C2 Domain Membrane Penetration by Group IVA Cytosolic Phospholipase A\textsubscript{2} Induces Membrane Curvature Changes

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Abbreviations: AA, arachidonic acid; BAR, Bin-Amphiphysin-Rvs167; BCA, bicinchoninic acid; CBL, calcium binding loop; C1P, ceramide-1-phosphate; CD, circular dichroism; cPLA\textsubscript{2}\textalpha, group IVA cytosolic phospholipase A\textsubscript{2}; EM, electron microscopy; FcR, Fc Receptor; GFP, green fluorescence protein; ITO, indium tin oxide; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine; RU, resonance unit; SPR, surface plasmon resonance; TEM, transmission electron microscopy.
Abstract

Group IVA cytosolic phospholipase A$_2$ (cPLA$_2$$\alpha$) is an 85-kDa enzyme that regulates release of arachidonic acid (AA) from the sn-2 position of membrane phospholipids. It is well established that cPLA$_2$$\alpha$ binds zwitterionic lipids such as phosphatidylcholine in a Ca$^{2+}$-dependent manner through its N-terminal C2 domain, which regulates its translocation to cellular membranes. In addition to its role in AA synthesis, it has been shown that cPLA$_2$$\alpha$ promotes tubulation and vesiculation of the Golgi and also regulates trafficking of endosomes. Additionally, the isolated C2 domain of cPLA$_2$$\alpha$ is able to reconstitute Fc receptor mediated phagocytosis suggesting that C2 domain membrane binding is sufficient for phagosome formation. These reported activities of cPLA$_2$$\alpha$ and its C2 domain require changes in membrane structure but to date the ability of the C2 domain to promote changes in membrane shape has not been reported. Here we demonstrate that the C2 domain of cPLA$_2$$\alpha$ is able to induce membrane curvature changes to lipid vesicles, giant unilamellar vesicles, and membrane sheets. Biophysical assays combined with mutagenesis of C2 domain residues involved in membrane penetration demonstrate membrane insertion by the C2 domain is required for membrane deformation suggesting that C2 domain induced membrane structural changes may be an important step in signaling pathways mediated by cPLA$_2$$\alpha$.

Keywords: calcium, C2 domain, cytosolic phospholipase A$_2$$\alpha$, lipid binding, membrane curvature, membrane penetration.
Introduction

Group IVA cytosolic phospholipase A\(_2\) (cPLA\(_2\)\(\alpha\)) is an 85-kDa enzyme, consisting of a N-terminal lipid-binding C2 domain (\(~120\) residues) and a C-terminal catalytic or lipase domain (\(~600\) residues) that is separated by a flexible linker (1, 2). cPLA\(_2\)\(\alpha\) regulates arachidonic acid release from the sn-2 positions of membrane phospholipids that is used in the synthesis of leukotrienes and prostaglandins in response to inflammatory agonists (3). cPLA\(_2\)\(\alpha\) has also been implicated in a number of pathological conditions including asthma (4), cancers (5), arthritis (6), cerebral ischemia (7), and heart disease (8). The general principles governing cPLA\(_2\)\(\alpha\) in vitro membrane binding (9, 10) and activation (11) as well as cellular translocation (12, 13) are well established where the C2 domain binds with high affinity to zwitterionic membranes in a Ca\(^{2+}\)-dependent manner (9), while the catalytic domain binds to membranes independent of Ca\(^{2+}\) albeit weakly (14). This functionality allows the C2 domain to act as a Ca\(^{2+}\) sensor in cells, which drives the cellular localization to the Golgi, ER, and nuclear membranes (2, 12, 13).

The C2 domain (\(~120\) amino acids) contains three calcium-binding loops (CBL), two of which, CBL1 and 3 harbor hydrophobic and aromatic amino acids (See Fig. 1) that promote binding to zwitterionic membranes (9). Ca\(^{2+}\) binding induces a dramatic change in electrostatic potential lowering the desolvation penalty associated with membrane insertion (15) and promoting the docking of the C2 domain to the membrane bilayer. This penetration into the membrane has been shown to be significant with a depth of \(~15\) Å (16), which extends extensively into the hydrocarbon region of the membrane. The significant membrane penetration of cPLA\(_2\)\(\alpha\) is important for its membrane targeting to zwitterionic membranes and also its ability to produce arachidonic acid (AA). Recently, two anionic lipids, ceramide-1-phosphate (C1P) (17, 18) and PtdIns(4,5)P\(_2\) (19, 20) have been demonstrated to bind and activate cPLA\(_2\)\(\alpha\) with emerging roles in the cellular translocation of the enzyme (21, 22).
Besides its role in eicosanoid biosynthesis, cPLA$_2$$\alpha$ is selectively activated upon Fc receptor (FcR)-mediated phagocytosis in macrophages where it rapidly translocates to the nascent phagosome (23). Unexpectedly, however, it was shown that membrane binding by the isolated C2 domain of cPLA$_2$$\alpha$ was sufficient to induce phagosome formation (23) suggesting that the C2 domain alone has membrane binding activity that regulates phagocytosis. This was further verified with a mutation in the C2 domain, D43N, which abrogates Ca$^{2+}$-binding and could not rescue phagocytosis (23). cPLA$_2$$\alpha$ also plays a role in membrane curvature generation through regulation of aberrant Golgi vesiculation (24), Golgi tubulation (25), and vesiculation of cholesterol-rich GPI-anchored, protein-containing endosomes (26). While it is speculated the C2 domain may induce changes to membrane structure (23), to date, direct evidence is lacking. These recent studies suggest that cPLA$_2$$\alpha$ and its C2 domain have membrane remodeling activity that is critical to biological signaling pathways.

Recently, a number of peripheral proteins, mainly attributed to their modular lipid binding domains have been found to induce membrane curvature changes (27) including ENTH (28), BAR (29), PH (30), ACCH (31), and C2 domains (32). Here, we investigate the ability of the isolated C2 domain of cPLA$_2$$\alpha$ to induce changes to lipid structure. A number of imaging assays are employed including electron microscopy (EM) of large unilamellar vesicles (LUVs), imaging of giant unilamellar vesicles (GUVs), and imaging of membrane sheets. In addition, biophysical assays including monolayer penetration analysis and surface plasmon resonance (SPR) were used to correlate membrane penetration and affinity with membrane remodeling activity. Results provide evidence that Ca$^{2+}$-dependent membrane insertion of CBL1 and 3 of the C2 domain drive membrane curvature changes.
Materials and Methods

Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylserine (POPS) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Octyl glucoside, (3-[3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS), Nunc Lab-Tek I Chambered Cover Glasses 8-well and bicinchoninic acid (BCA) protein assay kit were from Thermo Fisher Scientific (Waltham, MA). L1 sensor chips were from GE Healthcare (Piscataway, NJ). N-(3-(Triethylammoniumpropyl)-4-(4-(Diethylamino)styryl)Pyridinium Dibromide (FM® 2-10) lipophilic dye was from Life Technologies (Grand Island, NY). Restriction endonucleases and enzymes for molecular biology were obtained from New England Biolabs (Beverly, MA).

Cloning and Protein Expression

The QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to introduce mutations into the pET28a vector with a His₆ tag engineered into the N-terminus of the cPLA₂α C2 domain gene (9). All mutated constructs were sequenced to ensure presence of the desired mutation. The C2 domain and respective mutations were expressed and purified from E. Coli BL21(DE3) cells as previously described (9). Protein concentrations were determined by the BCA method and all protein aliquots were stored in 20 mM HEPES, pH 7.4, containing 160 mM KCl.

Electron Microscopy

200 µL of 1 mg/ml POPC LUVs were prepared as previously described (32). Briefly, the lipids were dried under nitrogen gas and resuspended in 20 mM HEPES, pH 7.4, containing 160 mM KCl and either 100 µM CaCl₂ or 100 µM EGTA. The vesicles were incubated at 37°C and extruded through an 800 nm filter (Avanti Polar Lipids, Alabaster, AL). The respective C2
domain and mutations were incubated at a concentration of 10 µM with the POPC vesicles for 30 minutes at 25°C. Samples were then applied to a carbon-formvar-coated copper grid and stained with 2% uranyl acetate. Liposome morphologies were then imaged at 80 kV on a FEI 80-300 D3203 electron microscope at 6300x magnification.

**Giant Unilamellar Vesicle Assay**

An aliquot of lipids containing POPC, POPE and POPS suspended in chloroform were prepared in a 60:20:20 molar ratio. The suspension was dried under nitrogen gas and resuspended in chloroform to a final concentration of 0.4 mg/mL. The lipid suspension was dried onto an indium tin oxide (ITO) coated slide and dehydrated under a vacuum for 1 hour. The GUV apparatus was assembled and a 350 mM D-sucrose solution was placed into the reservoir containing the dried lipids. Another glass plate was placed on top to eliminate air from the system then a sine wave generator was applied at 3V and 20 Hz for 5 hours at 25°C. The GUV solution was collected and stored at 25°C until use. The GUV solution was diluted 20-fold in 20mM HEPES, pH 7.4, containing 160 mM KCl and 10µM FM® 2-10 lipophilic dye. Samples were prepared with either 100 µM EGTA, 10 µM CaCl₂ or 500 nM CaCl₂ as necessary for experimental conditions. GUV vesiculation was assessed after a 5 min incubation with either 2 µM or 500 nM cPLA₂α-C2, 500 nM full-length cPLA₂α, or 2 µM of respective mutants and was subsequently imaged via confocal microscopy (Zeiss LSM 710) on Nunc Lab-Tek I Chambered Cover Glasses 8-well using a 63x 1.4 NA oil objective. Three replicates for each control or sample were quantified by counting 60-100 GUV’s per replicate. The number of vesiculated GUV’s were quantified separately and compared to the total number of GUV’s in each replicate. The degree of vesiculation was then expressed as a percentage, compared to the control, and quantified using an unpaired student t-test.

**Membrane Sheets**

2 µL of 10 mM POPC was prepared in chloroform and spotted onto Nunc Lab Tek I 8-well Chambered Cover Glasses then dried under a vacuum. The lipid was rehydrated with 20 mM
HEPES, pH 7.4, containing 160 mM KCl and 20 µM FM® 2-10 and allowed to rehydrate for 15 minutes. Samples were prepared with either 100 µM EGTA or 100 µM CaCl₂ as necessary for experimental conditions. The experiments contained either 2 µM cPLA₂α-C2 or respective mutants and were subsequently imaged after a 15 minute incubation with a confocal microscope (Nikon A1R-MP with a 100x 1.4 NA oil objective).

**Monolayer Penetration**

Surface pressure ($\pi$) of solution in a circular Teflon trough (2 mL) was measured using a wire probe connected to a Kibron MicroTrough X (Kibron, Inc., Helsinki) as previously described (33). Phospholipid solution (2 – 8 µL) in hexane/ethanol (9:1 v/v) was spread onto 2 mL of subphase to form a monolayer with a given initial surface pressure ($\pi_0$). The subphase was stirred continuously at 30 revolutions/min with a small magnetic stir bar. Following stabilization of the surface pressure of the monolayer (~ 5 min), the protein solution (typically 10 µL) was injected into the subphase and the change in surface pressure ($\Delta\pi$) was measured as a function of time. Generally, the $\Delta\pi$ value reached a maximum after 20 min. The maximal $\Delta\pi$ value depends upon the protein concentration and reached saturation at $[\text{cPLA}_2\alpha\text{-C2} > 1 \text{ µg/mL}]$ as previously reported (34). Protein concentrations in the subphase are maintained above such values to ensure the $\Delta\pi$ represents a maximal value. The $\Delta\pi$ versus $\pi_0$ plots were constructed from these measurements to obtain the x-intercept or critical pressure ($\pi_c$) defined as the value in which the protein penetrates up to (35).

**SPR Assays**

All SPR measurements were performed at 25 °C. A detailed protocol for coating the L1 sensor chip has been described elsewhere (34). Briefly, after washing the sensor chip surface, 90 µl of POPC vesicles were injected at 5 µl/min to give a response of 6500 resonance units (RU). An uncoated flow channel was used as a control surface. Under our experimental conditions, no binding was detected to this control surface beyond the refractive index change for the C2 domain of cPLA₂α as previously reported (18, 34). Each lipid layer was stabilized by
injecting 10 µl of 50 mM NaOH three times at 100 µl/min. SPR measurements were done at the flow rate of 5 µl/min. 50-90 µl of protein in 10 mM HEPES, pH 7.4, containing 160 mM KCl and 50 µM Ca\(^{2+}\) was injected to give an association time to reach saturation of binding signal \(R_{eq}\) (See Fig. 5C). The saturation responses for WT and mutations were normalized where maximum WT saturation response was set to 1 to compare the binding capacity of WT versus mutations. The lipid surface was regenerated using 10 µl of 50 mM NaOH. Each of the sensorgrams was corrected for refractive index change by subtracting the control surface response from the binding curve. A minimum of three data sets was collected for each protein at a minimum of five different concentrations for each protein within a 10-fold range of \(K_d\). \(R_{eq}\) values were then plotted versus protein concentration and the \(K_d\) value was determined by a nonlinear least-squares analysis of the binding isotherm using an equation, \(R_{eq} = R_{max}/(1 + K_d/C)\) (36). Each data set was repeated three times to calculate a standard deviation.

**Circular Dichroism (CD) Spectroscopy**

To ensure the WT and mutant proteins retained a stable structure, CD was utilized to assess the secondary structure of each recombinant protein used in the study. The spectra were taken on a JASCO 815 CD spectrometer scanned from 195-250 nm in a 1 mm quartz spectrophotometer cell (Starna Cells Inc. Atascadero, CA) at 20 °C in 10 mM Hepes, 80 mM KCl, pH 7.4. Each measurement was performed in triplicate and averaged to yield the representative scans shown in Figure S1A. Molar ellipticity was defined according to the JASCO software and was subtracted from a control buffer scan. WT and mutations displayed overlapping spectra consistent with \(\beta\)-sheet structure.

**Calcium Binding Assay**

To measure the calcium binding capacity of WT cPLA\(_2\alpha\)-C2 and the point mutants the calcium detector Bis-Fura-2 (Life Technologies, Carlsbad, CA) was employed according to the manufacture’s protocol. Briefly, 2 µM protein was incubated for 30 minutes with control or 10
µM Ca\textsuperscript{2+} standard in a black fluorescent plate with a clear bottom (Costar Life Science, Tewksbury, MA). The difference in the unknown Ca\textsuperscript{2+} concentration was determined in relation to a standard curve by measuring the ratio of the emission at 510 nm at excitation wavelengths of 350 nm and 380 nm, respectively. Percent binding was normalized to the average WT binding capacity. Measurements were performed in triplicate for WT and mutations to determine the standard deviation (Fig. S1B).

Results

Electron Microscopy of Liposome Morphology Changes Induced by the C2 domain

To date electron microscopy (EM) has been used to effectively characterize changes in liposome morphologies induced by ENTH (28), BAR (29), ACCH (31), and C2 domains (32). To assess the ability of the C2 domain of cPLA\textsubscript{2α} to induce changes to liposome morphologies we used transmission EM (TEM) with negative staining to visualize liposomes before and after incubation with the C2 domain. As shown in Figure 2, the C2 domain induced dramatic changes in POPC liposome structures as long tubules were extensively visualized through the grids. Moreover, the tubulation of liposomes induced by the C2 domain was Ca\textsuperscript{2+}-dependent as experiments performed in the presence of 100 µM EGTA in place of CaCl\textsubscript{2} did not display detectable changes in liposome morphology (Fig. 2). ENTH, BAR and ACCH domains insert into the hydrocarbon region of the membrane bilayer, which is a prerequisite for their ability to induce membrane curvature changes (31, 37, 38, 39). To test if hydrophobic and aromatic residues, which typically insert into membranes, were required for liposome morphology changes we prepared mutations of hydrophobic and aromatic residues in calcium binding loops 1 and 3 of the C2 domain (Fig. 1). Earlier studies have demonstrated the ability of these calcium-binding loops to penetrate deeply into membranes and monolayers where hydrophobic and aromatic residues in these loops protrude into the hydrocarbon region of the membrane (9, 16). Indeed, F35A/L39A and Y96A displayed a drastic reduction in liposome morphology
changes and displayed a lack of long thin tubules emanating from liposomes as seen for the WT C2 domain. M38A and M98A, which have been shown to have a lesser effect than F35A, L39A or Y96A on cPLA₂ membrane binding (9) still induced changes in liposome structure albeit to a lesser extent than WT.

Quantification of Membrane Curvature Changes Using GUVs

GUVs have served as an effective platform for monitoring changes to membrane structure as they can be fluorescently labeled, imaged with confocal microscopy and are more easily quantified than EM experiments. GUVs have been effective in monitoring membrane curvature changes for the ENTH domain (37) and viral matrix proteins (40). In addition, they are relatively flat (mean diameter ~ 30 µM) in comparison to LUVs (mean diameter ~ 400 nm) so they can be used to assess if proteins induce membrane curvature changes on different membrane surfaces. GUVs composed of POPC:POPE:POPS (60:20:20) were prepared and used to quantify membrane curvature changes for WT C2 domain and respective mutations. All experiments were performed in triplicate and at least 60 GUVs were counted in each experiment and assessed for membrane curvature changes in response to C2 domain binding. As shown in Figure 3A, WT C2 domain induced vesiculation of GUVs in the presence of Ca²⁺, which was not observed in the presence of 100 µM EGTA. Hydrophobic and aromatic mutations, F35A/L39A and Y96A, which greatly reduce alterations to liposome morphology in the EM assays significantly reduced GUV vesiculation for which their quantitative value was similar to that of the control. M38A and M98A displayed a statistically significant reduction in GUV vesiculation in line with the EM assays, which detected appreciable changes to liposome structure albeit to a lesser extent than WT. To assess the ability of the C2 domain to induce membrane curvature changes we assessed the ability of the C2 domain to induce vesiculation in GUVs at 200 nM WT C2 domain in the presence of 500 nM CaCl₂. Indeed, under these conditions, which are closer to physiological concentrations of cytoplasmic Ca²⁺ the C2 domain induced substantial GUV vesiculation (See Fig. 3C and D). Lastly, we assessed the ability of
full-length cPLA$_{2\alpha}$ to induce membrane curvature changes to GUVs at 200 nM protein in the presence of 500 nM CaCl$_2$. The full-length enzyme not only induced GUV vesiculation but also prompted extensive tubulation emanating from the GUVs.

**C2 Domain Induces Fragmentation of Membrane Sheets**

Membrane sheets labeled with fluorescent dye, which represent a relatively flat membrane surface, have been used to image membrane curvature changes for the PH domain of FAPP1 and 2 (30). Here we employed POPC membrane sheets labeled with FM® 2-10 dye to assess the ability of the C2 domain to induce changes to membrane sheet structure. Membrane sheets were imaged before and after introduction of C2 domain and mutants to observe changes in real-time (Figure 4A). In the presence of Ca$^{2+}$ the C2 domain induced rapid fragmentation of POPC membrane sheets (Fig. 4A). The specific nature of this finding was confirmed by adding the same volume of protein storage buffer to ensure that changes in volume did not induce membrane swelling or membrane fragmentation. Additionally, mutations that greatly reduced membrane structural changes in the EM or GUV assays, F35A/L39A and Y96A, also abolished membrane fragmentation of POPC sheets (Fig. 4B). M98A displayed similar membrane fragmentation as WT C2 but M38A did not induce detectable changes in membrane sheet structure up to 25 min after the addition of protein. Taken together, the C2 domain is able to induce changes to membrane structure for small highly curved membranes (LUVs) as well as less curved and relatively flat membranes (GUVs and membrane sheets).

**Membrane Penetration and Lipid Binding Affinity of C2 domain and Mutations**

Membrane penetration of C2 domain and mutations into POPC monolayers was detected by injecting protein into the subphase buffer at varying initial surface pressure ($\pi_0$) values (See Fig. 5A and B). This allows determination of the critical pressure ($\pi_c$), which is the pressure up to which the protein will penetrate (x-intercept) (35). As previously reported (9, 34), the WT C2 domain robustly penetrated a POPC monolayer with a value of ~ 36 mN/m. In
contrast, F35/L39A and Y96A, which abrogate membrane curvature changes also significantly reduce the ability of the C2 domain to penetrate POPC monolayers with \( \pi_c \) values of 23 and 20 mN/m, respectively. In addition, M38A and M98A, which had slightly reduced membrane-deforming capabilities also slightly reduced membrane penetration with \( \pi_c \) values of 30 and 31 mN/m, respectively. These results demonstrate that the C2 domain can effectively penetrate physiological bilayers since the surface pressure of cell membranes and LUVs is estimated to be in a range of 30-35 mN/m (41). Since monolayer penetration studies with WT and mutants are performed at saturating amounts of protein where maximal binding of WT or mutants occurs this signifies even at saturating conditions of F35A/L39A and Y96A the proteins are not significantly penetrating into the membrane while M38A and M98A have reduced penetration compared to WT. In the absence of Ca\(^{2+}\) the C2 domain did not insert into the monolayer, and the \( \pi_c \) value of POPC monolayers was essentially undetectable as previously reported (9, 34). Similarly, the mutation D43N in the C2 domain, which reduces Ca\(^{2+}\) binding and was unable to reconstitute FcR mediated phagocytosis (23) also reduced the \( \pi_c \) value (20 mN/m). Thus, membrane penetration is necessary to induce membrane curvature changes as observed in the EM, GUV, and membrane sheet assays. Likewise, phosphoinositides are necessary for the ENTH, PH, and ACCH domains to sufficiently penetrate membranes and induce membrane deformation (31, 39, 42).

To quantitatively assess the effect of mutations on the ability of the C2 domain to bind POPC vesicles, we performed SPR assays (Fig. 5 C and D). A blank surface was used as a control as it has been shown previously that the C2 domain of cPLA\(_2\)\(\alpha\) does not exhibit nonspecific binding to the L1 chip surface (32, 34). The dissociation constants (\( K_d \)s), obtained in triplicate, demonstrate that the C2 domain associated with 21 \( \pm \) 4 nM affinity to POPC vesicles at 50 \( \mu \)M CaCl\(_2\) but binding was not detectable up to 5 \( \mu \)M in the presence of 100 \( \mu \)M EGTA (data not shown). While mutations that abolish membrane-deforming activity, F35A/L39A and
Y96A, demonstrate >140- and 57-fold increases in $K_d$ (Figure 5D) consistent with their role in membrane penetration and in inducing alterations in membrane structure. Lastly, mutations that slightly reduced membrane curvature changes and membrane penetration, M38A and M98A, increase the $K_d$ by 6.2- and 4.4-fold, respectively. In order to rule out misfolded C2 domain mutations we used CD to determine the CD spectra of each mutant in comparison to wild type. As shown in Fig. S1A CD spectra’s from all mutations overlapped with that of wild type C2 domain and displayed a spectra indicative of $\beta$-sheet with an energy minima at ~ 215 nm. Next, in order to rule out changes in calcium binding for the mutants we quantified the calcium binding ability of wild type, F35A/L39A, M38A and M98A. As shown in Fig. S1B mutations had comparable calcium binding ability to wild type with M38A and M98A displaying slightly reduced binding that was not statistically significant. Taken together our data indicate that membrane affinity of the C2 domain and respective mutations correlates with the ability to penetrate membranes and induce membrane curvature changes.

Discussion

Trafficking of membrane vesicles, endocytosis, and lipid-enveloped viral egress are a few of the cellular pathways where major membrane curvature changes are necessary. These are highly dynamic processes that cannot occur spontaneously as a significant energy barrier has to be overcome to shape the lipid bilayer into a highly curved vesicle (43, 44). To overcome this energy barrier, protein-mediated effects or lipid bilayer asymmetry can mediate curvature changes. Protein induced changes are often facilitated by insertion of proteins into the membrane bilayer or scaffolding of proteins on the membrane surface through oligomerization (45). Lipid-mediated changes in bilayer structure can be attributed to lipid asymmetry mediated by cone-shaped and inverted-cone-shaped lipids, where lipid shape can induce positive or negative curvature changes (44).
Membrane curvature changes induced by lipid binding domains were first appreciated with the discovery of the ENTH domain and its ability to induce changes to liposome structure in a PI(4,5)P$_2$-dependent manner (28). The ENTH domain deeply penetrates membranes with a N-terminal amphipathic $\alpha$-helix and also forms oligomers on the membrane (37), both of which are necessary for effective membrane tubulation. This activity is essential to endocytosis and clathrin coated vesicle formation, which requires substantial changes to plasma membrane structure to form highly curved membrane vesicles (46). Subsequently, the discovery of the BAR domains of amphiphysin (29) and endophilin (47) lead to the notion that intrinsic curvature from the crescent moon shaped BAR domains is essential to remodeling membranes. This lead to further investigation, which demonstrated that BAR domains form elegant scaffolds on the membrane where mutation of residues that mediate scaffolding abrogates membrane curvature changes (48, 49). Additionally, as with the ENTH domain some BAR domains have a N-terminal $\alpha$-helix that can penetrate into the membrane and an insert on the membrane binding interface that inserts and is predicted to be a second amphipathic $\alpha$-helix (38). The depth and orientation of this penetration may also be important in regulating membrane curvature changes and membrane fission (50). For instance, it was recently shown that insertion of the amphipathic $\alpha$-helix drives vesiculation and thus scission by the ENTH domain. In contrast, an antagonistic relationship between the number and length of amphipathic helices in BAR domains was discovered where membrane fission can be restricted by the BAR domains’ crescent shape (50). Taken together, depth and area of insertion as well as inherent protein scaffold shape play a critical role in the type of membrane curvature generated and whether or not membrane fission will proceed.

PH domains (30) and C2 domains (32) have also been shown to induce membrane curvature changes. The FAPP1 and 2 PH domains require insertion of a turret loop adjacent to the PI(4)P binding pocket (42) to induce membrane remodeling where the inherent shape of
FAPP1 or 2 may also play a critical role (30). However, unlike the ENTH and BAR domain elegant models of membrane scaffolding and modes of membrane curvature induction for PH and C2 domains have not been investigated. In addition, the relationship between membrane penetration of C2 domains and membrane curvature changes is still unknown. Recently, it was shown that the C2B domain alone or the tandem C2AC2B domains of synaptotagmin 1, which can induce membrane tubulation (32) as well as vesicle aggregation (51) could induce lipid demixing of PS in POPC:POPS vesicles (52), which is thought to induce positive bilayer curvature changes.

Here we demonstrate that the C2 domain of cPLA2α, long appreciated as a high affinity target for zwitterionic lipids (9) with an ability to deeply penetrate the hydrocarbon core of zwitterionic membranes, is able to induce substantial changes to membrane structure. Imaging of liposomes with TEM, GUVs with confocal microscopy, or membrane sheets with confocal microscopy demonstrated dramatic changes in membrane structure induced in a Ca^{2+}-dependent manner by the cPLA2α C2 domain. Detectable changes in membrane structure correlated strongly with membrane penetrating ability and lipid binding affinity. As shown in figure 6, membrane penetration of the C2 domain generated positive membrane curvature as evidenced in the TEM and confocal assays. Positive curvature generation by the C2 domain is consistent with the role of cPLA2α in formation of the phagosome, Golgi tubulation and Golgi vesiculation, which occur by budding into the cytoplasm. It is also important to note the protein concentrations of C2 domain employed in the membrane curvature assays were similar or lower than most of the previous studies in the literature (28, 29, 32), supporting the specific nature of our findings. For the C2 domain it seems there may be a threshold affinity and depth or extent of penetration that is responsible for generating curvature as both M38A and M98A, which reduced membrane penetration and affinity still induced statistically significant changes in membrane curvature. In the sheet assay, M38A did not induce detectable membrane
fragmentation while M98A did. M38A is located in CBL1 and has lower affinity than M98A, which is located in CBL3 (See Fig. 1). It has been shown that CBL1 penetrates more deeply into the bilayer than CBL3 (53), which could perhaps play a role in the different observations in the membrane sheet assays. While the origin of this discrepancy is still unknown it leaves room for extensive biophysical studies of C2 domain parameters required for membrane curvature generation. It also appears M38A and M98A may cause some vesiculation in the liposome assays as visualized by EM as well as differences in the extent of vesiculation in the GUV assays suggesting the depth of penetration and/or membrane affinity may play an important role in the type of membrane curvature or membrane reorganization that is generated. Studying the C2 domain’s role in the type of curvature generation in conjunction with membrane fission (50) will be essential to better understanding the mechanism of curvature generation for this C2 domain. Penetration of hydrophobic residues by the C2 domain occurs into the hydrocarbon layer (~ 15 Å) reminiscent of the ENTH domain (37, 39) so its possible membrane fission and vesiculation (50) may occur, which is supported by membrane fragmentation in the membrane sheet assays. C2 domains and PH domains may also induce different types of curvature. For instance, in this study the C2 domain induced membrane fragmentation whereas studies with PH domains have demonstrated extensive positive curvature induction in the form of tubules in the membrane sheet assays (42).

Future studies will need to consider the role of C2 domain membrane binding and penetration in inducing or contributing to membrane curvature changes in conjunction with cPLA2α activity. The fact that the C2 domain alone is able to reconstitute FcR-mediated phagocytosis (23) suggests the cPLA2α C2 domain possibly has a high membrane remodeling activity that is essential to membrane reorganization. Additionally, inhibition of cPLA2α activity in cells with the inhibitor pyrrophenone generated an allosteric block that prevented cPLA2α translocation (23), which doesn’t allow one to account for C2 membrane binding and insertion in
assessing the generation of lysophospholipids in curvature generation (26). Nonetheless, the prior study demonstrated the C2 domain process is Ca\(^{2+}\) dependent as D43N, which abrogates calcium binding could not reconstitute phagocytosis. In conjunction with the current study this strongly suggests membrane penetration of the C2 domain is necessary for these effects as calcium is required for membrane penetration of the C2 domain (Fig. 1C and 5A) (9). It is also now well established the cationic β-groove of the C2 domain binds C1P (21), which is important for cPLA\(_2\alpha\) activity (21) and cellular translocation (54). Additionally, the role of ceramide kinase and its product C1P are key players in phagocytosis (55, 56). Thus, its been hypothesized that C1P has an important role in recruitment of cPLA\(_2\alpha\) in phagocytosis (57). To this end it’s tempting to speculate that cPLA\(_2\alpha\) may be able to induce reorganization or clustering of membranes harboring C1P.

The surface area of insertion for the C2 domain (58) is more substantial than that of the ENTH (39, 50) and PH domains (59), at least for a monomer, however, this alone may not account for the membrane mediated curvature changes. It was first thought the N-terminal α-helix insertion for ENTH domains and to some degree for BAR domains was the major cause of the membrane curvature induction, however, more recent and sophisticated studies have demonstrated the ability of these proteins to scaffold on the membrane (48, 49). This scaffolding is essential for both in vitro and cellular observations of membrane curvature changes (48, 49). Future studies will need to be directed towards resolving the molecular details of C2 oligomerization as well as the role of β-groove C1P binding in membrane curvature changes or membrane reorganization. Additionally, the type of membrane curvature generated by the C2 domain as well as full-length cPLA\(_2\alpha\) will require extensive analysis using a combination of biophysical, biochemical and cellular assays. Investigating how the C2 domain insertion and catalytic domain generation of lysophospholipids contribute to in vitro as well as
cellular membrane curvature changes should solve a number of burning questions in the fields of membrane trafficking and phagosome formation.

The type of curvature generated by the C2 domain or full-length enzyme may also be key to normal and aberrant physiological processes linked to cPLA$_{2\alpha}$ activity. cPLA$_{2\alpha}$ has been shown to function in generation of cholesterol rich, GPI-protein containing endosomes (26), Golgi tubulation and vesiculation (24, 25), and Golgi to PM trafficking (60). Additionally, cPLA$_{2\alpha}$ association with the Golgi has been show to regulate the function of endothelial cells (61, 62), which serve a barrier function in the luminal surfaces of blood vessels. Proliferation of endothelial cells has been shown to occur for formation of blood vessels in wound healing and tumor formation while blocking cPLA$_{2\alpha}$ activity through an inhibitor or siRNA blocks endothelial cell proliferation and cell cycle entry (61). In terms of pathophysiological states cPLA$_{2\alpha}$ has been linked to diseases such as asthma (4), arthritis (6) and cancers (5). Thus, up or downregulation of cPLA$_{2\alpha}$ enzyme levels may alter the transport of vesicles from the Golgi to the PM, modify intra-Golgi transport, or effect endothelial barrier function through the combined generation of fatty acids and lysophospholipids and membrane penetration of the C2 domain of cPLA$_{2\alpha}$. Additionally, because cPLA$_{2\alpha}$ enzyme inhibitors act as an allosteric block that reduces or precludes cPLA$_{2\alpha}$ membrane translocation it is difficult to rule out the C2 domain mediated effects. Thus, our data supports a model where vesiculation or tubulation of the Golgi may occur in response to C2 domain membrane penetration of the full-length enzyme under conditions of low cPLA$_{2\alpha}$ activity. Linkage of biochemical and biophysical studies in vitro and in cells with cellular and disease models that can tease apart the role of C2 domain penetration and cPLA$_{2\alpha}$ activity in these processes will be key to unraveling the full mechanism of membrane curvature generation.
Acknowledgements

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References


Figure Legends

**Fig. 1** Structural depiction of the C2 domain of cPLA$_2$α. A. The C2 domain (PDB ID 1CJY) is shown in grey in surface transparency mode to depict hydrophobic amino acids in calcium binding loops 1 and 3 (red). The two Ca$^{2+}$ ions bound to the C2 domain are shown in yellow. B. A close up view of the calcium binding and membrane penetration regions of the C2 domain of cPLA$_2$α. Amino acids mutated in this study to assess membrane penetration and membrane curvature are shown in red (Phe$^{35}$, Met$^{38}$, and Leu$^{39}$ in CBL1 and Tyr$^{96}$ and Met$^{98}$ in CBL3). Ca$^{2+}$ ions crystalized with the protein are shown in yellow. C. The C2 domain has been shown to deeply penetrate zwitterionic membranes. Here the C2 domain is shown with the depth of penetration and orientation previously resolved by EPR (16) deeply penetrating hydrophobic and aromatic residues are shown in red (Phe$^{35}$, Met$^{38}$, and Leu$^{39}$ in CBL1 and Tyr$^{96}$ and Met$^{98}$ in CBL3) and 2 Ca$^{2+}$ ions in yellow. The domain was docked to the membrane according to previous biophysical studies, which provided molecular insight into the depth and orientation of the C2 domain binding to membranes (16). The protein shown is docked to a POPC membrane, which displays the importance of Phe$^{35}$, Met$^{38}$, and Leu$^{39}$ in CBL1 and Tyr$^{96}$ and Met$^{98}$ in CBL3 in penetrating the lipid bilayer.

**Fig. 2** The C2 domain induces membrane tubulation of POPC LUVs. Transmission electron microscopy was used to assess the ability of the C2 domain and mutants to induce changes to liposome morphology. All measurements were done with 10 µM protein in 20mM HEPES, pH 7.4, containing 160 mM KCl, and either 100 µM CaCl$_2$ or 100 µM EGTA. WT C2 induced extensive tubulation of POPC liposomes in a Ca$^{2+}$-dependent manner. However, mutations F35A/L39A and Y96A greatly reduced changes in liposome morphology while M38A and M98A
induced formation of tubules from liposomes but to a lesser extent than WT. Incubation of the C2 domain in 100 µM EGTA in place of CaCl$_2$ with POPC liposomes did not induce appreciable changes in liposome morphology. Scale bars = 500 nm.

**Fig. 3** GUV assay demonstrates the C2 domain’s ability to induce membrane budding. A. Experiments with POPC:POPE:POPS (60:20:20) GUVs were performed in triplicate with a minimum of 60 GUVs assessed per measurement to provide a quantitative representation of membrane curvature changes shown in B. B. Quantitative representation of GUV vesiculation induced by C2 domain and respective mutants. WT induced significant vesiculation of GUVs in the presence of 10 µM CaCl$_2$ compared to control experiments performed in 100 µM EGTA. M38A and M98A did display some induction of GUV vesiculation and membrane reorganization but to a significantly lesser extent than WT. F35A/L39A and Y96A did not appreciably induce GUV vesiculation when compared to the control. C. WT C2 domain and full-length cPLA$_2$$^\alpha$ were assessed at 200 nM protein concentration for their ability to induce membrane curvature changes in the presence of 500 nM CaCl$_2$ to GUVs containing POPC:POPE:POPS (60:20:20). WT C2 domain induced substantial vesiculation while full-length cPLA$_2$$^\alpha$ induced vesiculation and long tubule formation from GUVs. D. Quantification of vesiculation in control versus WT C2 experiments shown in C. The p value for each protein was determined in comparison to the control in C and D (ns = not significant, * p < 0.001, ** p < 0.0001) using an unpaired student t-test. Scale bars = 5 µm.

**Fig. 4** C2 domain induces lipid fragmentation of membrane sheets. POPC membrane sheets were used to test the ability of the C2 domain to induce membrane fragmentation to relatively flat membrane surfaces. Membranes were hydrated then incubated with 2 µM WT or mutant C2 domain for 15 minutes. A. WT C2 domain induced extensive membrane fragmentation from
membrane sheets, which was not observed in control experiments with buffer alone. B. Hydrophobic and aromatic mutations reduced C2 domain membrane fragmentation. Only M98A displayed detectable membrane fragmentation compared to WT, F35A/L39A, M38A, and Y96A. All membrane sheets were imaged before and after protein incubation. All scale bars = 25 µm.

**Fig. 5** Mutations that reduce or abrogate membrane deformation reduce or abolish membrane penetration and membrane affinity. A. Insertion of the wild type C2 domain in the presence of Ca\(^{2+}\) (filled circles) or EGTA (open circles) into a POPC monolayer monitored as a function of \(\pi_0\). Insertion of F35A/L39A (filled squares), M38A (filled triangles), or D43N (filled diamonds) was also monitored in the presence of Ca\(^{2+}\). B. Insertion of the wild type C2 domain (filled circles), Y96A (filled squares) or M98A (filled triangles). All measurements performed in the presence of Ca\(^{2+}\). C. The normalized saturation response \((R_{eq})\) from WT cPLA\(_{2}\alpha\)-C2 (filled circles), M38A (filled triangles) or Y96A (filled squares) binding at each respective protein concentration was plotted versus [C2] to fit with a nonlinear least squares analysis of the binding isotherm \((R_{eq} = R_{max}/(1 + K_d/C))\) to determine the \(K_d\). D. \(K_d\) values for WT and respective mutations binding to POPC vesicles. The binding experiments were completed from independent experiments in triplicate and are listed with their respective \(K_d\) ± standard deviation.

**Fig. 6** Membrane penetration by the C2 domain of cPLA\(_{2}\alpha\) is sufficient to induce membrane curvature changes. The hydrophobic residues essential in penetrating the membrane are also key for membrane curvature generation. The deep ~ 15 Å penetration of these hydrophobic and aliphatic residues as well as a significant area of insertion (~ 2110 Å\(^2\)) are sufficient to reduce the energetic barrier to bend the membrane as deletion of one of these key residues abolishes this effect as shown for F35A/L39A and Y96A mutants. Although the overall mechanism is currently unknown, our data suggests that membrane penetration of the C2
domain is vital for membrane bending, tubulation, vesiculation and fragmentation depending on the initial curvature of the membrane.
Ward et al. Figure 2
A + 10µM CaCl₂

WT  M38A  M98A  F35A/L39A  Y96A  100µM EGTA

B

C

D

Ward et al. Figure 3
Ward et al. Figure 4A

A  

Control  + cPLA₂ α-C2

+ Buffer

Scale bar = 25 µm
**Kₐ measurements for the C2 domain of cPLA₂α.**
Measurements were determined with SPR at 50 µM Ca²⁺ in 10 mM HEPES, pH 7.4 containing 0.16 M KCl buffer.

<table>
<thead>
<tr>
<th>POPC</th>
<th>Protein Kₐ (M)</th>
<th>Fold Increase in Kₐ ²</th>
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<tbody>
<tr>
<td>WT</td>
<td>(2.1 ± 0.4) x 10⁻⁸</td>
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</tr>
<tr>
<td>F35/L39A</td>
<td>&gt; 3 x 10⁻⁶</td>
<td>&gt; 140</td>
</tr>
<tr>
<td>M38A</td>
<td>(1.3 ± 0.2) x 10⁻⁷</td>
<td>6.2</td>
</tr>
<tr>
<td>Y96A</td>
<td>(1.2 ± 0.3) x 10⁻⁶</td>
<td>57</td>
</tr>
<tr>
<td>M98A</td>
<td>(9.5 ± 0.7) x 10⁻⁸</td>
<td>4.4</td>
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²Fold increase in Kₐ relative to the binding of WT C2 to POPC vesicles.