A Structure-Function Study of the C2 Domain of Cytosolic Phospholipase A2

IDENTIFICATION OF ESSENTIAL CALCIUM LIGANDS AND HYDROPHOBIC MEMBRANE BINDING RESIDUES*

(Received for publication, November 25, 1998, and in revised form, January 11, 1999)

Lenka Bittova†, Marius Sumandea‡, and Wonhwa Cho§

From the Department of Chemistry, University of Illinois, Chicago, Illinois 60607-7061

The C2 domain of cytosolic phospholipase A₂ (cPLA₂) is involved in the Ca²⁺-dependent membrane binding of this protein. To identify protein residues in the C2 domain of cPLA₂ essential for its Ca²⁺ and membrane binding, we selectively mutated Ca²⁺ ligands and putative membrane-binding residues of cPLA₂ and measured the effects of mutations on its enzyme activity, membrane binding affinity, and monolayer penetration. The mutations of five Ca²⁺ ligands (D40N, D43N, N65A, D93N, N95A) show differential effects on the membrane binding and activation of cPLA₂, indicating that two calcium ions bound to the C2 domain have differential roles. The mutations of hydrophobic residues (F35A, M98A, L99A, Y96A, Y97A, M98A) in the calcium binding loops show that the membrane binding of cPLA₂ is largely driven by hydrophobic interactions resulting from the penetration of these residues into the hydrophobic core of the membrane. Leu¹⁹³ and Val¹⁹⁷ are fully inserted into the membrane, whereas Phe³⁵ and Tyr³⁶ are partially inserted. Finally, the mutations of four cationic residues in a β-strand (R57E/K58E/R59E/R61E) have modest and negligible effects on the binding of cPLA₂ to zwitterionic and anionic membranes, respectively, indicating that they are not directly involved in membrane binding. In conjunction with our previous study on the C2 domain of protein kinase C-α (Medkova, M., and Cho, W. (1998) J. Biol. Chem. 273, 17544–17552), these results demonstrate that C2 domains are not only a membrane docking unit but also a module that triggers membrane penetration of protein and that individual Ca²⁺ ions bound to the calcium binding loops play differential roles in the membrane binding and activation of their parent proteins.

Phospholipases A₂ (PLA₂; EC 3.1.1.4) are a large family of lipolytic enzymes that catalyze the hydrolysis of the fatty acid ester at the sn-2-position of phospholipids (1, 2). Among various mammalian PLA₂s, 85-kDa cytosolic PLA₂ (cPLA₂) can selectively liberate arachidonic acid from membrane phospholipids, which can be converted to potent inflammatory lipid mediators, prostaglandins, thromboxanes, leukotrienes, and lipoxins, collectively known as eicosanoids (3). Recent genetic studies showed that the deletion of the cPLA₂ gene results in loss of lipid mediator biosynthesis (4, 5). cPLA₂ is therefore an attractive target for developing specific inhibitors that can be used as novel anti-inflammatory drugs. cPLA₂ is translocated to endoplasmic reticulum membranes and nuclear envelopes in response to a rise in intracellular Ca²⁺ (6, 7). This membrane translocation of cPLA₂ is mediated by its amino-terminal C2 domain, which contains calcium and membrane binding sites (8, 9). However, the mechanism by which the C2 domain of cPLA₂ drives its Ca²⁺-dependent membrane targeting is not fully understood.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-Di-O-hexadecyl-sn-glycero-3-phosphocholine (DHPC) and 1,2-sn-dioleoylglycerol were from Sigma. All lipids were used without further purification. 1,2-Di-O-hexadecyl-sn-glycero-3-phosphoglycerol (DHPG) was prepared from DHPC by phospholipase D-catalyzed transphosphatidylation as described by Comfurius and Zwaal (17). Trinitiated POPC (1³¹⁴C)POPC was prepared from 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine and [9,10-³³C]Oleic acid (American Radiolabeled Chemicals) using rat liver microsomes as described (18, 19). Phospholipid concentrations were determined by phosphate analysis (20). 1-Stearyl-2-(1³¹⁴C)arachidonoyl-sn-glycero-3-phosphocholine ([1³¹⁴C]SAPC) (55 mCi/mmol) was from Amersham Pharmacia Biotech. Styrene-divinylben-
Assay mixtures contained small unilamellar vesicles of [14C]SAPC (10 μM), 16 μM BSA, 0.1 mM KCl, and varying concentrations of CaCl2 in 60 μM of 20 mM HEPES, pH 8.0. Free calcium concentration was adjusted using a mixture of EGTA and CaCl2 according to the method of Bers (25). 12 mM of 1,2-sn-dioleoylphosphatidylcholine was added to [14C]-SAPC vesicles, and then the free end of Scl activity assay in vesicle binding measurements (see below). Reactions were started by adding cPLA2 to the mixture (to a final concentration of ~20 nM) and quenched by adding 370 μL of chloroform/methanol/HCl (2:1:0.01) solution after a given period of incubation (3–5 min) at room temperature. Liberated [14C]arachidonic acid was separated from the reaction mixture on small silica gel columns as described by Ghomashchi et al. (22). Radioactivity of hydrolyzed arachidonic acid was measured by liquid scintillation counting.

cPLA2-Phospholipid Binding—The binding of cPLA2 to phospholipids was measured by a centrifugation assay using phospholipid-coated hydrophobic styrene-divinylbenzene beads. The beads were coated with DHPC or DHPG as described elsewhere (19). The concentration of phospholipids coated on beads was determined by measuring the radioactivity of a trace of [14C]POPC (0.1 mol %) included in all phospholipid mixtures. The bulk phospholipid concentration of phospholipid-coated beads was typically 50–100 μM. Binding mixtures contained a fixed concentration of phospholipid-coated beads in 20 mM Tris-HCl buffer, pH 7.5, 0.1 mM KCl, 0.5 mM Ca2+ and varying concentration of cPLA2 (0–250 nM). The control was added to minimize loss of protein due to nonspecific adsorption to tube walls. Controls contained the same mixtures minus beads. After the mixtures were incubated for 15 min, beads were pelleted (12,000 × g for 2 min), and the enzyme activity of each supernatant toward [14C]SAPC/1,2-sn-dioleoylglycerol liposomes was measured (see above). The concentration of bound enzyme ([E]b) was calculated from the difference of cPLA2 activity in control and binding mixtures. Parameters n and Kd were determined by nonlinear least-squares analysis of the bound ([E]b) versus total enzyme concentration ([E]t) plot using a standard binding equation,

\[
[E]_b = \frac{E_0 + [PL]/n}{(E_0 + [PL]/n)^2 + 4E_0[PL]/n} \\
(\text{Eq. 1})
\]

where [PL] represents total phospholipid concentration. This equation assumes that each enzyme binds independently to a site on the interface composed of n phospholipids with a dissociation constant of Kd. To determine the Ca2+ dependence of binding, cPLA2 (~60 nM) was incubated for 15 min with phospholipid-coated beads (60 μM) and 1 μM BSA in 150 μl of 10 mM HEPES (pH 7.0) containing 100 mM KCl and varying concentrations of Ca2+ (or EGTA) (see Fig. 2). After centrifugation (12,000 × g for 2 min), [E]b was determined by the activity assay (see above). The binding of Ca2+ ions to the isolated C2 domain was shown to be consistent with the cooperative Hill model (24). The concentration of Ca2+ giving rise to half-maximal binding (or activity) ([Ca2+]1/2) was determined from curve fitting of data to a Hill equation,

\[
y = \frac{a \cdot [\text{Ca}^{2+}]^{y}}{[\text{Ca}^{2+}]^{y} + [\text{Ca}^{2+}]} \\
(\text{Eq. 2})
\]

where y, a, h, and [Ca2+] are relative binding (or activity), arbitrary normalization constant, Hill coefficient, and free Ca2+ concentration, respectively.

The binding of isolated C2 domains to POPC vesicles was measured by a centrifugation assay using sucrose-loaded unilamellar vesicles (100-nm diameter) (25). Sucrose-loaded vesicles were prepared as follows. The lipid solution of POPC was added to a round-bottomed flask, and organic solvent was removed by rotary evaporation. The lipid film was suspended in 20 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM sucrose (to a final lipid concentration of ~20 mM) and vortexed vigorously. Unilamellar vesicles were prepared by multiple extrusion through a 0.1-μm polycarbonate filter (Millipore Corp.) in a LiposoFast microextruder (Avestin; Ottawa, Ontario). For binding measurements, the vesicle solution was diluted into 20 mM Tris-HCl buffer, pH 7.5, 0.1 mM KCl, and 0.5 mM Ca2+ (or EGTA) to yield a final phospholipid concentration of 0.5 mM. After adding wild type or a mutant C2 domain (final concentration of ~0.2 mM) to the vesicle suspensions, the suspensions were incubated for 15 min at room temperature and centrifuged at 100,000 × g for 30 min at 25 °C. Supernatants were decanted, and pellets were redissolved in 15 μl of 10 mM HEPES buffer, pH 7.0, containing 0.1 mM KCl and 0.5 mM EGTA. Resuspended pellets were loaded on 14% polyacrylamide gels, and C2 domains were separated by SDS-polyacrylamide gel electrophoresis. The amount of protein in each band was quantified using
RESULTS

Properties of Calcium Ligand Mutants—According to the structure of the C2 domain of cPLA2 shown in Fig. 1B, five Ca\(^{2+}\)-binding residues can be classified into three groups: Asn\(^{65}\), which primarily coordinates CA1; Asp\(^{93}\) and Asn\(^{95}\), which mainly coordinate CA2; and two CBR1 aspartates, Asp\(^{40}\) and Asp\(^{43}\), which coordinate both Ca\(^{2+}\) ions. Based on this Ca\(^{2+}\) binding geometry, the mutations of Asn\(^{65}\) and Asp\(^{93}/\)Asp\(^{95}\) would selectively affect the binding of CA1 and CA2, respectively, whereas the mutations of Asp\(^{40}/\)Asp\(^{43}\) would have effects on the binding of both Ca\(^{2+}\) ions. Thus, it is possible to systematically analyze the roles of the two Ca\(^{2+}\) ions in the membrane binding and activation of cPLA2 by selectively deactivating their ligands and separately measuring the effects of mutations on membrane binding and activity. For this purpose, we made five mutants that contain either Asp to Asn (e.g. D40N) or Asn to Ala (e.g. N65A) mutations. All five mutants exhibited full membrane binding affinity and enzyme activity at saturating Ca\(^{2+}\) concentrations (see Figs. 2 and 3), demonstrating the lack of deleterious conformational changes. We first measured the binding of wild type and mutants to hydrophobic beads coated with DHPC, which is a nonhydrolyzable ether analog of phospholipid, as a function of Ca\(^{2+}\) concentration. Phospholipid-coated hydrophobic beads have been shown to be useful in determining the membrane affinity of PLA2s (19). In particular, this model membrane allows for rapid and accurate measurement of PC affinity, which is normally difficult to achieve with PC vesicles due to their low pelleting efficiency at low concentrations.\(^2\) As shown in Fig. 2, all mutants exhibited increased Ca\(^{2+}\) requirements for PC binding, and [Ca\(^{2+}\)]\(_{50}\) values varied from 40 \(\mu\)M for N65A (7-fold increase) to 3.5 \(\mu\)M for D43N (614-fold increase) (see Table I). In general, Asp to Asn mutations had much larger effects than Asn to Ala mutations, because aspartates provide charged bidentate Ca\(^{2+}\) ligands, whereas asparagines provide neutral unidentate ones. Comparison among three Asp to Asn mutations and between two Asn to Ala mutations revealed a common theme. First, the mutational effect of Asp\(^{93}\) that only coordinates CA2 is comparable with those of Asp\(^{40}\) and Asp\(^{43}\) that coordinate both Ca\(^{2+}\) ions. This implies that mutational effects of Asp\(^{40}\) and Asp\(^{43}\) on membrane binding derive mainly from its coordination to CA2. Second, between the two Asn to

\(^2\) L. Bittova and W. Cho, unpublished observation.
The C2 Domain of cPLA2

Properties of Mutants of Membrane Binding Residues—Our previous study showed that cPLA2 has a unique ability to partially penetrate into membranes, which is essential for its membrane binding and interfacial catalysis (15). The Ca$^{2+}$ binding loops of cPLA2, CBR1, and CBR3 have a cluster of hydrophobic residues on the tips of their protruded structures (see Fig. 1A), suggesting that these residues might be involved in membrane penetration. We therefore mutated six hydrophobic residues in these regions to Ala: F35A, M38A, and L39A in CBR1 and Y96A, Y97A, and M98A in CBR3. To assess the contribution of electrostatic interactions to membrane binding of the C2 domain, we also mutated four cationic residues (R57E/K58E/R59E/R61E) that form a cationic patch along a β-strand and are implicated in interaction with anionic phospholipid head groups. First, we measured the relative enzyme activity of these mutants using [14C]SAPC vesicles as a substrate. The relative activity determined from the initial rates of hydrolysis is listed in Table II. All mutants have modestly lower activity than does wild type with two mutants in the CBR3, Y96A and Y97A, showing the lowest activity. This indicates that some of these residues are important in interfacial catalysis of cPLA2 but that none is critical. Since mutations of C2 domain residues would not significantly affect the catalytic efficiency of the enzyme, the relative activity of mutants should reflect their relative membrane binding affinity.

To quantitatively assess the effects of mutations on membrane binding, we measured dissociation constants for the binding of cPLA2 to DHPC-coated beads. Binding isotherms for cPLA2 and selected mutants are illustrated in Fig. 4, and n and Kd values determined from the curve fitting are summarized in Table II. Although there are several different ways to analyze the binding isotherms, we used a simple Langmuir-type equation (Equation 1) assuming the presence of the enzyme binding sites on the interface consisting of n phospholipids. Since Kd is expressed in terms of molarity of these binding sites, Kd/n is the dissociation constant in terms of molarity of lipid molecules, and the relative binding affinity can be best described in terms of relative values of (1/nKd) (Table II). The lateral mobility of phospholipids is not taken into account in this model, because little is known about the lateral mobility of phospholipids coated on hydrophobic beads. Since Kd was measured under conditions of enzyme crowding on beads, and because of possible protein-protein interaction, the value obtained might be somewhat different from the Kd obtained under conditions of low bead coverage.

In general, the relative affinity of mutants for DHPC-coated beads is consistent with their relative activity toward [14C]SAPC vesicles. For some mutants, there are changes in both n and Kd values, indicating that the mutations affect both their membrane affinity and binding mode. This effect is pronounced for those mutants with lower relative affinity, including F35A, L39A, and Y96A. Overall, all of the hydrophobic residues except for Met38 and Met39 appear to have significant and comparable contributions to hydrophobic membrane binding. Interestingly, the quadruple mutant of the cationic patch (R57E/K58E/R59E/R61E) exhibits lower activity and affinity for PC vesicles, suggesting that these cationic residues are

![Figure 2. Ca$^{2+}$ dependences of bead binding of cPLA2 and Ca$^{2+}$ ligand mutants.](image)

![Figure 3. Ca$^{2+}$ dependences of enzyme activity of cPLA2 and Ca$^{2+}$ ligand mutants.](image)

**TABLE I**

| cPLA2s   | DHPC bead binding | Enzyme activity |
|----------|-------------------|-----------------|
| Wild type | 6.7 ± 0.3) × 10^−6 | 1.7 ± 0.3) × 10^−6 |
| D40N     | 3.5 ± 0.3) × 10^−3 | 2.9 ± 0.1) × 10^−4 |
| N65A     | 4.0 ± 0.3) × 10^−5 | 3.3 ± 0.2) × 10^−5 |
| D93N     | 1.1 ± 1.6) × 10^−3 | 1.3 ± 0.3) × 10^−4 |
| N95A     | 9.4 ± 2.8) × 10^−5 | 1.2 ± 0.2) × 10^−5 |
The C2 Domain of cPLA<sub>2</sub>

Properties of Isolated C2 Domain and Its Mutants—For hydrophobic residues to contribute to the energetics of membrane binding, they must penetrate into the hydrophobic core of the membrane. Thus, mutants of essential hydrophobic residues of the C2 domain are expected to have significantly altered membrane penetrating ability. Phospholipid monolayers at the air-water interface serve as a highly sensitive tool to measure the membrane penetrating ability of protein (10, 15, 29). Monolayer measurements of mutant proteins were, however, hampered by generally low protein expression yields of cPLA<sub>2</sub> and mutants from our baculovirus-infected insect cells. To overcome this difficulty, we prepared the isolated C2 domain of cPLA<sub>2</sub> residues 1–137 and its mutants. Since these isolated C2 domains can be expressed in <i>E. coli</i> as inclusion bodies and readily refolded with high yields (~5 mg/liter of culture), we were able to obtain proteins sufficient for monolayer measurements. This also allowed us to compare the membrane penetrating ability of the C2 domain versus the whole cPLA<sub>2</sub> molecule. We first measured relative membrane binding affinity of isolated C2 domain mutants to see if these mutants behave similarly to their parent proteins. For these measurements, we used sucrose-loaded POPC vesicles at a high enough concentration (i.e., 0.5 mM) to ensure complete pelleting, and the relative affinity was estimated from the amount of protein bound to POPC vesicles. As listed in Table III, relative binding affinity of isolated C2 domain mutants is comparable with that of cPLA<sub>2</sub> mutants (see Table II), indicating that the mutational effects are essentially the same for the isolated C2 domain and the whole protein. Then we measured the penetration of these C2 domains into POPC monolayers. In these studies, a POPC phospholipid monolayer, and an extrapolation of the 

| cPLA<sub>2</sub> | SAPC vesicles (relative activity) | DHPC-coated beads | DHPG-coated beads |
|---------------|---------------------------------|------------------|------------------|
|               | n | K<sub>d</sub> | Relative affinity | ΔΔG<sub>2</sub>/ΔA | n | K<sub>d</sub> | Relative affinity |
| Wild type     | 1 | 130 ± 5 | 5 ± 1 | 1 | 440 ± 20 | 11 ± 3 | 1 |
| F35A          | 0.4 | 410 ± 9 | 12 ± 2 | 0.12 | 20 | 830 ± 60 | 25 ± 5 | 0.23 |
| M38A          | 0.5 | 350 ± 20 | 5 ± 3 | 0.31 | 15 | 1950 ± 190 | 3 ± 1 | 0.96 |
| L39A          | 0.3 | 370 ± 40 | 10 ± 4 | 0.16 | 30 | 930 ± 40 | 84 ± 32 | 0.06 |
| Y96A          | 0.2 | 250 ± 50 | 25 ± 7 | 0.09 | 19 | 710 ± 520 | 33 ± 15 | 0.20 |
| V97A          | 0.2 | 180 ± 10 | 21 ± 3 | 0.16 | 40 | 420 ± 60 | 17 ± 3 | 0.06 |
| M98A          | 0.8 | 180 ± 20 | 8 ± 4 | 0.43 | 11 | 820 ± 60 | 7 ± 1 | 0.86 |
| R57E/R58E/R59E/R61E | 0.4 | 180 ± 5 | 19 ± 4 | 0.29 | 11 | 370 ± 30 | 11 ± 1 | 1.2 |

somewhat involved in membrane binding. Because it was unclear how the cationic residues participate in binding to the zwitterionic membrane surface, we measured the binding of wild type and mutants including R57E/R58E/R59E/R61E to anionic DHPG-coated beads. It was previously shown that both cPLA<sub>2</sub> (27) and its isolated C2 domain (28) have lower affinity for monoanionic phospholipids than for zwitterionic phospholipids. Consistent with these findings, cPLA<sub>2</sub> showed ~7.5-fold lower affinity for anionic DHPG-coated beads than for DHPC-coated beads. Also, most of the mutations of hydrophobic residues decreased the binding affinity for DHPG-coated beads less significantly than that for DHPC-coated ones, suggesting that cPLA<sub>2</sub> might bind to phosphatidylglycerol membranes in a slightly different mode. Qualitatively, however, similar trends were observed for binding of the mutants to DHPC- and DHPG-coated beads. Surprisingly, R57E/R58E/R59E/R61E had a slightly higher affinity for DHPG-coated beads than the wild type did, demonstrating that these residues are not involved in binding to anionic membranes. This also suggests that the low affinity of the quadruple mutant of PC-coated beads might derive from a secondary effect unrelated to membrane binding.
challenge of calcium coordination between the two C2 domains. Thus, comparison of the role of a calcium ion in cPLA2 with that of its counterpart in protein kinase C-α should be made with caution. For cPLA2, the following results indicate that CA2 bound close to CBR3 is important for membrane binding and that CA1 bound close to CBR1 is involved in cPLA2 activation. First, mutations of Asp40, Asp43, Asp93, and Asn95, which coordinate CA2, have larger effects on membrane binding than that of Asn65, which coordinates CA1. Second, the mutation of Asp43, which is closest to CA2 (see Fig. 1B), has the largest impact on membrane binding. Third, the mutations of CA2 ligands show comparable effects on membrane binding and enzyme activity, whereas the mutation of Asn65 affects enzyme activity much more significantly. Notice that this assignment of roles is distinct from that given to calcium ions bound to protein kinase C-α. This also implies that the two C2 domains interact with membranes in different modes. A recent study of the isolated C2 domain of cPLA2 showed that two Ca2+ ions bind to the C2 domain sequentially with positive cooperativity, which in turn induces intradomain conformational changes and membrane translocation (24). Since the membrane binding of cPLA2 precedes its interfacial catalysis, it is likely that CA2 binding takes place first, which then promotes CA1 binding although the reverse order of calcium addition cannot be precluded. The 10-fold increase in [Ca2+]1/2 for the membrane binding of N65A (see Table II) indicates that CA1 binding also contributes to membrane binding, albeit to a lesser degree. Thus, it appears that the binding of both Ca2+ ions to the C2 domain takes place prior to the membrane binding of cPLA2. Since the calcium affinity of cPLA2 is much higher in the presence of membranes, it is reasonable to assume that binding of both calcium ions occurs in the vicinity of the membrane surface. Thus, it is likely that the binding of two Ca2+ ions and the membrane binding occur essentially simultaneously. In the case of protein kinase C-α, it was demonstrated that the binding of calcium ion(s) to CBR3 induces the membrane penetration of protein, which leads to activation. Because the measurements of membrane penetration and/or conformational changes for Ca2+ ligand mutants were hampered by low protein expression yields, it is unclear at present how exactly the two Ca2+ ions play their specific roles. Based on the coordination geometry of the two Ca2+ ions (see Fig. 1B) and the membrane binding properties of cPLA2, we speculate that CA2

| C2 domains | Relative affinity for POPC vesicles | POPC monolayer penetration (πc) |
|------------|-----------------------------------|-------------------------------|
| Wild type  | 1                                 | 34                            |
| F35A       | 0.12                              | 27                            |
| M36A       | 0.50                              | 29                            |
| L39A       | 0.25                              | 27                            |
| Y96A       | 0.07                              | 17                            |
| V97A       | 0.25                              | 26                            |
| M98A       | 0.75                              | 31                            |
| R57E/K58E/R59E/R61E | 0.89 | 33 |
induces the local conformational changes in the calcium-binding loops to expose hydrophobic residues for hydrophobic membrane binding (see below), whereas CA1 binds to one or more membrane phospholipids to properly orient the cPLA2 molecule for interfacial catalysis. This notion is based on the fact that CA2 has six coordinations to both CBR1 and CBR3, whereas CA1 has only four coordinations to CBR1 and CBR2 (see Fig. 1B). Thus, the higher affinity binding of CA2 to its ligands alter the conformation of essential hydrophobic side chains in CBR1 and CBR3 and possibly promote the lower affinity binding of CA1 (hence the order of addition). On the other hand, CA1, due to its incomplete coordination sphere in the C2 domain, should still be capable of coordinating to membrane phospholipids even after binding to the C2 domain. Since the membrane binding of cPLA2 is driven mainly by hydrophobic interactions (see below), the binding of CA2, if it indeed exposes all hydrophobic residues, would be more important for membrane binding than that of CA1, which would contribute less to conformational changes and thus primarily contribute to less important electrostatic interactions. The calcium-induced conformational changes of calcium binding loops containing essential hydrophobic residues are supported by a recent NMR study of the isolated C2 domain of cPLA2 (13). Also, the notion that CA1 binds to phospholipids and thus contributes to electrostatic interactions is supported by our finding that N65A has a considerably higher $[Ca^{2+}]_{15}$ value for anionic DHPC-coated bead binding than for DHPC-coated bead binding, whereas N95A shows the essentially the same calcium dependence for both types of beads (data not shown). Further investigation is needed to elucidate exact roles of the two calcium ions and determine the temporal and spatial sequences of calcium and membrane binding in the interfacial catalysis of cPLA2.

**Hydrophobic Residues**—Both electrostatic and hydrophobic interactions are involved in the membrane binding of most peripheral membrane binding proteins and their relative contributions vary with the type of proteins (see, for example, Refs. 30–33). This study shows that the binding of C2 domain of cPLA2 is mainly driven by hydrophobic interactions that are achieved by the penetration of hydrophobic residues in the calcium binding loops (CBR1 and CBR3) into the membrane core. To our knowledge, the C2 domain of cPLA2 has the highest membrane penetrating power than any other C2 domain studied, including that of protein kinase C-$\alpha$,$^3$ presumably due to its unique structure, containing a number of hydrophobic residues in the calcium binding loops. A combined contribution of six hydrophobic residues to binding energy, which can be estimated using the equation $\Delta G^0 = -RT \ln(\text{relative affinity})$ (see Table II), is 9.1 kcal/mol at 25 °C, assuming the additivity of individual contributions. This suggests that the hydrophobic interactions are strong enough to drive the membrane binding of cPLA2. Our monolayer data show that Ca$^{2+}$ is essential for the membrane penetration of the C2 domain. As described above, this is mainly because Ca$^{2+}$ triggers local conformational changes in the calcium binding loops, which exposes hydrophobic residues and thereby elicits their membrane penetration. Mutational effects of hydrophobic residues on activity, membrane binding, and monolayer penetration indicate that four hydrophobic residues, Phe$^{35}$ and Leu$^{39}$ of CBR1 and Tyr$^{96}$ and Val$^{97}$ of CBR3, are directly involved in membrane penetration and hydrophobic interactions. Although these data apparently indicate that Tyr$^{96}$ makes the largest contribution to the membrane penetration, it should be taken into account that the Tyr to Ala mutation involves a larger change in accessible surface area than any other mutation. To assess more quantitatively the contribution of each amino acid to membrane penetration and hydrophobic interactions, one thus has to determine the change in free energy of binding per change in accessible surface area $(\Delta \Delta G^0/\Delta A)$ caused by each mutation. These values determined for individual mutations are given in Table II. It was shown that an average $\Delta \Delta G^0/\Delta A$ value for transferring an amino acid from ethanol to water is $-24$ cal/mol/Å$^2$ (34). Thus, one can estimate from this value the degree of penetration of each hydrophobic residue in the calcium binding loops. For instance, Leu$^{39}$ and Val$^{97}$ the mutations of which yield high $\Delta \Delta G^0/\Delta A$ values of 30 and 40 cal/mol/Å$^2$, respectively, should be fully inserted into the hydrophobic core of membranes, whereas Phe$^{35}$ and Tyr$^{96}$ with smaller $\Delta \Delta G^0/\Delta A$ values would partially penetrate into the membrane during membrane binding. This notion is also consistent with a general observation that aromatic side chains have a high tendency to reside in the membrane-water interface (35). If the membrane binding of cPLA2 is driven primarily by hydrophobic

---

$^3$ M. Medkova and W. Cho, manuscript in preparation.
interactions, then its affinities for PC and phosphatidylglycerol membranes should be comparable. Thus, the PC preference of cPLA₂ and its C2 domain (28) suggests the presence of either a specific PC binding site(s) or an anionic patch on the membrane binding surface of the C2 domain. While the answer to this question entails further investigation, the PC preference of cPLA₂ makes it less likely that four cationic residues in the β3 strand are directly involved in membrane binding of cPLA₂. This is also consistent with the finding that wild type cPLA₂ and the quadruple mutant (R57E/K58E/R59E/R61E) have comparable affinities for DHPG-coated beads. The modestly lower binding affinity of the mutant for PC membranes might be due to local structural changes that affect PC binding more significantly than phosphatidylglycerol binding. Similar differential effects of mutations on PC and phosphatidylglycerol binding are also seen with mutations of hydrophobic residues (see Table II).

Based on these findings, we propose a membrane binding mode of the C2 domain of cPLA₂ as illustrated in Fig. 7. This model is different from one proposed by Williams and co-workers (12) in that both CBR1 and CRB3 are involved in membrane penetration, and the cationic cluster does not make direct contact with the membrane surface. The finding that the isolated C2 domain of cPLA₂ has higher penetrating power than cPLA₂ indicates the difference in membrane penetrating mode between the isolated C2 domain and the C2 domain in the intact cPLA₂ molecule. Presumably, the carboxyl-terminal domain of cPLA₂, which contains the active site, interferes with the maximal membrane penetration of the amino-terminal C2 domain. We previously showed that the partial membrane penetration of cPLA₂ is essential for its interfacial catalysis (15). Thus, it is possible that any membrane factors, such as diacylglycerols, which make up for the compromised membrane penetration power of cPLA₂ may induce its membrane translocation and activation.

In summary, this report represents the second part of our systematic structure-function analysis on the C2 domain of membrane-binding proteins. These studies reveal similarities and differences between the C2 domains of protein kinase C-α and cPLA₂ in terms of their mechanisms of membrane binding and activation. The two C2 domains are similar in that they are not only a membrane docking unit but also a module that triggers membrane penetration of protein and in that their individual Ca²⁺ ions bound to the calcium binding loops play differential roles in membrane binding and activation. On the other hand, individual Ca²⁺ ions of the two C2 domains have different specific roles, and the membrane penetration of hydrophobic residues plays a more important and direct role in the membrane binding of the C2 domain of cPLA₂ than in that of protein kinase C.

REFERENCES

1. Dennis, E. A. (1994) J. Biol. Chem. 269, 13057–13060
2. Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., and Kudo, I. (1997) Crit. Rev. Immunol. 17, 225–283
3. Leslie, C. C. (1997) J. Biol. Chem. 272, 16709–16712
4. Bonventre, J. V., Huang, Z., Taheri, M. R., O’Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1997) Nature 390, 622–625
5. Uozumi, N., Kume, K., Nagase, T., Nakatani, N., Ishii, S., Tashiro, F., Komagata, Y., Makii, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (1997) Nature 390, 618–622
6. Glover, S., de Carvalho, M. S., Bayburt, T., Jonas, M., Chi, E., Leslie, C. C., and Gelb, M. H. (1999) J. Biol. Chem. 274, 15359–15367
7. Schievella, A. R., Regier, M. K., Smith, W. L., and Lin, L. L. (1995) J. Biol. Chem. 270, 30749–30754
8. Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N., and Knopf, J. L. (1991) Cell 65, 1043–1051
9. Nalefski, E. A., Sultzman, L. A., Martin, D. M., Kriz, R. W., Towler, P. S., Knopf, J. L., and Clark, J. D. (1994) J. Biol. Chem. 269, 18239–18249
10. Medlkova, M., and Cho, W. (1998) Biochemistry 37, 4892–4900
11. Sutton, R. B., and Sprang, S. R. (1998) Structure 6, 1395–1405
12. Perisic, O., Fong, S., Lynch, D. E., Bycroft, M., and Williams, R. L. (1998) J. Biol. Chem. 273, 1596–1604
13. Xu, G. Y., McDonagh, T., Yu, H. A., Nalefski, E. A., Clark, J. D., and Cumming, D. A. (1998) J. Mol. Biol. 280, 485–500
14. Nalefski, E. A., and Falke, J. J. (1996) Protein Sci. 5, 2375–2390
15. Lichtenbergova, L., Yoon, E. T., and Cho, W. (1998) Biochemistry 37, 14128–14136
16. Davletov, B., Perisic, O., and Williams, R. L. (1998) J. Biol. Chem. 273, 19955–19966
17. Comfurius, P., and Zwaal, R. F. A. (1977) Biochim. Biophys. Acta 488, 36–42
18. Lands, W. E. (1960) J. Biol. Chem. 235, 2233–2237
19. Kim, Y., Lichtenbergova, L., Snitko, Y., and Cho, W. (1997) Anal. Biochem. 250, 109–116
20. Kates, M. (1986) in Techniques of Lipidology, 2nd Ed., pp. 114–115, Elsevier, Amsterdam
21. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–9
22. Ghomashchi, F., Schuttel, S., Jain, M. K., and Gelb, M. H. (1992) Biochemistry 31, 3814–3824
23. Bers, D. M. (1982) Am. J. Physiol. 242, C404–C408
24. Nalefski, E. A., Slazas, M. M., and Falke, J. J. (1997) Biochemistry 36, 12011–12018
25. Rebechi, M., Peterson, A., and McLaughlin, S. (1992) Biochemistry 31, 12742–12747
26. Verger, R., and Pattus, F. (1982) Chem. Phys. Lipids 30, 189–227
27. Mosior, M., Bix, D. A., and Dennis, E. A. (1998) J. Biol. Chem. 273, 2184–2191
28. Nalefski, E. A., McDonagh, T., Somers, W., Seehra, J., Falke, J. J., and Clark, J. D. (1998) J. Biol. Chem. 273, 1365–1372
29. Medlkova, M., and Cho, W. (1999) J. Biol. Chem. 274, 17544–17552
30. Dua, R., Wu, S.-K., and Cho, W. (1995) J. Biol. Chem. 270, 263–268
31. Han, S.-K., Yoon, E. T., Scott, D. L., Sigler, P. B., and Cho, W. (1997) J. Biol. Chem. 272, 3573–3582
32. Lee, B.-I., Yoon, E. T., and Cho, W. (1996) Biochemistry 35, 4231–4240
33. Snitko, Y., Koduri, R., Han, S.-K., Othman, R., Baker, S. F., Molini, B. J., Wilton, D. C., Gelb, M. H., and Cho, W. (1997) Biochemistry 36, 14325–14333
34. Chothia, C. (1974) Nature 248, 328–339
35. Yau, W. M., Wimley, W. C., Gawrisch, K., and White, S. H. (1998) Biochemistry 37, 14713–14718