Mapping the Phospholipid-binding Surface and Translocation Determinants of the C2 Domain from Cytosolic Phospholipase A₂*

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Olga Perisic‡, Hugh F. Paterson§, Georgina Mosedale‡, Samuel Lara-González‡, and Roger L. Williams¶

From the ‡Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom and the §Cancer Research Campaign Centre for Cell and Molecular Biology, Chester Beatty Laboratories, Fulham Road, London SW3 6BZ, United Kingdom

Cytosolic phospholipase A₂ (cPLA₂) plays a key role in the generation of arachidonic acid, a precursor of potent inflammatory mediators. Intact cPLA₂ is known to translocate in a calcium-dependent manner from the cytosol to the nuclear envelope and endoplasmic reticulum. We show here that the C2 domain of cPLA₂ alone is sufficient for this calcium-dependent translocation in living cells. We have identified sets of exposed hydrophobic residues in loops known as calcium-binding region (CBR) 1 and CBR3, which surround the C2 domain calcium-binding sites, whose mutation dramatically decreased phospholipid binding in vitro without significantly affecting calcium binding. Mutation of a residue that binds calcium ions (D43N) also eliminated phospholipid binding. The same mutations that prevent phospholipid binding of the isolated C2 domain in vitro abolished the calcium-dependent translocation of cPLA₂ to internal membranes in vivo, suggesting that the membrane targeting is driven largely by direct interactions with the phospholipid bilayer. Using fluorescence quenching by spin-labeled phospholipids for a series of mutants containing a single tryptophan residue at various positions in the cPLA₂ C2 domain, we show that two of the calcium-binding loops, CBR1 and CBR3, penetrate in a calcium-dependent manner into the hydrophobic core of the phospholipid bilayer, establishing an anchor for docking the domain onto the membrane.

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† To whom correspondence should be addressed. Tel.: 44-1223-412171; Fax: 44-1223-412178; E-mail: rlw@mrc-lmb.cam.ac.uk.

‡ The abbreviations used are: cPLA₂, cytosolic phospholipase A₂; cPLA₂-C₂, cytosolic phospholipase A₂ C₂ domain; SytI, synaptotagmin I; SytI-C₂A, synaptotagmin I C₂A domain; CBR, calcium-binding region; GFP, green fluorescent protein; FRET, fluorescence resonance energy transfer; dansyl-PE, N-(5-dimethylaminonaphthalene-1-sulfon-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphohanolamine; PC, phosphatidylcholine; doxyl-PC, 1-palmitoyl-2-stearoyl-(n-doxyl)-sn-glycero-3-phosphoholne.

NMR studies with dodecylphosphocholine micelles have identified several discrete regions in cPLA₂-C₂ that show large chemical shift perturbations in ¹³C heteronuclear single quantum correlation spectra upon micelle binding (20). These regions, which include residues within the three CBRs, residues at the ends of strands immediately adjacent to the CBRs, and

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residues of strands 2 and 3, could thus directly interact with phospholipids or have their conformation changed by phospholipid binding.

In this paper, we have employed a set of green fluorescent protein (GFP) fusions of cPLA₂ deletion variants to show that the C2 domain is both necessary and sufficient for translocation of the enzyme to internal membranes in response to calcium. We have also demonstrated that a set of hydrophobic residues present in the calcium-binding loops at one end of the domain is necessary for Ca²⁺-dependent membrane translocation in vivo and phospholipid binding in vitro. Membrane binding is accompanied by penetration of two loops, CBR1 and CBR3, into the hydrophobic core of the phospholipid bilayer.

**EXPERIMENTAL PROCEDURES**

**GFP-cPLA₂, Fusion Constructs—**Fig. 1 illustrates the various constructs that were employed in our studies. For in vitro translocation experiments, a series of human cPLA₂ constructs fused to the C terminus of GFP were made: full-length cPLA₂ (amino acids 1–749), a variant removing a short flexible N-terminal peptide (amino acids 17–749), a C2 domain-deleted variant (amino acids 148–749), or a C2 domain-only variant (amino acids 17–141). In the context of the full-length cPLA₂ construct, three single or multiple site-specific mutants were designed to examine the effect of mutations of a calcium-binding residue (D43N) and residues proposed to be important for phospholipid binding (M38N/L39A and Y96S/V97S/M98Q). Except for the GFP-cPLA₂-(17–141) fusion, all other GFP-cPLA₂ fusions had a His₆ tag directly fused to the C terminus of the construct. GFP-cPLA₂ fusions were cloned in a vector (26) with a cytomegalovirus immediate-early gene promoter and the GFP protein (preceded by the sequence MVSPVEK) containing several substitutions previously described to enhance fluorescence (S65T) and to increase solubility and thermostability of GFP (V163A/S175G/I167T) (27). All the cPLA₂ constructs except for cPLA₂-(17–141) were cloned as BamH₁/XbaI fragments into the GFP-containing vector cut with the same enzymes (the internal BamH₁ site in the cPLA₂ gene was mutated), and the resulting constructs had three residues (LVS) between the GFP and cPLA₂ sequences. The cPLA₂-(17–141) construct was cloned as a BgLII/EcoRI fragment into the vector, and the final construct had LGSMEKQLSIEELR5 between the GFP and cPLA₂ sequences.

*Escherichia coli Expression and Purification of Wild-type and Mutant cPLA₂, C2 Domains—*A plasmid expressing the C2 domain of cPLA₂ (residues 17–141 of the intact enzyme) was employed. For in vitro binding studies, site-specific mutants of this domain were prepared by polymerase chain reaction-directed mutagenesis and expressed in mini-pRSET, a version of pRSET (Invitrogen) modified to encode a 17-residue N-terminal tag (MRGSHHHHHHHGLVPRGS) containing a His₆ tag for affinity purification and a thrombin cleavage site. For the Trp mutants used for fluorescence quenching studies, the wild-type Trp residue at position 71 was mutated to Phe, and a single Trp residue present in the calcium-binding loops at one end of the C2 domain is necessary for Ca²⁺ binding affinity of recombinant C2 domains (wild type and M38N/L39A and Y96S/V97S/M98Q mutants) was measured by isothermal titration calorimetry with an Omega calorimeter (MicroCal Inc.) using the same conditions as described previously for the GFP-cPLA₂ C2 domain (20). All proteins used for titrations were dialyzed against 20 mM Tris, pH 7. Aliquots (4 μl each) of the protein solution (24 μM) were injected into the protein solution (40 μM) at a rate of 1.34 ml sample per minute at 27 °C. Control experiments were performed in the absence of protein to determine the heats of dilutions, which were subtracted from the apparent heat of binding prior to data analysis. Data were analyzed using the ORIGIN software package provided with the calorimeter.

**Protein Binding to Large Multilamellar Vesicles—**Binding of recombinant C2 domains to large unilamellar vesicles prepared with phosphatidylcholine from brain (Avanti Polar Lipids) was carried out as described previously (21). The reaction mixtures (400 μl) contained 5 μg of C2 domains (final concentration of 0.77 μM), 100 μg of large multilamellar vesicles (final concentration of 330 μM), and 2 mM EDTA or without 2.5 mM CaCl₂. After incubation for 5 min at room temperature, the reaction mixtures were centrifuged for 15 min at 4 °C, and the pellets were analyzed by SDS-polyacrylamide gel electrophoresis.

**Fluorescence Measurements—**Fluorescence measurements were carried out on a Hitachi F-4500 fluorescence spectrometer. The intrinsic fluorescence of Trp-71 that is present in the wild-type C2 domain and D43N, M38N/L39A, and Y96S/V97S/M98Q mutants was measured using an excitation wavelength of 284 nm, and emission scans were taken from 300 to 400 nm.

**Binding of C2 domains to large unilamellar vesicles was quantified by measuring fluorescence resonance energy transfer (FRET), using Tryp-71 as the donor and phosphatidylcholine vesicles containing dansyl-PE (5% mol/mol; Molecular Probes, Inc.) as the acceptor, at 20 °C. Phosphatidylcholine (PC from brain; Avanti Polar Lipids) and dansyl-PE in chloroform were mixed, dried under a stream of nitrogen and the residual chloroform evaporated. The mixture was suspended in buffer F (150 mM NaCl, 20 mM Tris, pH 7.5). Large unilamellar vesicles were prepared by extrusion through a polycarbonate filter (100-nm pore size). Binding reactions contained 0.5 μM C2 domain, 1 mM EDTA plus 2 mM CaCl₂ (or EDTA alone), and various amounts of phospholipids in 2 ml of buffer F. Samples were excited at 284 nm (5-nm slit width), and emission was monitored at 530 nm (10-nm slit width); a 450-nm long-pass filter was placed on the emission side. Relative FRET was calculated as (F₁₅₀ − F₄₄₀)/F₄₄₀, where F₁₅₀ represents the emission of the vesicles and the C2 domain in the presence of saturating levels of calcium, F₄₄₀ represents the emission of the vesicles and the protein in the absence of calcium, and F₄₄₀ represents the maximal energy transfer obtained from the binding curve. The data were analyzed by plotting relative FRET against the total lipid concentration and fitting the data to the binding equation y = n(x/Kd + x), where y represents relative FRET, x is the total lipid concentration, n is a normalization constant, and Kd is the apparent equilibrium dissociation constant.

**Fluorescence Quenching by Doxyl-labeled Phosphatidylcholine—**Large unilamellar vesicles were prepared by extrusion through a polycarbonate filter (100-nm pore size) using an Avanti Polar Lipids extruder. For C2 fusion proteins, the buffer was 100% buffer (or 90% buffer, 10% doxyl-labeled buffer, with a doxyl group at position 7 or 12 of the sn-2-acyl chain) (Avanti Polar Lipids). For Syt1-C2A binding assays, vesicles contained 50% phosphatidylserine and 50% PC (control) or 50% phosphatidylethanolamine, 40% PC, and 10% doxyl-PC. The protein sample (final concentration of 5 μM) was diluted into a 1.6-ml assay containing 250 μM total phospholipid in 20
mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM EDTA in a 3-ml quartz cuvette with a magnetic stirrer. Fluorescence spectra were taken before and after addition of calcium to a final concentration of 3 mM. Samples were excited at 288 nm (2.5-nm slit width), and emission spectra were collected from 300 to 450 nm (5-nm slit width) at 20 °C.

RESULTS

The C2 Domain of cPLA₂ Is Sufficient for Calcium-dependent Translocation in Vivo—To determine if the C2 domain is sufficient for membrane translocation in response to an increase in intracellular Ca²⁺ or whether other features of the intact enzyme are also essential, a series of GFP-cPLA₂ fusions were constructed (Fig. 1), and their translocation was observed in intact cells using confocal fluorescence microscopy. As shown in Fig. 2, the GFP fusion of full-length cPLA₂ (GFP-cPLA₂-(1–749)) translocates from the cytosol to internal membranes in a pattern that is consistent with binding to nuclear membranes, the endoplasmic reticulum, and the Golgi. This is in agreement with previous immunofluorescence studies of cPLA₂ using fixed cells (11–14). Structural studies have shown that the N-terminal 16 residues of the enzyme are flexible and do not contribute to the structural integrity of the N-terminal C2 domain (19, 20). The GFP-cPLA₂-(17–749) construct shows calcium-dependent translocation identical to the full-length enzyme. Using fixed cells, it was previously shown that an N-terminal truncation variant of cPLA₂ (amino acids 178–749) does not translocate in response to calcium (13). Fig. 2 shows that the deletion variant corresponding to removal of the C2 domain only, the GFP-cPLA₂-(148–749) construct, also does not translocate to internal membranes. However, a GFP fusion with only the C2 domain, GFP-cPLA₂-(17–141), is capable of Ca²⁺-dependent membrane translocation in vivo. These results show that the C2 domain consisting of residues 17–141 of cPLA₂ is both necessary and sufficient for Ca²⁺-dependent translocation. Although the C2 domain alone enables translocation, membrane interactions by the remainder of the enzyme including the putative pleckstrin homology domain may be important for the kinetics of translocation or for the fine-tuning of the enzyme activity once on the membrane surface (18, 29).

Mutations in Loops CBR1 and CBR3 Selectively Inhibit Phospholipid Binding by the C2 Domain in Vitro and by the Full-length Enzyme in Vivo—Several mutants were examined in the context of the isolated C2 domain to assess the contributions of various residues to membrane binding. The experimentally determined structure of the cPLA₂ C2 domain served as a guide to select residues that would be likely candidates for participation in membrane interactions. The double mutant M38N/L39A and the triple mutant Y96S/V97S/M98Q were ex-
amine that the wild-type C2 domain penetrates into membranes, whereas residues 49 and 63 remain in the polar region of the membrane. Taken together, the results show that residues in CBR1 and CBR3 insert into the hydrophobic portions of the membrane, whereas residues 49 and 63 remain in a polar environment.

Both Loops CBR1 and CBR3 Become Deeply Immersed in the Hydrophobic Portion of the Membrane—We had previously shown that the cPLA2 C2 domain penetrates into membranes upon binding (28). The change in intrinsic fluorescence upon vesicle binding suggests that CBR1 and CBR3 are interacting with the membrane. To determine the extent to which the domain penetrates into the membrane and its orientation on the membrane, we examined quenching of the intrinsic fluorescence of the Trp mutants by doxyl probes covalently attached at either position 7 or 12 of the membrane, we examined quenching of the intrinsic fluorescence of these mutants, it is possible to ascertain the importance of the exposed hydrophobic residues in CBR1 and CBR3, respectively, for membrane binding. In vitro binding studies were carried out for the isolated C2 domains, whereas the effect of the mutants on in vivo translocation was assayed in the context of the full-length enzyme. All of the C2 domain constructs used for in vitro binding assays were monomers as analyzed by gel filtration (data not shown) and correctly folded as judged by intrinsic fluorescence spectra (Fig. 3). The membrane binding properties of these mutants were compared with those of the D43N mutant, in which the acidic residue shown crystallographically to bind both Ca2+ ions was replaced by a neutral analogue.

The ability of C2 domain mutants to bind Ca2+ was first evaluated using a rapid gel-shift assay, which is based on a large difference in the overall charge between a Ca2+-bound and Ca2+-free form of cPLA2-C2 and, consequently, differential migration on a native gel. Neither the mutations in CBR1 nor CBR3 significantly affected Ca2+ binding at the high concentration of free Ca2+ (0.5 mM) that was used in the Ca2+-dependent gel-shift assay, whereas, as expected, the D43N mutant did not bind Ca2+ (Fig. 4A). The calcium binding affinities of the CBR1 and CBR3 mutants were further examined by isothermal titration calorimetry and compared with those of the wild-type C2 domain. For wild-type cPLA2-C2, Kd of 2.4 μM was determined, in excellent agreement with the Kd of 1.7 μM that was determined previously for a similar cPLA2-C2 construct (residues 1–138) by isothermal titration calorimetry under the same experimental conditions (20). Mutations in CBR1 and CBR3 had only a modest effect on calcium binding affinities, with Kd values of 9.2 and 7.6 μM, respectively. Despite their abilities to bind Ca2+, the CBR1 and CBR3 mutants did not bind PC vesicles at saturating Ca2+ concentrations as shown by both multilamellar large vesicle sedimentation assays (Fig. 4B) and FRET assays (Fig. 4C). Using the FRET analysis, an apparent Kd for PC binding of 11 ± 2 μM was obtained for the wild-type C2 domain, in agreement with previously reported affinities (25, 29). In contrast, none of the mutants showed any measurable FRET at the highest lipid concentration used (250 μM) and 1 mM Ca2+. Higher phospholipid concentrations could not be tested due to an inner filter effect of the lipid vesicles. As a consequence of its impaired Ca2+ binding, the D43N mutant also showed no detectable binding to PC vesicles (Fig. 4, B and C). All three mutations that inhibited in vitro vesicle binding also abolished in vivo membrane translocation of the full-length protein in response to Ca2+ (Fig. 5).

Mapping the Phospholipid-binding Surface by Shifts in Intrinsic Fluorescence Maxima—A series of mutants were constructed to replace single surface-exposed hydrophobic residues (usually phenylalanine) with tryptophan. By monitoring the intrinsic fluorescence of these mutants, it is possible to infer which of these residues enters a more non-polar environment upon vesicle binding. These Trp replacements were constructed in the context of a mutant enzyme in which the only endogenous Trp residue in the wild-type enzyme (Trp-71) was replaced by phenylalanine. The positions at which Trp replacement mutants were created are illustrated in Fig. 6 and include F35W in CBR1, F49W at the end of strand β2, F63W at the beginning of CBR2, and Y96W and V97W in CBR3. In the presence of PC vesicles, the addition of Ca2+ to the CBR1 or CBR3 Trp mutants resulted in a blue shift of the fluorescence spectrum (Fig. 7A and Table I), whereas the wild type and F49W showed no significant change in the emission wavelength maximum, and the F63W mutant had its maximum slightly red-shifted. The large blue shift for the CBR1 (F35W) and CBR3 (Y96W and V97W) Trp mutants indicates that these residues are in an environment that is much more protected from solvent when the domain is bound to vesicles. This would be consistent with these residues being directly involved in vesicle binding. For the wild-type enzyme, the single Trp present (Trp-71) is deeply buried in the core of the domain and shows only a minimal shift upon addition of Ca2+ and PC vesicles. The F49W mutant also showed no blue shift, and F63W showed a slight red shift, suggesting that these residues do not enter a more non-polar environment upon vesicle binding. The maximum emission wavelength of F63W upon vesicle binding is nearly the same as that of free tryptophan, suggesting an extreme solvent exposure. The red shift and the pronounced change in fluorescence intensity displayed by the CBR2 mutant (F63W) may be indicative of conformational changes in the domain or may indicate interaction with the polar region of the membrane. Taken together, the results show that residues in CBR1 and CBR3 insert into the hydrophobic portions of the membrane, whereas residues 49 and 63 remain in a polar environment.
SytI-C2A and found that Phe-234 and, to a lesser extent, Phe-231 (both in CBR3) penetrate into the phospholipid membrane. Fig. 7 and Table I show that in cPLA2, both CBR1 and CBR3 Trp mutants have their fluorescence quenched by the doxyl-PC lipids. In contrast, the wild-type (Trp-71), F49W, and F63W C2 domains were unaffected by the presence of doxyl-PC. Because both 7- and 12-doxyl-PC show equivalent fluorescence quenching, the residues of CBR1 and CBR3 must be immersed so that they are 10–11 Å from both positions 7 and 12 of the sn-2-acyl chain (31). Our results indicating that CBR3 and CBR1 of cPLA2-C2 penetrate into the membrane whereas CBR2 does not are consistent with the character of these loops. In all C2 domains of known structure, there is at least one exposed hydrophobic residue at the tip of CBR3. In cPLA2-C2, CBR1 has a much more hydrophobic character than in other C2 domains. Our results with doxyl-PCs indicate that CBR2 does not penetrate into the membrane, consistent with the red shift observed for the F63W mutant observed upon binding PC vesicles.

**DISCUSSION**

The importance of translocation to membranes as an activating mechanism for various enzymes in cells is becoming increasingly apparent. A variety of ubiquitous protein modules function as mediators of translocation. For example, the role of pleckstrin homology domains in recognizing specific phospholipids and bringing about translocation to membranes has been demonstrated for a range of signaling proteins (reviewed in Ref. 32). Although C2 domains that are present in a great number of proteins have been implicated in membrane binding and vesicle fusion (33, 34), the contribution of these domains to cellular localization has been established for only a few proteins. For example, it has been shown by deletion analysis that the C2 domain is necessary for calcium-dependent transloca-
tion of protein kinase Cγ and Nedd4 to plasma membranes (35, 36) and of cPLA2 to nuclear membranes and the endoplasmic reticulum (13). However, we have demonstrated here that the cPLA2 C2 domain alone is sufficient for calcium-dependent translocation in intact cells. We have furthermore demonstrated that the epitopes of the cPLA2 C2 domain that penetrate into the lipid bilayer are essential for membrane binding.

**Fig. 5.** Mutations of the C2 domain prevent translocation of GFP-cPLA2 in PtK2 cells. Mutants of the full-length GFP-cPLA2 construct (amino acids 1–749) were assayed for translocation in vivo. The left panel for a given mutant illustrates fluorescence of the GFP fusion for a field of transfected cells. The right panel illustrates the same field 3–5 min after addition of 4-bromo-A23187 calcium ionophore at a concentration of 20 μM. All three of the C2 domain mutants abolish translocation.

**Fig. 6.** Tryptophan replacement mutants used for intrinsic fluorescence quenching studies. The schematic illustrates the positions of the exposed hydrophobic residues that were selected for Trp replacement mutants. Each mutation was done in the context of a W71F mutant of the wild-type cPLA2 C2 domain so that each mutant would have a single Trp residue. The Ca2+ sites observed structurally are shown as black spheres. The three loops at one end of the domain that are involved in calcium binding are referred to as CBR1, CBR2, and CBR3 (19, 47). The figure was prepared with BOBSCRIPT (48).

**Fig. 7.** Intrinsic fluorescence spectra of the wild-type and Trp replacement mutants. A, the intrinsic fluorescence (arbitrary units) of the wild-type (WT) and mutant cPLA2 C2 domains with large unilamellar PC vesicles either in the absence (solid lines) or presence (dashed lines) of 1 mM free CaCl2. B, intrinsic fluorescence of the cPLA2 C2 domains with 1 mM free CaCl2 in the presence of large unilamellar vesicles of 100% PC (solid lines), 90% PC and 10% 12-doxyl-PC (dotted lines), or 90% PC and 10% 7-doxyl-PC (dashed lines).

**Table I** Probing membrane binding and penetration of single tryptophan cPLA2 C2 domain mutants by fluorescence measurements

For the cPLA2 C2 domains containing a single tryptophan residue at a different position on the surface of the C2 domain, maximum emission wavelengths corresponding to the spectra presented in Fig. 7 are listed. The fluorescence was measured first in the presence of PC vesicles and 2 mM EDTA and then following addition of 3 mM CaCl2. The integrated values for the fluorescence spectra of the mutants in the presence of calcium and vesicles containing 100% PC were compared with the spectra obtained in the presence of calcium and vesicles composed of 90% PC and 10% 7-doxyl- or 12-doxyl-PC. The percent quenching in the presence of doxyl-labeled PC relative to the 100% PC vesicles is listed.

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<td>EDTA + PC</td>
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<td>Trp-71 (wild type)</td>
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<td>F35W</td>
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and translocation. Other parts of the intact cPLA₂ beyond the C2 domain may modify lipid specificity, calcium sensitivity, and enzyme activity at the target site (18, 29). In particular, the ability of the intact enzyme to bind phosphatidylinositol 4,5-bisphosphate at low calcium concentrations (18, 37) has been attributed to the presence of a pleckstrin homology domain. The binding of the anionic phosphatidylmethanol to the intact enzyme in the absence of calcium, but not to the C2 domain alone (29), also indicates that there are multiple sites of membrane binding on the enzyme. Although our results show that the C2 domain is sufficient for membrane translocation in response to calcium ionophore, other pathways of activation of cPLA₂ may rely on these additional sites of membrane interaction.

Our mutagenesis results show that membrane interaction for the cPLA₂ C2 domain is driven largely by hydrophobic forces. We have demonstrated that the CBR1 and CBR3 mutants, which replace hydrophobic residues with polar ones, M38N/L39A and Y96S/V97S/M98Q, have functional Ca²⁺ binding, but show no detectable binding to phospholipid membranes in vitro or in vivo. Our results are consistent with the observation that cPLA₂ C2 domain binding to PC vesicles is enhanced at high ionic strengths (38). Calcium binding is clearly another critical factor in membrane interaction. The importance of Asp-43, which makes interactions with each of the two Ca²⁺ ions bound to cPLA₂-C2, is confirmed by the loss of membrane binding by the D43N mutant. By preventing Ca²⁺ neutralization of the negatively charged residues, there may be an electrostatic repulsion between the protein and negative charges in the membrane. For the protein kinase CβII C2 domain, it was shown that replacing acidic calcium-binding residues with basic residues did not result in calcium-independent membrane binding (39), suggesting that charge neutralization is not calcium’s only role in membrane binding. Another role for Ca²⁺ ions might be acting as direct ligands of the phosphate groups in the membrane such as seen for annexin V (40).

Given the importance of the hydrophobic residues in CBR1 and CBR3 for membrane binding, we sought to determine whether these residues penetrate into the hydrophobic portions of the lipid bilayer and, if so, to what extent they are immersed in the membrane. The ability of the doxyl labels at positions 7 and 12 of the sn-2-acyl chains to quench fluorescence from residues in CBR3 indicates that this loop is immersed in the

**Fig. 8.** Model of the cPLA₂ C2 domain bound to a phospholipid membrane. A, shown is the chemical structure of spin-labeled phospholipids used in fluorescence quenching experiments (1-palmitoyl-2-stearoyl-(n-doxyl)-sn-glycero-3-phosphocholine) with a doxyl group at position 7 or 12 of the sn-2-acyl chain. Values for the depths of doxyl groups are taken from Ref. 49. B, the crystallographically observed cPLA₂ C2 domain structure was placed onto the crystal structure of a dimyristoyl-PC monolayer (one layer from the bilayer crystal structure is shown) (50). The crystal structure of the dimyristoyl-PC has the lipids arranged in two staggered levels. Positions 7 and 12 of the sn-2-acyl chains in each of the two levels are indicated by red and blue arrows. The domain was positioned so that Trp residues at positions 96, 97, and 35 (rendered as green CPK models) would be immersed into the membrane to approximately equal depths and <10 Å from positions 7 and 12 of the sn-2-acyl chains. The domain was also positioned so that residues 49 and 63 (purple CPK models) were farther than 10 Å from position 7 of the sn-2-acyl chains in the lower of the two staggered PC levels. The solvent-exposed areas of the bound Ca²⁺ ions (cyan) were positioned to form a direct interaction with the phosphate of the phospholipid head group. The figure was prepared with BOBSCRIPT and Raster3D (51).
hydrophobic core of the membrane. The CBR3 loops in SytI-C2A (30) and in protein kinase Cα (41) have also been shown to penetrate into lipid bilayers, suggesting that C2 domains in general may be similarly oriented when bound to membranes. A more surprising result from the doxyl-PC quenching is that CBR1 is immersed in the hydrophobic portion of the membrane to a similar depth as CBR3. Although CBR1 in cPLA2-C2 is longer and more hydrophobic than in most C2 domains, other C2 domains also have hydrophobic residues in CBR1, and it may be that membrane penetration by this loop is a general feature of C2 domain-membrane interaction. For cPLA2, it has been recently shown that calcium-dependent membrane penetration plays a critical role in the enzyme activity and greatly contributes to membrane binding and arachidonate specificity of the enzyme (42).

The critical interaction distance between a tryptophan and a membrane-embedded nitroxide spin-labeled phospholipid at which a quenching can be observed has been estimated to be 10–11 Å (31). This enables us to place Phe-49 (at the end of strand β2) and Phe-63 (at the beginning of CBR2) outside the hydrophobic core of the lipid bilayer. Consistent with this, there is no calcium-dependent blue shift in the emission maximum upon liposome binding for either of these two mutants. Nevertheless, there is a pronounced change in the intensity of fluorescence for residue 63 upon binding PC vesicles. This might upon liposome binding for either of these two mutants. Nevertheless, there is a pronounced change in the intensity of fluorescence for residue 63 upon binding PC vesicles. This could mean that CBR2 is forming interactions with the polar head groups of the membrane or that its environment is changing due to a conformational change in the protein induced by lipid binding. Either of these interpretations would be consistent with the observation that micelle binding by cPLA2-C2 induces NMR chemical shift changes for residues in CBR2 (20) and similar observations for short-chain phosphatidylserine (di-C4-phosphatidylserine) binding to the SytI C2A domain (43).

A model of the cPLA2 C2 domain bound to a lipid membrane is presented in Fig. 8. This model, based on our fluorescence quenching results, requires that Phe-35 in CBR1 and Tyr-96 and Val-97 in CBR3 are immersed to approximately the same depth in the hydrophobic core of the membrane and that Phe-49 and Phe-63 are at a very different level, i.e., outside the hydrophobic region. A further requirement was to place the C2 domain in such a position that the phosphates of the phospholipids could make direct interaction with the exposed coordination sites of the Ca2+ ions in the crevice formed by CBR1 and CBR3. This constraint may be reasonable in light of other protein-phospholipid interactions that have been characterized such as for annexin V, but has no experimental evidence in the context of C2 domains. In the model shown in Fig. 8, residues in CBR2 would lie just at the interface between the solvent and the head group region of the membrane. This arrangement with CBR1 and CBR3 penetrating and CBR2 at the interface is somewhat different from what was proposed by Chae et al. (43) for the SytI C2A domain based on NMR chemical shifts caused by binding to short-chain phosphatidylserine. These workers proposed that a hydrophobic ridge consisting of residues from CBR2 and CBR3 forms the phospholipid-interacting surface of the C2 domain with CBR1 directed away from the membrane. The apparent lack of involvement of CBR1 in membrane binding by SytI-C2A may mean that cPLA2-C2 and SytI-C2A bind membranes very differently, given that we observe CBR1 to penetrate into the membrane to a similar degree as CBR3, but the difference may also be related to the use of soluble short acyl chain phospholipids for the NMR study. A heteronuclear single quantum correlation NMR study of cPLA2-C2 in the presence and absence of dodecylphosphocholine micelles (20) has shown that residues in all three CBRs undergo the greatest changes in 15N/1H chemical shifts.

A feature of the cPLA2 C2 domain that has eluded understanding is its selective translocation to nuclear membranes and the endoplasmic reticulum. This specific translocation results in cPLA2 activity in the vicinity of other enzymes of the eicosanoid pathway and is therefore critical to the role that this enzyme plays in inflammatory responses. Specific translocation may be the consequence of higher local calcium concentration or specific lipid composition at the translocation site, or it may be related to more general features of internal membranes such as membrane fluidity and head group packing. For example, it has been shown that diacylglycerol enhances arachidonic acid release in vivo in a phospholipase C2-activated signaling pathway (44) and cPLA2 membrane penetration and enzyme activity in vitro (42) in a manner consistent with its ability to increase phospholipid head group spacing. Another source of complexity is related to the ability of calcium to induce rapid transbilayer redistribution of membrane phospholipids (45, 46). Specific interactions between residues in the CBRs and the phospholipid head groups have been proposed to account for the differences in affinities of cPLA2-C2 for various phospholipids, but no experimental evidence has been reported to substantiate these models. The relative importance of specific interactions and more general hydrophobic interactions that could be modulated by changes in membrane structure remains to be demonstrated. Only when the relative importance of these interactions is understood will we begin to have an insight into the molecular basis for targeting of cPLA2 to specific membranes.

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REFERENCES
Phospholipid Binding and Translocation of the cPLA₂ C2 Domain