_2$α catalytic activity and vesicle binding, affinity-purified His-tagged wild-type and cPLA$_2$α mutants expressed in *Sf*9 cells were used. Assessment of the purity by SDS−PAGE (Figure 1) showed that all preparations were more than 90% pure. When assayed at saturating concentration of Ca^{2+}, the S111P mutant had a slightly decreased catalytic activity compared to that of wild-type cPLA$_2$α (Table 1 and Figure 2). Single mutant R485H and double mutant R485H/K651R were nearly devoid of catalytic activity (Table 1 and Figure 2). The K651R mutant exhibited 1.7-fold higher activity than wild-type cPLA$_2$α, indicating that the R485H mutation is the determinant of the overall effect of the double mutation on enzyme activity. A comparison of the enzymatic properties of the wild type and the cPLA$_2$α mutants assayed as a function of calcium concentration revealed that the apparent K_{Ca} (concentration of Ca^{2+} that supports half-maximal activity obtained at a saturating Ca^{2+} concentration) of the K651R and S111P mutants (0.4 μM) is similar to that of wild-type cPLA$_2$α (0.5 μM) (Figure 2 and Table 1).

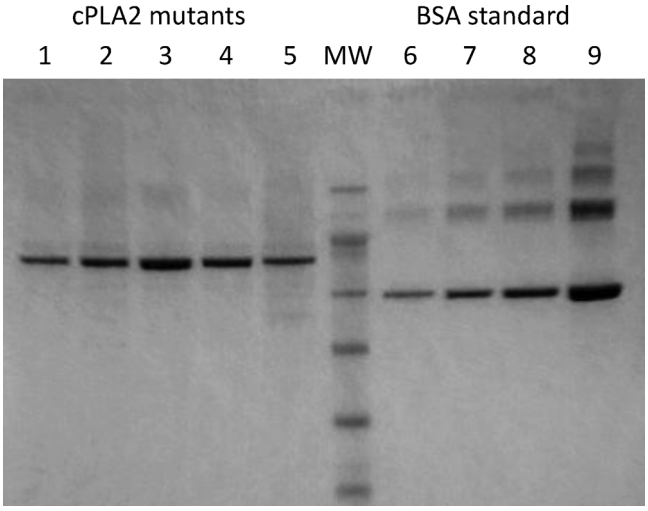

FIGURE 1: SDS−PAGE of purified mutants of cPLA$_2$α. cPLA$_2$α mutants were expressed and purified as described in Materials and Methods. One microliter of each purified mutant was loaded on a SDS−PAGE gel and stained with Coomassie blue. A standard curve of bovine serum albumin (BSA) was loaded on the same gel (lanes 6−9) at concentrations of 0.3, 0.6, 1.25, and 2.5 μg/mL. Lanes 1−5 contained the wild type, R485H, R485H/K651R, K651R, and S111P, respectively. MW denotes molecular weight markers.

When interfacial binding of wild-type cPLA$_2$α and the mutants to PAPC vesicles at a saturating calcium concentration (20 μM) was assayed, the results show that a smaller fraction (~68%) of the S111P mutant is bound compared to the wild type (Table 1). Because the catalytic activity of the S111P mutant is ~73% of that of the wild type at a saturating calcium concentration (Table 1), the results suggests that the S111P mutation affects calcium-dependent binding and not catalytic activity. The R485H mutant binds vesicles at a saturating calcium concentration to an extent similar to that of wild-type cPLA$_2$α, indicating that the lack of enzymatic activity of the R485H mutant is due to a modification of the catalytic domain rather than an effect on calcium-dependent binding through the C2 domain (Table 1).

Effect of the Mutations on Translocation of cPLA$_2$α in Cells. We had previously defined the intracellular calcium signals regulating translocation of cPLA$_2$α to the Golgi in MDCK cells (*14*). This cell model was used initially to determine the effect of the mutations on the ability of cPLA$_2$α to translocate to the Golgi in response to increases in intracellular Ca^{2+}

Table 1: In Vitro Analysis of Wild-Type and cPLA$_2$α Mutant Proteins[a]

protein	specific activity at saturating $[Ca^{2+}]$ [nmol min^{-1} (mg of protein)$^{-1}$]	apparent K_{Ca} (μM)	fraction enzyme bound to vesicles at 20 μM Ca^{2+}
wild-type cPLA2α	140	0.5 ± 0.2	0.85
cPLA2α R485H	7		0.70
cPLA2α K651R	233	0.4 ± 0.2	
cPLA2α R485H/K651R	7		
cPLA2α S111P	109	0.4 ± 0.2	0.58

[a]Each value is the average of duplicate determinations.

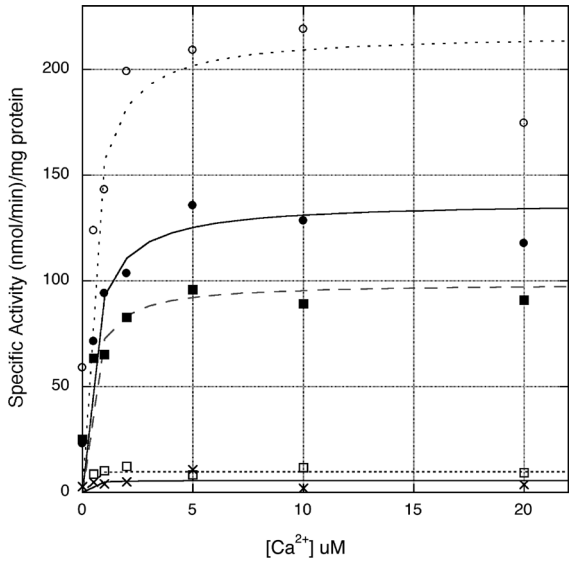

FIGURE 2: Specific activities of wild-type cPLA$_2$α and mutants as a function of calcium concentration. Hydrolysis of ^{14}C-PAPC vesicles by affinity-purified wild-type cPLA$_2$α (●) and S111P (■), K651R (○), R485H (×), and K651R/R485H (□) mutants was assessed as described in Materials and Methods.

concentrations. Wild-type ECFP-cPLA$_2$α was coexpressed with either EYFP-cPLA$_2$α S111P, EYFP-cPLA$_2$α R485H, or EYFP-cPLA$_2$α K651R/R485H, which allows a direct comparison of the translocation properties of the wild-type and mutant enzymes in the same cell (*12*, *14*). Translocation was monitored by dual live cell imaging in response to 1 μM ionomycin, which induces a calcium transient that correlates in a time-dependent manner with translocation of ECFP-cPLA$_2$α to the Golgi in MDCK cells (*14*). Translocation of wild-type ECFP-cPLA$_2$α to the perinuclear region occurs rapidly as shown in the images of cellular fluorescence (Figure 3A). In contrast, there is less translocation of EYFP-cPLA$_2$α S111P than of wild-type ECFP-cPLA$_2$α when expressed in the same cell (Figure 3A). Analysis of several individual cells revealed less translocation of cPLA$_2$α with the C2 domain mutation (S111P) than wild-type cPLA$_2$α depicted graphically for three individual cells in Figure 3B (panel A). The EYFP-cPLA$_2$α S111P mutant translocates at a slighty slower rate in response to calcium increase, and a smaller fraction of the mutant translocates to the membrane compared to the fraction of the wild type. cPLA$_2$α containing the SNP allele K651R exhibits translocation properties identical to those of wild-type cPLA$_2$α (Figure 3B, panel B). Translocation of cPLA$_2$α containing single mutant R485H or double mutant R485H/K651R alleles in the catalytic domain is also similar to that of wild-type cPLA$_2$α (Figure 3B, panels C and D). We also compared translocation of wild-type ECFP-cPLA$_2$α and the EYFP-cPLA$_2$α S111P mutant in response to a higher concentration of ionomycin, which induces a higher and more sustained increase in the intracellular Ca^{2+} concentration in MDCK cells (*14*). There is greater translocation of EYFP-cPLA$_2$α S111P to the Golgi than with lower levels of ionomycin, but it still remains below the level of translocation observed with wild-type ECFP-cPLA$_2$α as seen in the images (Figure 4A) and depicted graphically (Figure 4B).

Effect of the Mutations on cPLA$_2$α Translocation and AA Release upon Reconstitution in cPLA$_2$α-Deficient Lung Fibroblasts. To directly compare the translocation properties and the ability of the cPLA$_2$α mutants to release AA, they were expressed in IMLF$^{-/-}$, a cell type that lacks endogenous cPLA$_2$α. We previously reported that expression of wild-type cPLA$_2$α in IMLF$^{-/-}$ reconstitutes AA release and allows evaluation of cPLA$_2$α function without interference of endogenous cPLA$_2$α (*12*). In addition, we can use the physiological agonist serum to stimulate these cells. When expressed in IMLF$^{-/-}$, cPLA$_2$α is potently activated by serum, which induces a capacitative increase in the intracellular Ca^{2+} concentration and activation of mitogen-activated protein kinases (*12*). Serum stimulates less translocation of EYFP-cPLA$_2$α S111P than wild-type cPLA$_2$α when coexpressed in IMLF$^{-/-}$, as observed in MDCK cells (Figure 5A, top three panels). The extent of translocation of the S111P mutant relative to that of wild-type cPLA$_2$α is variable from cell to cell. In some cells, translocation of S111P is undetectable, and in others, a low level of translocation to the Golgi is observed. In contrast, the EYFP-cPLA$_2$α R485H/K651R mutant actually translocates to a greater extent than the wild type in response to serum (Figure 5A, bottom three panels).

To determine the effect of the mutations on AA release, parallel cultures of IMLF$^{-/-}$ expressing wild-type or mutant forms of cPLA$_2$α were compared (Figure 5B). For each experiment, Western blot analysis of the cell lysates was conducted to confirm that wild-type and mutant forms of cPLA$_2$α are expressed at equivalent levels as previously described (*12*). The R485H/K651R mutant did not release significant amounts of AA above basal levels in response to serum, indicating that although calcium-dependent translocation is not defective it is catalytically inactive. The level of release of AA by the S111P mutant is reduced by approximately 72% compared to that of wild-type cPLA$_2$α in response to serum (Figure 5B). The low but significant level of AA released by the S111P mutant in response to serum suggests that it is catalytically active, consistent with the in vitro activity assays.

DISCUSSION

cPLA$_2$α activity is regulated by complex mechanisms involving binding of intracellular calcium, posttranslational phosphorylation, and interaction with specific membrane lipids (*3*, *16*, *17*). Given the importance of cPLA$_2$α in platelet function (*4*), and its ubiquitous expression in tissues, structure−function studies are

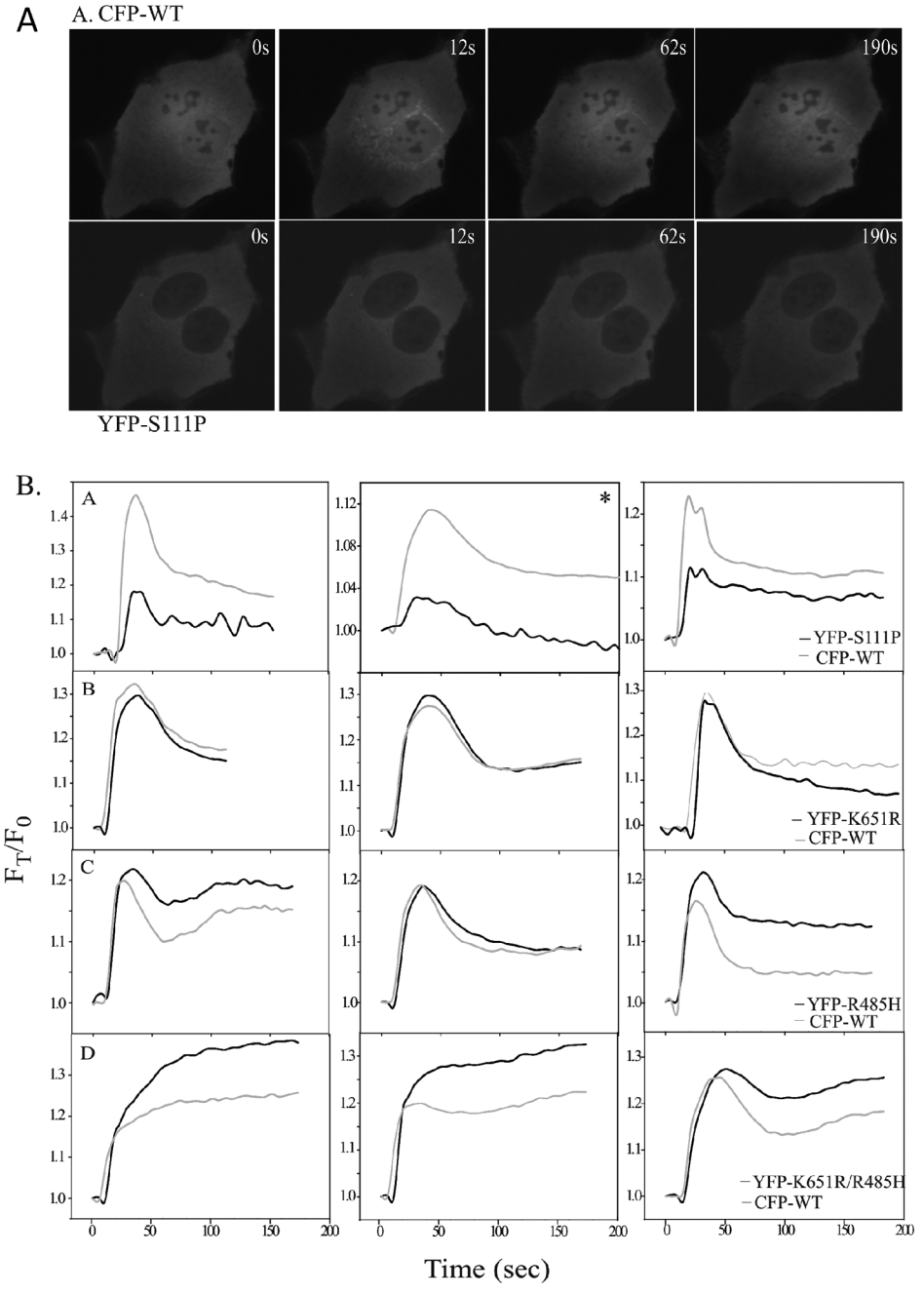

FIGURE 3: Translocation of wild-type cPLA$_2$α and mutants in MDCK cells stimulated with 1 μM ionomycin. (A) Images of a representative cell coexpressing wild-type ECFP-cPLA$_2$α (top panels) and EYFP-cPLA$_2$α S111P (bottom panels) are shown at the indicated times after addition of 1 μM ionomycin. (B) MDCK cells coexpressing wild-type ECFP-cPLA$_2$α and either EYFP-cPLA$_2$α S111P (A), EYFP-cPLA$_2$α K651R (B), EYFP-cPLA$_2$α R485H (C), or EYFP-cPLA$_2$α K651R/R485H (D) were stimulated with 1 μM ionomycin, and images were collected every 3 s as a function of time as indicated. The three graphs shown side by side in panels A−D depict translocation data for three individual cells. Translocation data of individual cells shown graphically in each panel were calculated on the basis of the average fluorescence intensity of a mask of the Golgi in each cell. Values are corrected for background fluorescence and differential bleaching at each wavelength throughout the duration of the imaging. Data are presented relative to time zero (F_T/F_0).

needed to shed light on the mechanisms regulating cPLA$_2$α in humans. We recently described the first case of a patient devoid of cPLA$_2$α activity because of mutations in both alleles encoding the enzyme (*4*). One allele encodes a nonhomologous mutation, S111P. The other allele encodes two nonhomologous mutations: R485H and K651R. In this paper, we investigated the functional consequences of these three mutations with regard to catalytic activity, Ca^{2+} requirement, and affinity for the phospholipid membrane in vitro and in cells in culture.

All mutants express well at the expected molecular weight, and none of the mutations have a significant impact on the recognition of the mutated enzymes by the cPLA$_2$α specific antibody used for Western blot analysis. We previously observed that although no cPLA$_2$α activity was present in the proband's platelets, ~50% of the protein was expressed. Taken together with our results, these data suggest that, in contrast to insect cells, one of the mutations is associated with lower levels of protein expression in humans (*4*). The effects of the mutations on catalytic activity were assessed in vitro in the presence of saturating concentrations of calcium to remove any confounders due to different calcium requirements of each mutant. S111P has no significant effect on the mutant's catalytic activity as measured by

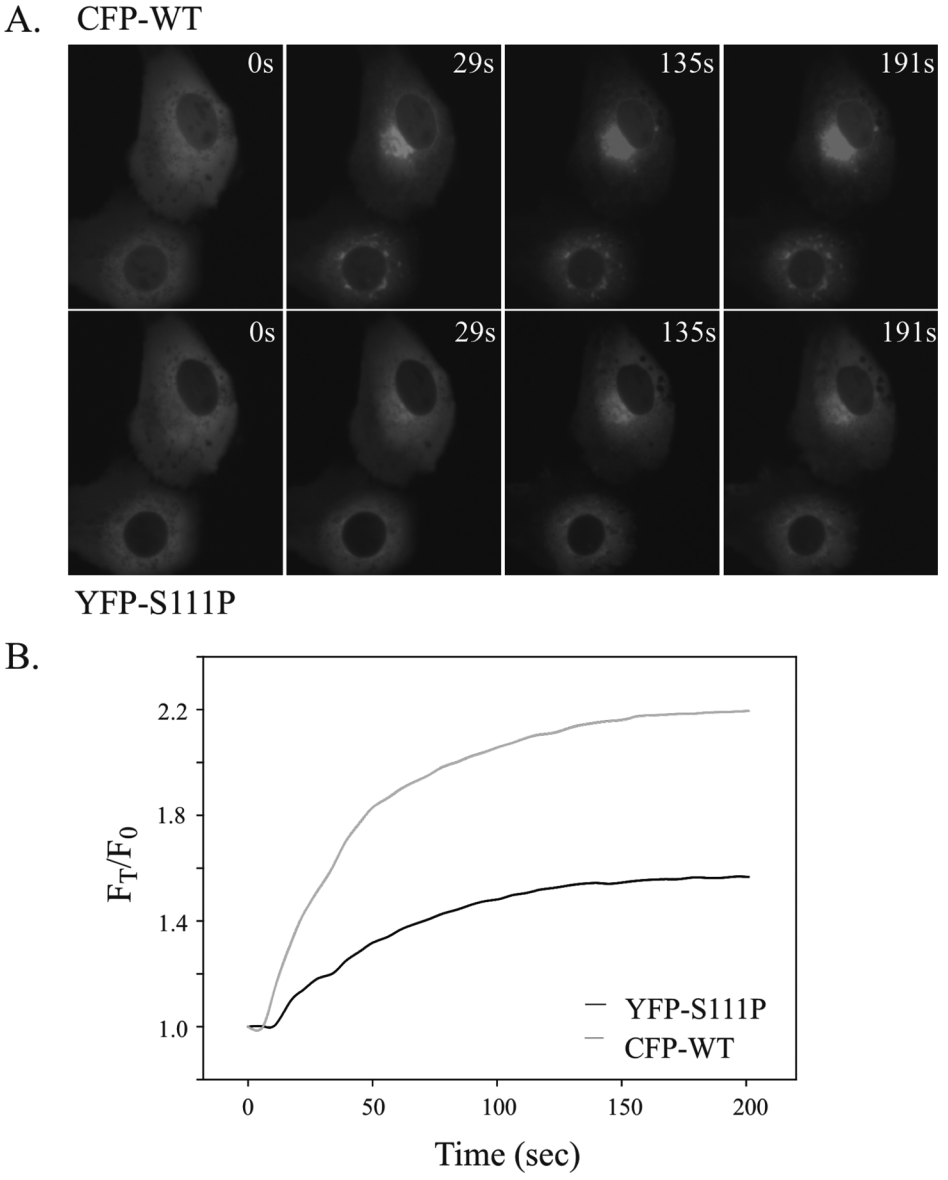

FIGURE 4: Translocation of wild-type cPLA$_2\alpha$ and cPLA$_2\alpha$ S111P in MDCK cells at a saturating calcium concentration. MDCK cells coexpressing wild-type ECFP-cPLA$_2\alpha$ and EYFP-cPLA$_2\alpha$ S111P were stimulated with 10 μM ionomycin, and images were collected as a function of time as indicated. (A) Images of a representative cell coexpressing wild-type ECFP-cPLA$_2\alpha$ (top panels) and EYFP-cPLA$_2\alpha$ S111P (bottom panels) are shown at the indicated times after addition of 10 μM ionomycin. (B) Ionomycin (10 μM)-stimulated translocation is shown graphically in a representative MDCK cell coexpressing wild-type ECFP-cPLA$_2\alpha$ and EYFP-cPLA$_2\alpha$ S111P. Live cell imaging was conducted as described in the legend of Figure 3.

hydrolysis of AA from phospholipid vesicles, suggesting that the integrity of the catalytic active site is not affected by this mutation. The R485H mutant is nearly completely devoid of activity, indicating that R485 is important for the catalytic activity of cPLA$_2\alpha$. This amino acid is in the vicinity of the positively charged lid that covers the active site (*4*). Mutation of other basic residues (K483, K541, K543, and K544), which constitute the site for activation by PI-(4,5)-P_2, to asparagines slightly enhances activity against PAPC but abolishes activation by PI-(4,5)-P_2 (*11*, *18*, *19*). In contrast, the substitution of R485 with a histidine results in inactivation perhaps by an adverse conformation effect. The K651R mutation confers slightly increased activity of cPLA$_2\alpha$ when compared to the wild type. This observation was unexpected as the residue is in the cytosolic C-terminal loop and not obviously associated with the catalytic active site of the enzyme. We can hypothesize that this residue might be involved in the overall interaction of the enzyme with the phospholipids or have a stabilizing effect on the catalytic domain through three-dimensional interactions or by stabilizing the active conformation of cPLA$_2\alpha$. Interestingly, this mutation is a previously described polymorphism of the enzyme (rs2307198) with an allele frequency of 2−6% according to the population studied (http://uswest.ensembl.org/index.html). Our results raise the question of whether this polymorphism is associated with increased cPLA$_2\alpha$ activity in humans.

An increase in the intracellular Ca^{2+} concentration is essential in promoting translocation of cPLA$_2\alpha$ to the membrane where it can catalyze release of AA from phospholipids. We investigated the effects of the different mutations on calcium-dependent binding to phospholipid vesicles in vitro. Our results show that S111P binding at a saturating Ca^{2+} concentration does not reach the maximal level of association exhibited by the wild-type enzyme. The loss of a H-bonding interaction due to substitution of a proline for S111 could potentially affect the conformation of

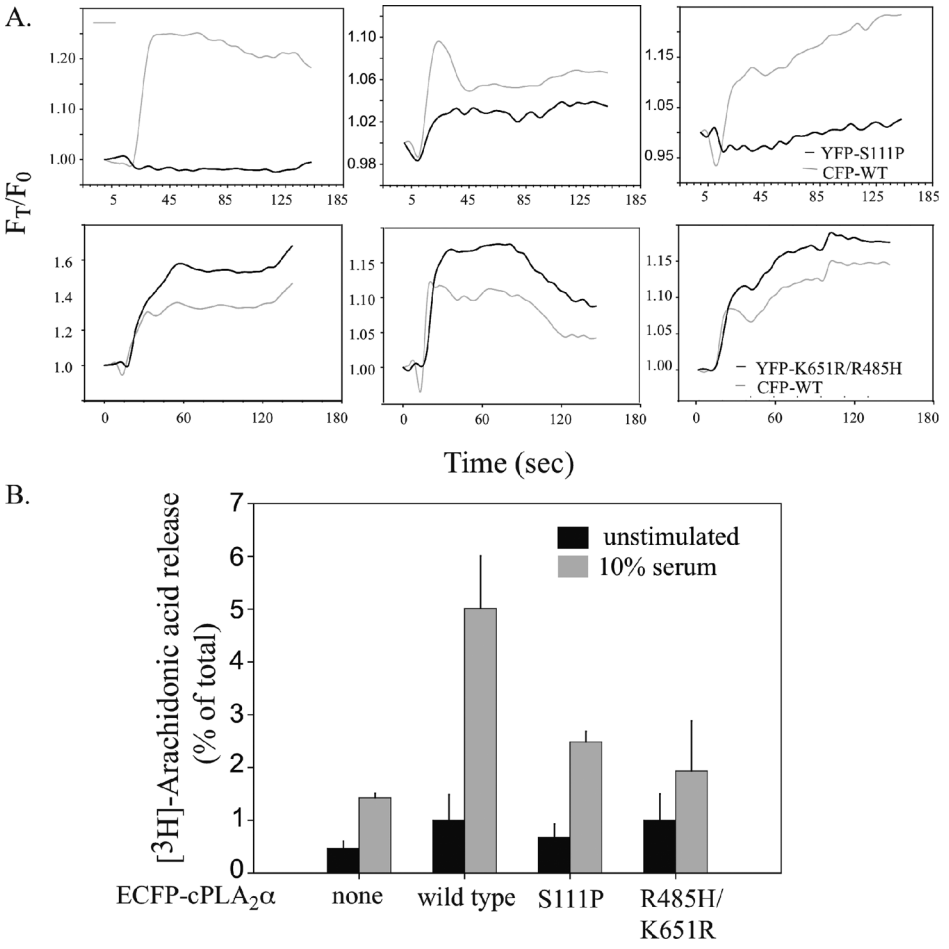

FIGURE 5: Translocation of wild-type cPLA$_2$α and mutants in IMLF$^{-/-}$ stimulated with serum. (A) Translocation is shown in three individual IMLF$^{-/-}$ coexpressing wild-type ECFP-cPLA$_2$α with EYFP-cPLA$_2$α S111P (top three panels) or with EYFP-cPLA$_2$α K651R/R485H (bottom three panels) as a function of time after stimulation with 10% serum. Live cell imaging was conducted as described in the legend of Figure 4. (B) Parallel cultures of [^{3}H]AA-labeled IMLF$^{-/-}$ expressing either wild-type ECFP-cPLA$_2$α, EYFP-cPLA$_2$α S111P, EYFP-cPLA$_2$α K651R/R485H, or no cPLA$_2$α (none) as indicated were stimulated for 10 min with 10% serum. [^{3}H]AA released into the medium is expressed as a percentage of the total cellular radioactivity in each well. Immunoblotting of cell lysates was conducted to confirm equivalent levels of expression of wild-type and mutant cPLA$_2$α in each well.

β-strands 6 and 7 because of unfavorable steric effects. This could affect the conformation of CBL-3, which is connected to strand B6, resulting in effects on calcium-dependent membrane association. In contrast, the R485H mutant exhibits association with vesicles similar to that of the wild type at a saturating calcium concentration.

We investigated the effects of the mutations on the translocation of cPLA$_2$α to membranes by monitoring the association of the different mutants with the Golgi in MDCK cells stimulated with ionomycin because we had extensively characterized the calcium-dependent translocation properties of wild-type cPLA$_2$α in this cell model (*14*, *20*). At 1 μM ionomycin, a smaller fraction of the cellular S111P mutant expressed in MDCK cells translocates to the Golgi compared to the fraction of the wild type. However, when the calcium transient is more sustained with higher levels of ionomycin (*14*), more wild type and S111P mutant accumulate on the Golgi over time than with a lower level of ionomycin, but there is still less S111P mutant cPLA$_2$α associated with Golgi than wild type, consistent with our in vitro data. A defect in the translocation of the S111P mutant is also observed when it is coexpressed with wild-type cPLA$_2$α in IMLF$^{-/-}$ in response to serum. As we previously reported, serum is a physiological agonist that induces a typical capacitative calcium increase (*12*). Although there is cell-to-cell variability that may be due to heterogeneity in the calcium responses (*21*), the level of translocation of the S111P mutant is lower than that of the wild type. The defect in translocation results in less release of AA by the S111P mutant when it is expressed at levels equivalent to that of wild-type cPLA$_2$α in IMLF$^{-/-}$ treated with serum (~70% less) for 60 min.

The R485H mutation causes a profound defect in cPLA$_2$α catalytic activity. It has no catalytic activity measured as a function of calcium concentration in vitro and does not release AA above control levels from IMLF$^{-/-}$ in response to increases in calcium levels induced by serum. The R485H mutant translocates to the Golgi at a rate similar to that of wild-type cPLA$_2$α, suggesting that it responds like wild-type cPLA$_2$α to increases in calcium levels in cells. The R485H mutant has a tendency to accumulate to a greater extent on Golgi than wild-type cPLA$_2$α. This also occurs with cPLA$_2$α mutations in the basic residues of the PI-(4,5)-P$_2$ binding site, although the basis for this enhanced accumulation on the Golgi is not known. The substitution of R485 with a histidine is predicted to disrupt hydrogen bonding interactions and create a destabilizing cavity (*4*).

Our results showing that S111P has catalytic activity when assayed in vitro and releases a low level of AA from cells contrast with the in vivo data showing that cPLA$_2$α activity in our patient is inhibited by more than 95% compared to that in control

individuals (*4*). One contributing factor is the observation that in the proband's platelets, ~50% of the wild-type levels of the cPLA$_2\alpha$ protein are expressed, suggesting that one of the mutations is associated with a lower level of protein expression. Another possibility is a dominant negative effect by which simultaneous expression of both R485H/K651R and S111P leads to more robust inhibition of cPLA$_2\alpha$ activity than expression of S111P alone. We can hypothesize that binding of the inactive R485H mutant to the membrane competes for binding of S111P, further inhibiting the phospholipase activity.

Conclusion. In this work, we have characterized the biochemical consequences of the three nonsynonymous mutations described in a patient devoid of cPLA$_2\alpha$ activity. Our results show that the S111P mutation hampers calcium binding, leading to decreased affinity for phospholipid membranes, but does not diminish the mutant's catalytic activity. The common K651R polymorphism increases the catalytic activity of the enzyme, suggesting a role for this residue in favoring a catalytically active conformation of the enzyme. Finally, the R485H mutant does not adversely affect translocation but completely inactivates the enzyme, suggesting that this positively charged residue regulates the catalytic function of cPLA$_2\alpha$. Our results explain how the presence of these mutations in the two alleles of the patient led to the loss of cPLA$_2$ activity and describe roles for residues previously unstudied, S111, R485, and K651.

REFERENCES

1. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) Role of cytosolic phospholipase A2 in allergic response and parturition. *Nature 390*, 618–622.
2. Bonventre, J. V., Huang, Z., Taheri, M. R., O'Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1997) Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. *Nature 390*, 622–625.
3. Ghosh, M., Tucker, D. E., Burchett, S. A., and Leslie, C. C. (2006) Properties of the Group IV phospholipase A2 family. *Prog. Lipid Res. 45*, 487–510.
4. Adler, D. H., Cogan, J. D., Phillips, J. A., III, Schnetz-Boutaud, N., Milne, G. L., Iverson, T., Stein, J. A., Brenner, D. A., Morrow, J. D., Boutaud, O., and Oates, J. A. (2008) Inherited human cPLA(2α) deficiency is associated with impaired eicosanoid biosynthesis, small intestinal ulceration, and platelet dysfunction. *J. Clin. Invest. 118*, 2121–2131.
5. Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L., and Clark, J. D. (1994) Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca^{2+}-dependent lipid-binding domain and a Ca^{2+}-independent catalytic domain. *J. Biol. Chem. 269*, 18239–18249.
6. Dessen, A., Tang, J., Schmidt, H., Stahl, M., Clark, J. D., Seehra, J., and Somers, W. S. (1999) Crystal structure of human cytosolic phospholipase A2 reveals a novel topology and catalytic mechanism. *Cell 97*, 349–360.
7. Perisic, O., Fong, S., Lynch, D. E., Bycroft, M., and Williams, R. L. (1998) Crystal structure of a calcium-phospholipid binding domain from cytosolic phospholipase A2. *J. Biol. Chem. 273*, 1596–1604.
8. Perisic, O., Paterson, H. F., Mosedale, G., Lara-Gonzalez, S., and Williams, R. L. (1999) Mapping the phospholipid-binding surface and translocation determinants of the C2 domain from cytosolic phospholipase A2. *J. Biol. Chem. 274*, 14979–14987.
9. Dessen, A. (2000) Structure and mechanism of human cytosolic phospholipase A_2. *Biochim. Biophys. Acta 1488*, 40–47.
10. Das, S., and Cho, W. (2002) Roles of catalytic domain residues in interfacial binding and activation of group IV cytosolic phospholipase A2. *J. Biol. Chem. 277*, 23838–23846.
11. Six, D. A., and Dennis, E. A. (2003) Essential Ca^{2+}-independent role of the group IVA cytosolic phospholipase A_2 C2 domain for interfacial activity. *J. Biol. Chem. 278*, 23842–23850.
12. Tucker, D. E., Ghosh, M., Ghomashchi, F., Loper, R., Suram, S., St. John, B., Girotti, M., Bollinger, J. G., Gelb, M. H., and Leslie, C. C. (2009) Role of phosphorylation and basic residues in the catalytic domain of cytosolic phospholipase A2α in regulating interfacial kinetics and binding and cellular function. *J. Biol. Chem. 284*, 9596–9611.
13. Hixon, M. S., Ball, A., and Gelb, M. H. (1998) Calcium-dependent and -independent interfacial binding and catalysis of cytosolic group IV phospholipase A2. *Biochemistry 37*, 8516–8526.
14. Evans, J. H., Spencer, D. M., Zweifach, A., and Leslie, C. C. (2001) Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes. *J. Biol. Chem. 276*, 30150–30160.
15. Stewart, A., Ghosh, M., Spencer, D. M., and Leslie, C. C. (2002) Enzymatic properties of human cytosolic phospholipase $A_2\gamma$. *J. Biol. Chem. 277*, 29526–29536.
16. Clark, J. D., Schievella, A. R., Nalefski, E. A., and Lin, L. L. (1995) Cytosolic phospholipase A2. *J. Lipid Mediators Cell Signalling 12*, 83–117.
17. Leslie, C. C. (2004) Regulation of the specific release of arachidonic acid by cytosolic phospholipase A2. *Prostaglandins, Leukotrienes Essent. Fatty Acids 70*, 373–376.
18. Leslie, C. C., and Channon, J. Y. (1990) Anionic phospholipids stimulate an arachidonoyl-hydrolyzing phospholipase A2 from macrophages and reduce the calcium requirement for activity. *Biochim. Biophys. Acta 1045*, 261–270.
19. Mosior, M., Six, D. A., and Dennis, E. A. (1998) Group IV cytosolic phospholipase A2 binds with high affinity and specificity to phosphatidylinositol 4,5-bisphosphate resulting in dramatic increases in activity. *J. Biol. Chem. 273*, 2184–2191.
20. Evans, J. H., and Leslie, C. C. (2004) The cytosolic phospholipase A2 catalytic domain modulates association and residence time at Golgi membranes. *J. Biol. Chem. 279*, 6005–6016.
21. Williamson, J. R., and Monck, J. R. (1989) Hormone effects on cellular Ca^{2+} fluxes. *Annu. Rev. Physiol. 51*, 107–124.