Independent Folding and Ligand Specificity of the C2 Calcium-dependent Lipid Binding Domain of Cytosolic Phospholipase A2

(Received for publication, October 31, 1997)

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The Ca\(^{2+}\)-dependent lipid binding domain of the 85-kDa cytosolic phospholipase A2 (cPLA\(_2\)) is a homolog of C2 domains present in protein kinase C, synaptotagmin, and numerous other proteins involved in signal transduction. NH\(_2\)-terminal fragments of cPLA\(_2\) spanning the C2 domain were expressed as inclusion bodies in Escherichia coli, extracted with solvent to remove phospholipids, and refolded to yield a domain capable of binding phospholipid vesicles in a Ca\(^{2+}\)-dependent manner. Unlike other C2 domains characterized to date, the cPLA\(_2\) C2 domain bound preferentially to vesicles comprised of phosphatidylcholine in response to physiological concentrations of Ca\(^{2+}\). Binding of the cPLA\(_2\) C2 domain to vesicles in the presence of excess Ca\(^{2+}\) chelator was induced by high concentrations of salts that promote hydrophobic interactions. Despite the selective hydrolysis of arachidonoyl-containing phospholipid vesicles by cPLA\(_2\), the cPLA\(_2\) C2 domain did not discriminate among phospholipid vesicles containing saturated or unsaturated sn-2 fatty acyl chains. Moreover, the cPLA\(_2\) C2 domain bound to phospholipid vesicles containing sn-1 and -2 ether linkages and sphingomyelin at Ca\(^{2+}\) concentrations that caused binding to vesicles containing ester linkages, demonstrating that the carbonyl oxygens of the sn-1 and -2 ester linkages are not critical for binding. These results suggest that the cPLA\(_2\) C2 domain interacts primarily with the headgroup of the phospholipid. The cPLA\(_2\) C2 domain displayed selectivity among group IIA cations, preferring Ca\(^{2+}\) approximately 50-fold over Sr\(^{2+}\) and nearly 10,000-fold over Ba\(^{2+}\) for vesicle binding. No binding to vesicles was observed in the presence of greater than 10 mM Mg\(^{2+}\). Such strong selectivity for Ca\(^{2+}\) over Mg\(^{2+}\) reinforces the view that C2 domains link second messenger Ca\(^{2+}\) to signal transduction events at the membrane.

Much of the interest in the 85-kDa cytosolic PLA\(_2\) (cPLA\(_2\)) is in its ability to release arachidonic acid from membranes selectively, thus initiating the biosynthesis of prostaglandins, leukotrienes, and platelet-activating factor (for review, see Ref. 1). Maximal activation of cPLA\(_2\) in intact cells requires phosphorylation by a member of the mitogen-activated protein kinase family on Ser-505 (2–5). However, in the absence of an increase in cytosolic Ca\(^{2+}\), even stoichiometrically phosphorylated cPLA\(_2\) fails to release arachidonic acid because Ca\(^{2+}\) is obligatory for binding to the membrane substrate (3–8). In cells, the increase in Ca\(^{2+}\) results in the selective translocation of cPLA\(_2\) to the membranes of the nuclear envelope and endoplasmic reticulum (9–11), resulting in the colocalization of cPLA\(_2\) with the downstream enzymes responsible for metabolizing arachidonic acid to prostaglandins and leukotrienes. We have shown previously that the domain responsible for the membrane association is encoded at the NH\(_2\) terminus of cPLA\(_2\) (6, 7) and serves to bring a Ca\(^{2+}\)-independent catalytic domain to the membrane substrate in response to increases in second messenger Ca\(^{2+}\). This Ca\(^{2+}\)-dependent lipid binding domain is homologous to the regulatory C2 domain originally described in the classical isoforms of protein kinase C (12) but now recognized to be present in numerous proteins, and it may serve as a paradigm to explain both the features common to these domains as well as those that provide specificity among the domains.

Homologs of the protein kinase C C2 domain have been identified in at least four classes of eukaryotic proteins that carry out critical functions at cellular membranes (1, 6, 13–15). These proteins include lipid-modifying enzymes (e.g. cPLA\(_2\), phosphoinositide-specific phospholipase C (PLC), yeast phosphatidylserine decarboxylase-2, several isoforms of the catalytic subunit of phosphatidylinositol 3-kinase, and a plant phospholipase D), protein kinases (e.g. α, β, γ, δ, ε, η, and θ isoforms of protein kinase C and certain related protein kinase C), GTPase-activating proteins (ras-GTPase-activating protein and its relatives), and regulators of vesicle transport (e.g. synaptotagmin, raphphilin, DOC2, UNC-13, and perforin). Although the functions of the proteins that contain C2 domains are in many cases well defined, the roles that C2 domains play in these proteins are poorly understood. The generalized function of the C2 domain appears to be membrane association; the ligands for C2 domains identified to date comprise various components of cellular membranes, including phospholipids, palmitoyl-sn-glycero-3-phosphoethanolamine; PO-PC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PO-PE, -phosphoethanolamine; PO-PA, -phosphate; PO-PS, -phosphoserine; PG, phospho-rac-(1-glycero); mixed acyl-PI, phosphatidylinositol containing a mixture of acyl chains; PA-PC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; PL-PC, 1-palmitoyl-2-linoyleoyl-sn-glycero-3-phosphocholine; sn-1 and -2 ether-linked phospholipid, 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine; PIPES, 1,4-piperazinediethanesulfonic acid.

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**Phospholipid and Cation Specificity of the cPLA<sub>2</sub> C2 Domain**

inotol polyphosphates, and other membrane-associated proteins (for review in detail, see Ref. 15). Interactions between C2 domains and these ligands are predicted to regulate the various protein-specific biochemical activities. For example, the C2 domain of PLC-δ1 has been proposed to orient or “fix” the catalytic domain of the enzyme to the membranes after it has been “tethered” by the binding of the pleckstrin homology domain to phosphatidylinositol bisphosphate (16).

To study the ligand specificity of the cPLA<sub>2</sub> C2 domain without the effects of lipid binding to the catalytic domain of cPLA<sub>2</sub>, we have expressed the cPLA<sub>2</sub> C2 domain initially as a fusion protein and subsequently as a polypeptide refolded from inclusion bodies free of lipid contamination. This domain binds zwitterionic phospholipid vesicles composed of phosphatidylcholine preferentially over anionic vesicles in the presence of physiological levels of Ca<sup>2+</sup>. This domain is highly selective for Ca<sup>2+</sup> over Sr<sup>2+</sup>, Ba<sup>2+</sup>, and Mg<sup>2+</sup> with respect to vesicle binding. In addition, this domain binds to phospholipid vesicles lacking sn-1 or -2 carbonyl oxygens and is insensitive to changes in the length and degree of saturation of the sn-2 fatty acyl chain, suggesting that the specific interactions with the ligand are limited to the headgroup of the phospholipid. Based on these results, we compare the properties of the cPLA<sub>2</sub> C2 domain with other C2 domains and other proteins that bind phospholipids in a Ca<sup>2+</sup>-dependent manner.

**EXPERIMENTAL PROCEDURES**

**DNA Constructions**—Constructions were carried out using conventional protocols (17) to either generate DNA encoding proteins that could be expressed in mammalian COS or Chinese hamster ovary (CHO) cells or in Escherichia coli either as fusion proteins with glutathione S-transferase (GST) or as independent polypeptides in inclusion bodies. DNA fragments encoding cPLA<sub>2</sub> residues 1–126, 1–138, 1–155, and 1–178 were generated by polymerase chain reaction (PCR) using full-length human cPLA<sub>2</sub> CDNA as a template and antisense primers incorporating a 3'-stop codon and EcoRI/SalI restriction sites. For construction of GST fusion proteins, 5'-PCR sense primers incorporated a BglII restriction site, codons for Gly and Ser residues as part of a thrombin cleavage site, and the first seven codons of cPLA<sub>2</sub> cDNA, PCR fragment generated from these PCRs were subcloned into the BamHI/EcoRI poly linker site of the vector (Pharmacia) (Pha4-3, Pharmacia). Consequently, cPLA<sub>2</sub> polypeptides liberated from GST fusion proteins by cleavage with thrombin contain additional NH<sub>2</sub>-terminal Gly-Ser residues. For construction of cPLA<sub>2</sub> polypeptides produced in inclusion bodies, cDNA that had been modified previously by substitution of several 5'-codons with codons preferentially utilized in bacteria served as template DNA for the PCR; 5'-PCR primers incorporated these silent changes to avoid PCR primers being the same as those used in GST fusion protein constructions. These PCR products were subcloned into the vector (Pha4-3) of the vector (Pha4-3) of the vector (Pha4-3) of the vector (Pha4-3) of the vector (Pha4-3) of the vector (Pha4-3) of the vector (Pha4-3) of the vector (Pha4-3).

**Protein Purification**—Bacteria expressing the appropriate constructs were fermented to late log phase and induced with 1 mM isopropyl β-D-thiogalactopyranoside at 25 °C for GST constructs and 37 °C for inclusion body proteins. For isolation of GST fusion proteins, bacterial cell pellets were lysed by nitrogen cavitation. Supernatant was added to a solution of 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris (pH 7.4), 5 mM dithiothreitol, and 5 mM EDTA. Correctly folded protein was concentrated by vacuum dialysis and passed over a Mono Q 650S column (Toso-Haas) equilibrated with buffer containing 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and eluted in a single step with salt-free buffer. Eluted protein was concentrated by vacuum dialysis and dialyzed against storage buffer. Protein purity was evaluated by SDS-PAGE on 4–20% and 14% Tris-glycine and 10% Tricine gels (18).

**Natural Membrane Binding Assay**—The Ca<sup>2+</sup>-dependent binding of recombinant proteins to natural membranes isolated from CHO cells was determined as described in previous detail (6, 7). In short, at least 2 μg protein, was mixed with CHO cell membranes in the presence of 150 mM NaCl, 20 mM HEPES (pH 7.4), and Ca<sup>2+</sup>/EGTA buffers to maintain the desired free Ca<sup>2+</sup> concentration and incubated for 15 min at 30 °C. After centrifugation at 100,000 × g, equal proportions of supernatant and pellet fractions were subjected to SDS-PAGE on 4–20% Tris-glycine gels. Gels were blotted onto nitrocellulose, and test proteins appearing in supernatant and pellet fractions were detected by immunostaining with cPLA<sub>2</sub>, antisera and visualized by chemiluminescence.

**Synthetic Phospholipid Binding Assays**—The binding of the cPLA<sub>2</sub> C2 domain, which acts as the fluorescence donor, to small unilamellar phospholipid vesicles containing the fluorescent probes dansylphosphatidylethanolamine (dansyl-PE) (Molecular Probes, Eugene, OR), the fluorescence acceptor, was measured as described previously (7) with modification. A total of 25–50 μg of test protein was diluted into a 2-mL solution containing 60 μg of test liposomes (composed of 5%–10% dansyl-PE) in 2 M NaCl, 20 mM HEPES (pH 7.4), and 1 mM EGTA in 3-mL quartz cuvettes. In experiments comparing different divalent cations, binding reactions were carried out in buffer containing 100 mM KCl, 20 mM HEPES (pH 7.4), and 1 mM EDTA. KCl was diluted from a Ca<sup>2+</sup>-free stock solution (Orion Research Incorporated, Boston). Reaction mixtures were stirred continuously, maintained at 20 °C, and illuminated with 284 nm wavelength light; emission of dansyl-PE was recorded at a wavelength of 520 nm. Levels of free divalent cations were raised by the addition of concentrated stocks of cations: for most experiments, a Ca<sup>2+</sup> atomic absorption standard (VHG Laboratories, Manchester, NH) was used. Free Ca<sup>2+</sup> levels were calculated using the Chelator program (19) taking into account the pH of the binding reactions, measured separately. For experiments testing different divalent cations, the chloride salt of the metal ions (Fluka, puriss grade) was used; free cation levels were calculated according to Raafaub (20). The fluorescence emission of dansyl-PE in the presence of EGTA or EDTA (5) was subtracted from that in the presence of added metal ion (I) to determine energy transfer induced by the added metal ion, which was normalized to give (I – I<sub>0</sub>)/I<sub>0</sub> expressed as a percentage. Vesicle compositions were varied according to the experiment. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PO-PC), -phosphatidylethanolamine (PO-PE), -phosphate (PO-PA), -phosphoserine (PO-PS) and -phospho-rac(1-glycerol-PO-PE), -a-phosphatidylinositol (mixed acyl-PI), 1-palmitoyl-2-palmitoyl-sn-glycero-3-phosphocholine (PP-PC), 1-palmitoyl-2-oleoyl-snglycerol-3-phosphocholine (PA-PC), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PL-PC), 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine (as sn-1 and -2 ether-linked phospholipid) and brain sphingomyelin were purchased from Avanti Polar Lipids (Alabalaster, AL).

In experiments testing the effect of salt on vesicle binding, reactions were carried out in buffer containing 20 mM HEPES (pH 7.4) and 5 mM EGTA to determine potential salt effects. Vesicles and protein was reciprocally diluted by a like solution containing concentrated salts to increase the salt concentration and to keep the concentration of vesicles and protein constant. For each salt tested, a parallel series of experiments was carried out using stock solutions free of protein. Fluorescence emission of dansyl-PE in binding reactions free of protein (I<sub>0</sub>) was subtracted from that containing protein (I) to deter-
mine energy transfer due to protein at each salt concentration, which was normalized to give \( I - I_0 \), expressed as a percentage. NaCl, (NH₄)₂SO₄, and NHCl were greater than 99.5% pure (Fluka), and Na₂SO₄ was greater than 99% pure (Aldrich). All salts were specified by manufacturer to contain less than 0.001% Ca²⁺.

Binding of the C2 domain to synthetic vesicles was tested additionally as described above for natural membranes with modification. Synthetic phospholipid vesicles were prepared by brief sonication and collected by centrifugation at 100,000×g. 10 μg of C2 domain was mixed with 150 mM NaCl, 20 mM Tris (pH 7.4), and 5 mM EDTA plus 1 mM CaCl₂ (to maintain 100 μM free Ca²⁺) in 100 μl for 5 min at room temperature. After centrifugation, pellet fractions were washed once with reaction buffer and resuspended in buffer containing 0.2% Triton X-100. Equal proportions of supernatant and pellet fractions were subjected to SDS-PAGE on 15% gels. Gels were stained with Coomassie Brilliant Blue R-250, and the amount of protein was quantified by scanning densitometry. Results were expressed as the ratio of the amount of protein present in the pellet fraction to the total amount in pellet and supernatant fractions. Total phosphate analysis of the resuspended vesicles verified that equivalent amounts of PO-PE, -PA, -PS, and -PG and mixed acyl-PI were present in the assay. The amounts of PO-PE which could be collected by centrifugation were very low; therefore, PO-PE was not used in the experiment.

**Fluorescence Spectroscopy**—A total of 2 μg of the C2 domain, diluted into a 1-ml solution, with or without denaturant, containing 150 mM NaCl, 20 mM Tris (pH 7.4), and 5 mM EDTA, was excited with 280 nm wavelength light in a 1.5-ml quartz cuvette at room temperature. Fluorescence emission wavelengths were scanned from 300 to 400 nm. Excitation and emission slit widths were both 5 nm. Similar emission wavelengths light in a 1.5-ml quartz cuvette at room temperature. Fluorescence emission wavelengths were scanned from 300 to 400 nm.

**RESULTS**

The NH₂-termimal 178 residues of cPLA₂ (cPLA₂(1–178)) contain a C2 domain, a sequence motif that functions as a Ca²⁺-dependent lipid binding domain (6, 7). Previously, cPLA₂(1–178) was expressed as an independent polypeptide in COS cells and bound natural membranes in vitro at Ca²⁺-concentrations that activated enzymatic activity of cPLA₂ (7). In addition, these residues conferred upon a heterologous fusion protein the ability to bind to natural membranes and synthetic phospholipid vesicles in the presence of physiological levels of Ca²⁺ in vitro (7). We sought to define better the minimal residues of the C2 domain. Based on the structure of the first C2 domain of synaptotagmin (21, 22) or coordinate lanthanide ions in PLC-81 (30). Residues highlighted in black denote nonpolar (A, V, M, F, I, L, P, C, or G) or aromatic (F, W, or Y) residues; those highlighted in gray denote polar or charged (R, K, D, E, N, Q, H, W, or Y) residues.

**FIG. 1. Sequence alignment of the cPLA₂ C2 domain.** The sequence of cPLA₂ was aligned with residues from the C2 domains of synaptotagmin I (syna/I) and PLC-61, which are prototypical of type I and type II C2 topologies (15), respectively. The respective secondary structures of these C2 domains are indicated (16, 21). The letter b above the alignment indicates buried residues in synaptotagmin (21). Boxes indicate positions that coordinate Ca²⁺ (dashed box) or coordinate lanthanide ions in PLC-81 (30). Residues highlighted in black denote nonpolar (A, V, M, F, I, L, P, C, or G) or aromatic (F, W, or Y) residues; those highlighted in gray denote polar or charged (R, K, D, E, N, Q, H, W, or Y) residues. Below the alignment is a “consensus” sequence for C2 domains which records identical residues present in at least 50% of C2 domains examined (15). Dashes indicate gaps introduced to maximize the alignment. GenBank™ accession numbers are: M72383 (human cPLA₂), X52772 (rat synaptotagmin I), and M20637 (rat PLC-81).

**FIG. 2. Reversible Ca²⁺-dependent binding of the cPLA₂ C2 domain to phospholipid vesicles.** cPLA₂(1–138) (circles) and cPLA₂(1–155) (squares) liberated from GST fusion proteins were incubated with phosphatidylcholine vesicles (90% PP-PC and 10% dansyl-PE) and Ca²⁺/EGTA buffers to maintain free Ca²⁺. The change in vesicle fluorescence caused by protein binding (\( I - I_0 \)) was normalized to the starting fluorescence of the vesicle/protein mixture in the presence of 1 mM EGTA (U), and was plotted as a function of free Ca²⁺. Fluorescence intensities returned to base line upon addition of excess EGTA (data not shown), demonstrating that the binding is reversible.

Val-138, two exon boundaries, to ascertain whether they demarcated minimal functional boundaries. cPLA₂ was also truncated in a polar stretch of the polypeptide at Lys-155 to test the importance of residues between 138 and 178. These polypeptides were expressed in COS or CHO cells as independently expressed polypeptides, or they were expressed in E. coli as fusion partners of GST or as inclusion body proteins that could be refolded.

Recombinant fragments containing, at a minimum, residues 1-155, were expressed in both COS and CHO cells as soluble polypeptides that bind cellular membranes in a Ca²⁺-dependent manner (data not shown). In contrast, both cPLA₂(1–126) and (1–138) were expressed as insoluble polypeptides in COS cells. We turned to expression of recombinant protein in E. coli, reasoning that expression of cPLA₂(1–126) and (1–138) in bacteria grown at lower temperatures in fusion to a highly soluble partner (GST) might overcome the solubility problems associated with protein overexpression in COS cells cultured at 37°C. Indeed, recombinant fragments containing, at a minimum, residues 1–126 attached to the COOH terminus of GST were expressed as soluble fusion proteins in E. coli. However, GST-cPLA₂(1–126) was considerably more insoluble than the other three fusion proteins; the little soluble cPLA₂(1–126) that could be liberated from GST-cPLA₂(1–126) bound in a Ca²⁺-independent manner to cellular membranes (data not shown). GST-cPLA₂(1–138), (1–155), and (1–178) were finally functional, binding in a Ca²⁺-dependent manner in vitro to cellular mem-
brane (data not shown). When liberated from GST-fusion proteins by thrombin cleavage and purified, cPLA₂(1–138) and (1–155) reversibly bound to phosphatidylcholine vesicles (Fig. 2) and cellular membranes (data not shown) at low micromolar Ca²⁺ levels. The additional Gly-Ser residues attached to the NH₂ terminus of cPLA₂(1–138) and (1–155) to create a thrombin cleavage site (see “Experimental Procedures”) thus do not hamper binding to phospholipid vesicles. Although cPLA₂(1–138) represented the minimal, fully functional C2 domain, we chose to characterize cPLA₂(1–155) initially because of its higher yields.

**FIG. 3.** Reversible denaturation of the cPLA₂ C2 domain monitored by its intrinsic fluorescence. Intrinsic fluorescence of cPLA₂(1–155) liberated from GST fusion protein was monitored in the presence of chaotrophic agents. In panel A emission spectra (excitation at 280 nm) of protein diluted in buffer or 8 mM urea after buffer subtraction were recorded. Similar results were obtained when tryptophan was excited selectively at 294 nm. In panel B, wavelength maxima of cPLA₂(1–155) diluted into increasing concentrations of urea (circles) or GndHCl (squares) were recorded. In panel C are the wavelength maxima of cPLA₂(1–155) initially diluted into 10 mM urea (circles) or 6 mM GndHCl (squares) and subsequently diluted serially into buffer without denaturant to give the indicated concentration of denaturant.

cPLA₂(1–155) contains several aromatic residues, including a single tryptophan, Trp-71, which serves as a useful spectroscopic probe. Excitation of cPLA₂(1–155) at 280 nm resulted in a strong fluorescence emission wavelength maximum at approximately 325 nm (Fig. 3A), which is characteristic of tryptophan buried in a relatively nonpolar environment (23). When diluted into high concentrations of urea or GndHCl, this fluorescence wavelength maximum red-shifted to approximately 345 nm, characteristic of tryptophan exposure to polar solvent. This indicates that Trp-71 of native cPLA₂ is buried in a relatively nonpolar environment and that upon denaturation it becomes exposed to solvent. The spectroscopic properties of Trp-71 in cPLA₂ are consistent with the location of the homologous residues Phe-206 of synaptotagmin I (21) and Trp-684 of PLC-δ1 (16), which are buried in the β₆- and β₄-strands of type I and II C2 domains, respectively (see Fig. 1). Titration of cPLA₂(1–155) with urea or GndHCl revealed a single major transition in unfolding (Fig. 3B). When cPLA₂(1–155) was first denatured in urea or GndHCl and then serially diluted into buffer without denaturant, the fluorescence wavelength maximum blue-shifted back to approximately 325 nm (Fig. 3C), revealing that denaturation by these chaotropic agents is reversible. Further, cPLA₂(1–155), which first had been subjected to urea or GndHCl concentrations that denatured the protein then was refolded by 100-fold dilution into buffer without denaturant, exhibited nearly the same Ca²⁺ dependence for binding to phosphatidylcholine vesicles as native cPLA₂(1–155) (Fig. 4). However, it appeared that denaturation and refolding under these conditions reduced the yield of the protein (see Fig. 4, inset).

Given the ability of cPLA₂(1–155) derived from GST fusion protein to refold into functional protein, cPLA₂(1–126), (1–138), (1–155), and (1–178) were expressed in E. coli inclusion bodies, extracted with a chloroform and methanol mixture (2:1) to remove endogenous lipids, purified, and refolded. Partially purified, refolded cPLA₂(1–126) displayed a fluorescence maximum near 331 nm and failed to bind vesicles in response to micromolar levels of Ca²⁺ (data not shown), suggesting that it had either refolded improperly or that a critical functional
EGTA, fluorescence intensities obtained for PO-PC and PO-PE reversible binding of cPLA2(1–138) to vesicles containing phosphatidylcholine to a variety of synthetic vesicles was measured. Reversed ethanolamine followed the same Ca$^{2+}$ dependence as PO-PA (Fig. 5B) and PO-PE (Fig. 5A). This figure shows vesicles composed of PO-PA (circles) and PO-PE (squares). Panel B shows vesicles composed of PO-PA (circles), PO-PS (squares), PO-PG (triangles), and mixed acyl-PI (diamonds). Upon addition of excess EGTA, fluorescence intensities obtained for PO-PC and PO-PE returned to within <1% of $I_0$ for PO-PA fluorescence returned to within 18% of $I_0$ (data not shown). Results are averages of three independent experiments ($\pm$ S.D.).

FIG. 5. Reversible Ca$^{2+}$-dependent binding of cPLA$_2$ C2 domain to vesicles containing select headgroups. Binding of cPLA$_2$(1–138), which was refolded from E. coli inclusion bodies, to vesicles composed of 5% dansyl-PE and 95% zwitterionic or acidic phospholipids as a function of free Ca$^{2+}$ was measured. Tested in panel A are vesicles composed of PO-PC (circles) and PO-PE (squares). Panel B shows vesicles composed of PO-PA (circles), PO-PS (squares), PO-PG (triangles), and mixed acyl-PI (diamonds). Upon addition of excess EGTA, fluorescence intensities obtained for PO-PC and PO-PE returned to within <1% of $I_0$ for PO-PA fluorescence returned to within 18% of $I_0$ (data not shown). Results are averages of three independent experiments ($\pm$ S.D.).

FIG. 6. Direct Ca$^{2+}$-dependent binding of cPLA$_2$ C2 domain to vesicles containing select headgroups. Binding of cPLA$_2$(1–138) to pure vesicles composed of the indicated phospholipid vesicles (PO-PC, PO-PA, PO-PS, PO-PG, and mixed acyl-PI) was measured in the presence of EDTA (1 mM; open bars) or 100 $\mu$m free Ca$^{2+}$ (1 mM CaCl$_2$; filled bars). The percentage of total protein that bound to vesicles after centrifugation was determined by scanning densitometry of supernatant and pellet fractions subjected to SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. Results are averages of three independent experiments ($\pm$ S.D.).

FIG. 7. Reversible Ca$^{2+}$-independent binding of the cPLA$_2$ C2 domain to vesicles induced by salts. Binding of cPLA$_2$(1–138) to phosphatidylcholine vesicles (95% PO-PC and 5% dansyl-PE) as a function of Na$_2$SO$_4$ (circles), (NH$_4$)$_2$SO$_4$ (squares), NaCl (triangles), and NH$_4$Cl (diamonds) was measured in the presence of 5 mM EGTA. The increase in vesicle fluorescence caused by protein binding ($I - I_0$) was normalized to vesicle fluorescence in the absence of protein ($I_0$). Results are averages of three independent experiments ($\pm$ S.D.).

To ascertain if the cPLA$_2$ C2 domain interacts directly with the phospholipid headgroup, the binding of the cPLA$_2$ C2 domain to a variety of synthetic vesicles was measured. Reversible binding of cPLA$_2$(1–138) to vesicles containing phosphatidylethanolamine followed the same Ca$^{2+}$ dependence as binding to phosphatidylcholine; however, the maximal Ca$^{2+}$-induced fluorescence transfer observed (approximately 10%) was much lower than that observed for phosphatidylcholine (approximately 80%). Because the maximal signal observed for phosphatidylethanolamine could not be increased by increasing the concentration of vesicles (data not shown), the lower maximal signal observed for phosphatidylethanolamine vesicles was likely the result of a lower efficiency of fluorescence resonance energy transfer rather than a lower affinity of C2 domain binding. Differences in the ability of dansyl-PE to pack into the two types of vesicles might account for differences in their ability to accept fluorescence energy from bound proteins. cPLA$_2$(1–138) also bound to vesicles containing phosphatidic acid (Fig. 5B); however, approximately 10-fold greater free Ca$^{2+}$ was required for half-maximal binding to these vesicles compared with phosphatidylcholine and phosphatidylethanolamine. In addition, because not all of the binding to vesicles containing phosphatidic acid was reversed by EGTA treatment (see legend to Fig. 5), a fraction of the binding observed to these vesicles was Ca$^{2+}$-independent. No significant Ca$^{2+}$-dependent binding of cPLA$_2$(1–138) to vesicles containing phosphatidylserine, phosphatidylglycerol, or phosphatidylinositol was observed (Fig. 5B), even at higher vesicle concentrations (data not shown). Thus, the cPLA$_2$ C2 domain displays dramatic selectivity for zwitterionic phospholipid headgroups in the presence of physiological levels of Ca$^{2+}$.

The phospholipid headgroup selectivity of the cPLA$_2$ C2 domain was confirmed further using a direct binding assay (Fig. 6). In this assay, the cPLA$_2$ C2 domain complexed to phospholipid vesicles was isolated by centrifugation, subjected to SDS-PAGE, and then quantified by staining with Coomassie Brilliant Blue.
liant Blue and densitometry. Synthetic vesicles composed of phosphatidylcholine, phosphatidic acid, phosphatidylycerine, phosphatidylglycerol, and phosphatidylinositol were tested for their ability to bind to the C2 domain in the presence or absence of Ca$^{2+}$. We could not test phosphatidylethanolamine vesicles in this manner because PO-PE vesicles could not be collected in comparable yields by centrifugation (see "Experimental Procedures"). As observed by fluorescence resonance energy transfer, the cPLA$_2$ C2 domain bound preferentially to phosphatidylcholine vesicles in a Ca$^{2+}$-dependent manner. In addition, Ca$^{2+}$-independent binding to phosphatidic acid vesicles and little or no Ca$^{2+}$-dependent binding to vesicles composed of phosphatidyletherine, phosphatidylglycerol, and phosphatidylinositol were observed.

Because molar concentrations of neutral salts have been shown to promote cPLA$_2$ activity in vitro (24, 25), the ability of several salts to promote vesicle binding by the cPLA$_2$ C2 domain was investigated. High concentrations of Na$_2$SO$_4$ promoted reversible Ca$^{2+}$-independent binding of cPLA$_2$(1–138) to vesicles containing phosphatidylcholine (Fig. 7). Half-maximal binding was observed above 1 M Na$_2$SO$_4$. High concentrations of (NH$_4$)$_2$SO$_4$ (approximately 2 M) and NaCl (above 3 M) also promoted binding. At greater than 4 M NH$_4$Cl no binding of cPLA$_2$(1–138) was observed. Ca$^{2+}$-independent binding of the cPLA$_2$ C2 domain to vesicles elicited by these salts did not depend as dramatically on the identity of headgroup of the phospholipid, in contrast to Ca$^{2+}$-dependent binding (Figs. 5 and 6), since we observed salt-induced binding to vesicles containing phosphatidylethanolamine, phosphatidic acid, phosphatidylycerine, phosphatidylglycerol, and phosphatidylinositol (data not shown). These results demonstrate that phospholipid vesicles containing phosphatidyletherine, phosphatidylglycerol, and phosphatidylinositol are capable of acting as fluorescence acceptors for cPLA$_2$(1–138) and serve as controls for the Ca$^{2+}$-induced vesicle binding assay described above. However, vesicles containing phosphatidylethanolamine displayed significantly better binding at lower salt concentrations and greater efficiency of fluorescence energy transfer at saturating salt concentrations compared with vesicles composed of these other phospholipids.

Since cPLA$_2$ cleaves the unsaturated arachidonoyl sn-2 chain (20:4) at least 20-fold more efficiently than the shorter saturated palmitoyl chain (16:0) (6, 26, 27), the dependence of C2 domain activity on length and degree of saturation of the fatty acyl chain was tested. In contrast to the headgroup selectivity noted above, cPLA$_2$(1–138) reversibly bound to phosphatidylcholine vesicles containing sn-2 oleoyl (18:1), linoleoyl (18:2), arachidonoyl (20:4), and palmitoyl (16:0) chains, exhibiting similar Ca$^{2+}$ dependences (Fig. 8). The weaker maximal fluorescence signals obtained for PA-PC and PP-PC were likely caused by a lower efficiency of energy transfer rather than a lower affinity for binding, since an increase in vesicle concentration had no effect (data not shown). Therefore, the cPLA$_2$ C2 domain does not display selectivity for the structure of the sn-2 acyl chain in Ca$^{2+}$-dependent binding to phosphatidylcholine.

Given that the ternary Ca$^{2+}$-phospholipid-protein complex observed in crystal structures of the low molecular weight secreted by PLA$_2$ show a direct interaction between the sn-2 carbonyl and Ca$^{2+}$ (28, 29), the effect of altering the linkages between the glycerol backbone and the fatty acyl chain was probed. cPLA$_2$(1–138) reversibly bound in a Ca$^{2+}$-dependent manner to vesicles composed of brain sphingomyelin (Fig. 9), which contains an amide linkage at the sn-1 position and a free hydroxyl at the sn-1 position. cPLA$_2$(1–138) also bound reversibly in a Ca$^{2+}$-dependent manner to vesicles composed of phospholipids containing ether linkages in place of ester linkages at the sn-1 and -2 position (Fig. 9). The same Ca$^{2+}$ dependence was observed for the binding of cPLA$_2$(1–138) to vesicles composed of sphingomyelin or glycerophospholipids bearing ester or ether linkages; however, the maximal signal obtained for sphingomyelin was not as great as that for the ester- or ether-linked phospholipids, possibly because of lower energy transfer efficiency. Thus, the sn-1 and sn-2 carbonyl oxygens are not critical for Ca$^{2+}$-dependent binding of the cPLA$_2$ C2 domain to phospholipid vesicles.

To ascertain the cation selectivity of the cPLA$_2$ C2 domain, vesicle binding was tested in the presence of variousivalent metals. cPLA$_2$(1–138) bound to phosphatidylethanolamine vesicles in the presence of Ca$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$; this binding was reversible upon cation chelation (Fig. 10). However, approximately 50-fold greater concentration of Sr$^{2+}$ and nearly 10,000-fold greater Ba$^{2+}$ over Ca$^{2+}$ was required for half-maximal binding (Fig. 10). No significant binding to vesicles was observed in the presence of greater than 10 mM Mg$^{2+}$. Therefore, the cPLA$_2$ C2 domain displays at least 10,000-fold selectivity for Ca$^{2+}$ over the other physiological cation Mg$^{2+}$ in binding to phosphatidylethanolamine.
FIG. 10. Reversible binding of the cPLA₂ C2 domain to vesicles induced by select divalent cations. Binding of cPLA₂(1–138) to phosphatidylcholine vesicles (95% PO-PC and 5% dansyl-PE) as a function of free Ca²⁺ (circles), Sr²⁺ (squares), Ba²⁺ (triangles), or Mg²⁺ (diamonds) was measured. Free metal levels were maintained using metal salts/EDTA buffers and were increased by the addition of concentrated stocks of metal salts. Upon addition of excess EDTA, fluorescence intensities obtained in Ca²⁺ and Sr²⁺ titrations returned to within <2% of I₀ for Ba²⁺ titrations, they returned to within <7% of I₀ (data not shown). Results are averages of three independent experiments (±S.D.).

DISCUSSION

In this report we characterize cPLA₂(1–138) as a minimal NH₂-terminal fragment of cPLA₂ that functions as a Ca²⁺-dependent lipid binding domain. Interestingly, Val-138 corresponds to the end of a short exon that begins with Val-127 (1, 6). Shortening this domain to the next nearest COOH-terminal exon boundary (after Gln-126) produced a polypeptide that is likely to be folded improperly when expressed in E. coli for the following reasons: (a) a greater fraction of GST-cPLA₂(1–126) was expressed as insoluble material in E. coli compared with other GST-C2 domain fusion proteins; (b) when liberated from the limited GST fusion protein that could be isolated, cPLA₂(1–126) bound CHO cell membranes in a Ca²⁺-independent manner; (c) cPLA₂(1–126) refolded from inclusion bodies failed to bind to vesicles in response to Ca²⁺ or high concentrations of Na₂SO₄ (data not shown). These results support the hypothesis that the cPLA₂ C2 domain forms the alternative type II C2 domain topology represented by PLC-δ1 (16, 30), which was predicted by sequence alignment (15).

The metal-dependent phospholipid binding of the cPLA₂ C2 domain displays selectivity among group IA cations in the order of preference Ca²⁺ > Sr²⁺ > Ba²⁺ > Mg²⁺. Notably, the same preference was observed for hydrolysis of phospholipid vesicles by the full-length cPLA₂ (24, 31). The pattern of metal-selective vesicle binding by the cPLA₂ C2 domain is identical to that observed for the binding of the first C2 domains of synaptotagmin to phosphatidylserine vesicles (32, 33). The cation selectivity of raphphilin has not been reported in detail; however, under the assay conditions reported, Ca²⁺, but not Ba²⁺, Sr²⁺, or Mg²⁺, promoted phospholipid binding (34). Because Ca²⁺ is the sole metal capable of promoting membrane binding at physiological concentrations, these C2 domains must possess metal ion binding sites optimized for divalent ions the size of Ca²⁺, as has been observed in other Ca²⁺ binding sites that exclude Mg²⁺ (35). As observed for phospholipid binding by the synaptotagmin C2 domain (32, 33, 36), vesicle binding by the cPLA₂ C2 domain induced by Ca²⁺ is positively cooperative, suggesting the presence of multiple Ca²⁺ ions in the phospholipid-Ca²⁺-C2 domain ternary complex. This finding is consistent with NMR and crystallographic studies of the C2 domains of synaptotagmin and PLC-δ1, which support the presence of multiple Ca²⁺ binding sites (16, 20, 21, 30).

The phospholipid preference of the cPLA₂ C2 domain is distinct from that described for isolated C2 domains of other proteins characterized to date. Whereas C2 domains of most synaptotagmins, raphphilin, DOC2 (and full-length protein kinase C-β) require the presence of anionic but not zwitterionic phospholipid in vesicles for binding (7, 32, 34, 36–38), at physiological ionic strength and Ca²⁺, the cPLA₂ C2 domain binds preferentially to vesicles comprised of phosphatidylcholine. It is worth noting that the residues separating the aspartates in the second Ca²⁺ binding loop (the loop between strands β6 and β7 in type I C2 domains) of synaptotagmin, raphphilin, DOC2, and protein kinase C-β contain positively charged residues, whereas the corresponding short stretch in cPLA₂ (the loop between strands β5 and β6 in type II C2 domains) contains hydrophobic residues (see Fig. 1). In addition, in place of the histidine immediately preceding the last Ca²⁺ coordinating residue in synaptotagmin, the C2 domain of cPLA₂ contains an aspartate. Perhaps the choline headgroup interacts with these acidic and hydrophobic residues in this loop of cPLA₂, but is repelled by the basic residues of C2 domains which interact with anionic vesicles. Interestingly, the C2 domain of cPLA₂ binds to vesicles containing the small anionic headgroup phosphate of phosphatidic acid to some extent at high Ca²⁺ concentrations.

To date, little information has been published addressing the critical interactions between the phospholipid and C2 domains. Because we were able to refold the cPLA₂ C2 domain from inclusion bodies, we had the opportunity to extract first any endogenous lipids that might have compromised our studies. The preference of the cPLA₂ C2 domain for the neutral headgroup phosphocholine contrasts sharply with the headgroup preference at the active site of cPLA₂, where less than 4-fold selectivity has been observed among phosphocholine, phosphoethanolamine, phosphate, phosphoserine, and phosphoinositol, despite their large structural differences (26, 27). Because phosphatidyl ethanol was used to trap cPLA₂ in these experiments, it is noteworthy that we have observed Ca²⁺-dependent binding of the cPLA₂ C2 domain to vesicles containing this nonphysiological headgroup (data not shown).

The lack of selectivity for the sn-2 acyl chain in vesicle binding by the cPLA₂ C2 domain is strikingly different from the selectivity observed in liposome hydrolysis by the full-length enzyme. The CPLA₂ C2 domain does not distinguish among acyl chains in the sn-2 position, whereas the catalytic center prefers the unsaturated arachidonoyl (20:4) chain by 20–40-fold over the saturated palmitoyl (18:0) chain (6, 26, 27, 39–43).

We have also demonstrated that vesicle binding by the cPLA₂ C2 domain does not require the carbonyl oxygens of the ester linkage at either the sn-1 or -2 positions by showing equivalent binding to either natural diacylphosphatidylcholine or synthetic phosphatidylcholine containing ether linkages at the sn-1 and -2 positions. We also showed that the cPLA₂ C2 domain binds to sphingomyelin, which contains an amide linkage at the sn-2-like position. Together these results indicate that the C2 domain does not coordinate Ca²⁺ via the sn-2 carbonyl oxygen. Similarly, acyl linkages are not required for the binding of annexin V to phospholipid vesicles in the presence of Ca²⁺; in the crystal structure of annexin V bound to Ca²⁺ and a phospholipid analog, Ca²⁺ was coordinated via an sn-3 phosphoryl oxygen (44). In contrast, the sn-2 carbonyl oxygen (in conjunction with an sn-3 phosphoryl oxygen) coordinates Ca²⁺ when bound to the structurally and mechanistically dissimilar secreted PLα₂ (28, 29).

Our results indicate that hydrolysis of phospholipid vesicles by cPLA₂ induced by high salt concentrations in the absence of Ca²⁺ as observed previously (24, 25) is likely due to salt-
induced Ca\(^{2+}\)-independent phospholipid binding by its C2 domain and Ca\(^{2+}\)-independent hydrolysis of the substrate. We observed previously that a recombinant cPLA\(_2\), lacking the C2 domain failed to hydrolyze phospholipid vesicles in the presence of high concentrations of salt, even though this protein hydrolyzed monomeric substrates at wild type rates in the absence of Ca\(^{2+}\) (7). We show here that the C2 domain of cPLA\(_2\) binds to phospholipid vesicles in the absence of Ca\(^{2+}\) when subjected to high concentrations of select salts. The extreme steepness of the binding curves as a function of salt concentration and potency in the molar range suggest that salt-induced phospholipid binding results from the effects of multiple ionic interactions with the protein or solvent (or vesicle) rather than substitution of a cation for Ca\(^{2+}\) in the metal binding site. These salts are likely to stabilize a hydrophobic interface of the C2 domain because the relative potency of these ions (Na\(^{+} > NH_4^{+} > SO_4^{2-} > Cl^-\)) follows exactly the Hofmeister series of cations and anions that promote hydrophobic interactions (45). This hydrophobic interface may also be stabilized by Ca\(^{2+}\) binding to the C2 domain and might interact directly with a hydrophobic portion of the phospholipid vesicle or form an allosteric switch to induce vesicle binding elsewhere in the C2 domain. C2 domains that bind phospholipids in a Ca\(^{2+}\)-independent manner have evolved to stabilize this interface constitutively.

Our experiments have not addressed the reason why full-length cPLA\(_2\) translocates selectively to the nuclear and endoplasmic reticulum membranes over plasma membranes (9–11, 46) or even if this preference is encoded within the C2 domain. The preference may be caused by interaction of cPLA\(_2\) with a docking protein localized to these membranes, or it may be the result of differences in the composition of membranes. For example, the C2 domain may translocate to these membranes selectively over the plasma membrane because the latter contains high levels of sphingomyelin and cholesterol, both of which promote order and tight packing of phospholipids. Nevertheless, the cPLA\(_2\) C2 domain was shown to bind with high affinity to the vesicles composed of sphingomyelin. Similarly, in experiments not shown, we have observed that high levels of cholesterol also do not block binding to the membrane. Interestingly, sphingomyelin has been reported to inhibit a partially purified cPLA\(_2\) (47). This result, together with the finding that full-length cPLA\(_2\) binds more tightly to membranes containing products of phospholipid hydrolysis (31), suggests that sphingomyelin content may influence binding, not by blocking the initial binding event but by inhibiting the PLA\(_2\) reaction that promotes increased binding.

In conclusion, we have demonstrated that the first 138 amino acids of cPLA\(_2\), ending at an exon border, constitute a minimal NH\(_2\)-terminal fragment of cPLA\(_2\) which contains a fully active C2 domain. This finding suggests that the cPLA\(_2\) C2 domain possesses a topology similar to the C2 domain of PLC-\(\delta\)-1 rather than that of the first synaptotagmin C2 domain. By observing a selectivity among headgroups and yet no preference in the fatty acyl chains or the linkages to the glycerol backbone, we have also determined the portions of the phospholipid which are likely to intimately contact the C2 domain. Finally, we have demonstrated that Ca\(^{2+}\) is the only divalent metal that promotes binding at physiological concentrations, thus reinforcing the concept that the cPLA\(_2\) C2 domain and the Ca\(^{2+}\)-dependent C2 domains in general link second messenger Ca\(^{2+}\) to protein function.

Acknowledgments—We thank Dale Cumming for advice, Neil Schauer for large scale bacterial fermentation, Elliott Nickbarg for mass spectroscopic analysis, and John Knopf for continuing support and advice.