The Molecular Basis of Differential Subcellular Localization of C2 Domains of Protein Kinase C-α and Group IVa Cytosolic Phospholipase A₂*

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The C2 domain is a Ca²⁺-dependent membrane-targeting module found in many cellular proteins involved in signal transduction or membrane trafficking. C2 domains are unique among membrane targeting domains in that they show a wide range of lipid selectivity for the major components of cell membranes, including phosphatidylycerine and phosphatidylcholine. To understand how C2 domains show diverse lipid selectivity and how this functional diversity affects their subcellular targeting behaviors, we measured the binding of the C2 domains of group IVa cytosolic phospholipase A₂ (cPLA₂) and protein kinase C-α (PKC-α) to vesicles that model cell membranes they are targeted to, and we monitored their subcellular targeting in living cells. The surface plasmon resonance analysis indicates that the PKC-α C2 domain strongly prefers the cytoplasmic plasma membrane mimic over the cytoplasmic plasma membrane. Asn₁₈₉ plays a key role in this specificity. In contrast, the cPLA₂ C2 domain has specificity for the nuclear membrane mimic over the cytoplasmic plasma membrane mimic due to high phosphatidylcholine content in the former and that Asn₁₈₉ plays a key role in this specificity. In contrast, the cPLA₂ C2 domain has specificity for the nuclear membrane mimic over the cytoplasmic plasma membrane mimic due to high phosphatidylcholine content in the former and aromatic and hydrophobic residues in the calcium binding loops of the C2 domain are important for its lipid specificity. The subcellular localization of enhanced green fluorescent protein-tagged C2 domains and mutants transfected into HEK293 cells showed that the subcellular localization of the C2 domains is consistent with their lipid specificity and could be tailored by altering their in vitro lipid specificity. The relative cell membrane translocation rate of selected C2 domains was also consistent with their relative affinity for model membranes. Together, these results suggest that biophysical principles that govern the in vitro membrane binding of C2 domains can account for most of their subcellular targeting properties.

The agonist-induced subcellular targeting of protein is an important process in cell signaling and regulation. Recently, the membrane targeting of peripheral proteins (e.g. phospholipases, lipid-dependent protein kinases, lipid kinases, and lipid phosphatases) by Ca²⁺ and lipid mediators, including phosphoinositides, has received much attention as an important event in cell signaling and membrane trafficking. It has been shown that the subcellular targeting of peripheral proteins is driven by a growing number of membrane targeting domains. These domains include protein kinase C (PKC)1 conserved 1 (C1) domain, PKC conserved 2 (C2) domain, pleckstrin homology (PH) domain, Fab1, YOTB, Vac 1 and EEA1 (FYVE) domain, band four-point-one, ezrin, radixin and moesin (FERM) domain, epin amino-terminal homology (ENTH) domain, and phox (PX) domain (1–5). The C2 domain has been identified in many cellular proteins involved in signal transduction or membrane trafficking (5–7). A majority of C2 domains bind the membrane in a Ca²⁺-dependent manner and thereby play an important role in Ca²⁺-dependent membrane targeting of peripheral proteins. Structural analyses of Ca²⁺-dependent membrane binding C2 domains have demonstrated similar tertiary structures in which three Ca²⁺-binding loops are located at an end of an extended antiparallel β-sandwich (8–14). Despite similar structural folds, C2 domains exhibit great functional diversities due to local structural variations, particularly in the Ca²⁺-binding loops. Most Ca²⁺-dependent membrane binding C2 domains have higher affinity for anionic membranes than for zwitterionic ones. In particular, the C2 domains of PKC-α (PKC-α-C2) and phospholipase C-64 show selectivity for phosphatidylserine (PS) (15–17). In contrast, the C2 domains of group IVa cytosolic phospholipase A₂ (cPLA₂) (18) and 5-lipooxygenase (19) strongly favor zwitterionic phosphatidylcholine (PC). Furthermore, C2 domains show distinct subcellular localization patterns. For instance, the C2 domains of PKC-α (20) and phospholipase C-51 (17) translocate to the plasma membrane, whereas the C2 domains of cPLA₂ (cPLA₂-C2) (21, 22) and 5-lipooxygenase (19) translocate to the perinuclear region. Although recent studies indicate that the Ca²⁺-binding loops are involved in lipid selectivity of C2 domains (17, 19, 23, 24), the mechanisms of differential lipid selectivity and distinct subcellular localization of C2 domains have not been fully elucidated. To understand these mechanisms, we performed biophysical studies using purified recombinant proteins and vesicles whose lipid compositions resemble those of cell membranes and measured the spatiotemporal dynamics of enhanced green fluorescent protein (EGFP)-tagged C2 domains.

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1 The abbreviations used are: PKC-α, protein kinase C-α; BSA, bovine serum albumin; cPLA₂, cytosolic phospholipase A₂; DMEM, Dulbecco’s modified Eagle’s medium; EGFP, enhanced green fluorescence protein; FBS, fetal bovine serum; PC, phosphatidylcholine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; POPI, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol; POPs, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; PS, phosphatidyserine; RU, resonance units; SPR, surface plasmon resonance; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
and mutants in live cells. These studies not only identify the residues that are responsible for their distinct lipid selectivity but also demonstrate that in vitro membrane binding properties of C2 domains are quantitatively correlated with their subcellular targeting behaviors.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol (POPI), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Phospholipid concentrations were determined by ultraviolet spectrophotometry (25). The Liposofast microextruder and 100-nm polycarbonate filters were from Avestin (Ottawa, Ontario). Fatty acid-free bovine serum albumin was from Bayer Inc. (Kankakee, IL). Triton X-100 was obtained from Pierce Chemical Co. (Rockford, IL). Restriction endonucleases and enzymes for molecular biology were obtained from either Roche Molecular Biochemicals or New England BioLabs (Beverly, MA). CHAPS and octyl glucoside were from Sigma and Fisher Scientific, respectively. Pioneer L1 sensor chip was from Biacore AB (Piscataway, NJ).

**Mutagenesis and Protein Expression**—The isolated cPLA2-α-C2 and mutants (15) were expressed and purified as previously described. Mutants of PKC-α-C2 were generated by the overlap extension PCR (27) using pVL1932-PKC-α plasmid as a template (28). Briefly, four primers, including two complementary oligonucleotides introducing a desired mutation and two additional oligonucleotides complementary to the 5’-end of the C2 domain (residue 154) and the 3’-end (284) of the PKC-α gene, respectively, were used for PCR performed in a DNA thermal cycler (PerkinElmer Life Sciences) using Pfu DNA polymerase (Stratagene). Two DNA fragments overlapping at the mutation site were first generated and purified on an agarose gel. These two fragments were then annealed and extended to generate an entire C2 domain gene containing a desired mutation, which was further amplified by PCR. The product was subsequently purified on an agarose gel, digested with NcoI and XhoI, and subcloned into the PET21d vector digested with the same restriction enzymes. The mutagenesis was verified by DNA sequencing using a T7 Sequenase kit (U.S. Biochemical, Cleveland, OH).

**Construction of Gene Constructs of cPLA2 and PKC-α**—Fused with EGFP—EGFP in pEGFP vector (Clontech) was modified by PCR to remove the first methionine and to add two amino-terminal glycines and an EcoRI site in the modified EGFP gene was inserted into a modified pIND vector (Invitrogen) between the EcoRI and XhoI sites to yield a plasmid, pIND/EGFP. PKC-α was cloned by PCR to remove the stop codon and add two carboxyl-terminal glycines and an EcoRI site. The PKC-α gene, which was further amplified by PCR, was subsequently purified on an agarose gel, digested with NcoI and XhoI, and subcloned into the PET21d vector digested with the same restriction enzymes. The mutagenesis was verified by DNA sequencing using a T7 Sequenase kit (U.S. Biochemical, Cleveland, OH).

**Membrane Targeting by C2 Domains**

**Construction of Gene Constructs of cPLA2 and PKC-α**—Unfused with EGFP—Fused with EGFP in pEGFP vector (Clontech) was modified by PCR to remove the first methionine and to add two amino-terminal glycines and an EcoRI site in the modified EGFP gene was inserted into a modified pIND vector (Invitrogen) between the EcoRI and XhoI sites to yield a plasmid, pIND/EGFP. PKC-α was cloned by PCR to remove the stop codon and add two carboxyl-terminal glycines and an EcoRI site. The PKC-α gene, which was further amplified by PCR, was subsequently purified on an agarose gel, digested with NcoI and XhoI, and subcloned into the PET21d vector digested with the same restriction enzymes. The mutagenesis was verified by DNA sequencing using a T7 Sequenase kit (U.S. Biochemical, Cleveland, OH).

**Cell Culture**—A stable HEK293 cell line expressing the edcsyne receptor (Invitrogen) was used for all experiments. Briefly, cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and penicillin, streptomycin, and amphotericin B. Cells were maintained in cell culture water immersion objective was used for all experiments. EGFP fluorescence imaging was performed using a high-performance argon laser and a 500-550 nm filter was used to monitor EGFP emission on channel 1. We used mag-indo-1 for real-time monitoring of changes in intracellular calcium concentration ([Ca2+]i) in the micromolar range. For simultaneous monitoring of EGFP and [Ca2+]i, mag-indo-1 was excited using the 488-nm line of a UV laser, and the emitted light was collected using band pass filters (395–425 nm) and long pass filters (500–590 nm). The LSM 510 imaging software was used to control the time intervals for imaging. Ca2+-dependent translocation of C2 domains was monitored as follows: Thirty minutes before imaging, cells were treated with 2 μM of mag-indo-1 (10 μM final concentration) (Molecular Probes). Immediately
ately before imaging, the induction media was removed and the cells were washed with 150 μl of 2 mM EDTA and then overlaid with 150 μl of HEK buffer (1 mM HEPES, pH 7.4, containing 2.5 mM MgCl₂, 1 mM NaCl, 0.6 mM KCl, 0.67 mM D-glucose, 6.4 mM sucrose). After initially imaging cells, 150 μl of HEK buffer containing 10 μM ionomycin and 1 mM CaCl₂ was added to the cells. Protein translocation was monitored by time scanning at 4-s intervals for PKC-α-C2 and at 15-s intervals for cPLA₂-C2, respectively. The cytoplasm was simultaneously measured at 4- to 15-s intervals using the equation, $\frac{[Ca^{2+}]}{max} = K_d \times Q \times (R - R_{min})/(R_{max} - R)$, where $R$ represents the fluorescence intensity ratio $F_{ magn-indo-1}/F_{max}$ that was determined from the average background-corrected pixel values of mag- indo-1 fluorescence from the cytoplasmic area of individual cells at 410 and 470 nm, respectively; $R_{max}$ and $R_{min}$ correspond to minimum and maximum fluorescence intensity ratios at no $Ca^{2+}$ and saturating calcium levels (i.e., 1 mM free $Ca^{2+}$, respectively). $Q$ is the ratio of the minimum fluorescence intensity at zero free calcium ($F_{max}$) to the maximum fluorescence intensity at 1 mM saturating calcium ($F_{max}$) at 470 nm, and $K_d$ is the calcium dissociation constant for mag-indo-1. $R_{max}$, $R_{min}$, $Q$, and $K_d$ values were determined by in vitro calcium calibration using 1 μM mag-indo-1 and a set of standard calcium buffers (Molecular Probes) containing 0–1 mM free $Ca^{2+}$. The calculated $K_d$ (34 ± 3 μM) was the same as the reported value of 35 μM (35).

**Cell Imaging Data Analysis**—Images were analyzed using the analysis tools provided in the Zeiss biological software package. Using these tools, regions of interest in the cytosol were defined, and the average intensity in a square (1 μm × 1 μm) was obtained with time. Membrane intensities were determined for each frame in individual cells by extending a line from the cytosol to the outside of the cell and recording the intensity with distance along the line. By cross-checking markers on the diagram with a table of intensity data, four intensity values corresponding to the place on the line indicating the edge of the cell were averaged. Lines were drawn in at least three places in each cell, and membrane intensity values were determined and averaged. Intensity values outside the photomultiplier tube’s linear range were discarded. The resultant intensity values were converted to a ratio of intensity at membrane to the sum of intensities at membrane and at cytosol for each time frame. Membrane translocation rates of individual C2 domains were then calculated from the slopes of these plots by linear regression. Each experiment was repeated at least twice on at least two different days with different transfections. Cellular distribution of EGF intensity throughout the cell was obtained using the profile function on the software package. A line was drawn through the cell from an arbitrary point, and the EGF intensity from each point along the line was plotted versus arbitrary distance to correlate the difference in EGF intensities at different subcellular locations.

**RESULTS**

**PS Selectivity of PKC-α-C2**—The C2 domain of PKC-α has been shown to have PS selectivity with extremely low affinity for zwitterionic phospholipids (i.e., PS > PG > PC) (15). Our structure-function analysis of PKC-α (15) and its isolated C2 domain (36) and the crystal structure of PKC-α-C2 (12) suggested that the PS selectivity derive from the specific recognition of PS by a C2-domain-bound calcium ion and several residues located in the calcium binding loops (Fig. 1). In particular, a recent mutation study on PKC-α showed that Asn₁⁸₉, Arg²¹⁶, Arg²⁴⁹, and Thr²⁵¹ in the calcium binding loops are involved in PS binding (24). However, it is still unclear whether these residues are involved in specific headgroup recognition or in binding to other parts of phospholipid molecules. We therefore measured the effect of the mutations of these residues on the lipid headgroup selectivity. We also mutated another cationic residue (Arg²³₂) that has been implicated in membrane binding (28). PS selectivity of wild type and mutants was determined by comparing their binding affinity for immobilized POPC/POPS (7:3) and POPC/POPG (7:3) vesicles by SPR analysis. PKC-α-C2 shows extremely low affinity for POPC vesicles. This allowed the use of the physiologically more relevant mixed vesicles in lieu of 100% POP or POPG vesicles for identifying the residues directly involved in specific lipid headgroup recognition (i.e., serine versus glycerol headgroup).

We have shown that the SPR analysis allows direct determination of membrane association ($k_a$) and dissociation ($k_d$) rate constants for peripheral proteins (29, 31). As summarized in Table I, PKC-α-C2 has higher affinity (in terms of $K_d$) for POPC/POPS (7:3) than for POPC/POPG (7:3), and the PS selectivity of PKC-α-C2 expressed in term of the ratio of 1/$K_d$ for POPC/POPS (7:3) to 1/$K_d$ for POPC/POPG (7:3) is about 11. Interestingly, the higher affinity for PS is almost entirely ascribed to the lower $k_d$ value. Our previous study indicated that nonspecific electrostatic interactions primarily accelerate the association of protein to anionic membrane surfaces, whereas hydrophobic interactions and short-range specific interactions (electrostatic or hydrogen bonds) mainly slow the membrane dissociation (29). Thus, the present data support the notion that PS forms specific interactions with the PKC-α-C2.

The previous study showed that the T251A mutation had the most detrimental effect on binding affinity for PS-containing vesicles and micelles, whereas N189A, R216A, and R249A exhibited lesser effects (24). Our SPR data also showed that T251A reduced the affinity for POPC/POPS (7:3) vesicles more significantly than N189A, R216A, and R249A/R252A. T251A has 18-fold lower affinity than wild type, whereas N189A, R216A, and R249A/R252A have 5- to 6-fold lower affinity. Most important, T251A, R216A, and R249A/R252A have essentially the same PS selectivity as the wild type, because the mutations reduced the affinity of PKC-α-C2 for POPC/POPS (7:3) and POPC/POPG (7:3) vesicles to comparable degrees. In contrast, the N189A mutation reduced the PS affinity of PKC-α-C2 5-fold without affecting its PG affinity. As a result, N189A exhibited much reduced 2-fold PS selectivity. Together, these data indicated that Asn¹⁸⁹ is involved in specific PS headgroup binding, whereas other mutated residues are involved in either binding to a non-headgroup part of an anionic phospholipid, such as sn-1 or -2 acyl group, or nonspecific binding to the anionic membrane surface.

Examination of effects of mutations on $k_a$ and $k_d$ provided a further insight into their roles. For binding to POPC/POPS (7:3) vesicles, the N189A, R216A, and T251A mutations changed $k_d$ more than $k_a$, whereas the R249A/R252A mutation primarily reduced the $k_a$ value (see Table I). This indicates that Arg²¹⁶ and Thr²⁵¹ are involved in specific short-range interactions with a non-headgroup part of PS molecule and that Arg²⁴⁹ and Arg²³₂ participate in nonspecific electrostatic interactions with the anionic membrane surface.

**PC Selectivity of cPLA₂-C2**—The C2 domain of cPLA₂ con-
contains multiple aliphatic and aromatic residues, including Phe\textsuperscript{85}, Leu\textsuperscript{89}, Tyr\textsuperscript{96}, and Val\textsuperscript{97}, in its calcium binding loops (see Fig. 1) that have been shown to partially penetrate the membrane during the membrane binding of cPLA\textsubscript{2}-C2 (26). It has also been known that cPLA\textsubscript{2}-C2 has PC selectivity (18). However, the origin of this selectivity has yet to be elucidated. To identify the residues in the cPLA\textsubscript{2}-C2 that are responsible for its PC selectivity, we mutated aromatic and aliphatic residues in the Ca\textsuperscript{2+} binding loops and measured their membrane binding by SPR analysis. In this case, we measured the affinities of wild type and mutants for 100% POPC and POPG vesicles. As listed in Table II, cPLA\textsubscript{2}-C2 prefers POPC to POPG by a factor of 11 in terms of \(K_d\), confirming PC selectivity of cPLA\textsubscript{2}-C2. The mutations of all four hydrophobic residues reduced the affinity of cPLA\textsubscript{2}-C2 for membrane by more than two orders of magnitude. Furthermore, the mutations of two aromatic residues (Phe\textsuperscript{85} and Tyr\textsuperscript{96}) affected \(k_d\) and \(k_s\) to comparable degree, whereas those of two aliphatic residues (Leu\textsuperscript{89} and Val\textsuperscript{97}) primarily increased \(k_d\). This again underscores the difference between aliphatic and aromatic residues in their interfacial interactions. Most importantly, all mutants showed dramatically reduced PC selectivity: their PC selectivity expressed in terms of the ratio of \((1/K_d)/K_d\), for POPC ranges from 0.6 to 1.1, indicating that all four residues in the calcium binding loops are involved in PC selectivity.

**Binding of cPLA\textsubscript{2}-C2 and PKC-α-C2 to Cell Membrane Mimic Vesicles**—It has been shown that the C2 domains of conventional PKCs (\(\alpha, \beta\), and \(\gamma\)) translocate to the plasma membrane (20, 37) and cPLA\textsubscript{2}-C2 to the perinuclear region in response to Ca\textsuperscript{2+} import (21, 22). Also, this subcellular localization pattern of isolated C2 domains correlates with that of peripheral proteins harboring the C2 domains; i.e. conventional PKCs translocate to plasma membrane (38–41) while cPLA\textsubscript{2} moves to perinuclear region (42, 43). Although vesicles used in the preceding measurements serve well for determining the lipid selectivity of the C2 domains and mutants, they do not fully represent the cytoplasmic plasma membrane and the cytoplasmic nuclear envelope that PKC-α-C2 and for cPLA\textsubscript{2}-C2, respectively, are targeted to. To better understand the subcellular targeting behaviors of the C2 domains, we measured their binding to immobilized vesicles whose lipid compositions recapitulate those of cellular membranes.

The lipid compositions of the cytoplasmic plasma membrane and the cytoplasmic nuclear envelope were predicted from the reported lipid compositions of rat liver cell membranes (44) and by making a few experimentally supported but simplifying assumptions. The assumptions were: (i) that half of the lipid molecules are present in each leaflet; (ii) that a large majority of phosphatidylethanolamine (80%), phosphatidylinositol (80%), and PS (95%) are present in the cytoplasmic leaflet of the plasma membrane, whereas a majority of PC (70%) and sphingomyelin (>90%) are in the outer leaflet (45); and (iii) that all phospholipids are equally distributed between the cytoplasmic and the nucleoplasmic leaflets of nuclear membranes. This simple approximation yielded POPC/POPG/POPS/POPI/cholesterol (12:35:22:9:12, molar ratio) as a cytoplasmic plasma membrane mimic and POPC/POPG/POPS/POPI/cholesterol (61:21:4:7:7, molar ratio) as a cytoplasmic nuclear membrane mimic, respectively. Table III summarizes the parameters for binding of the C2 domains and their respective mutants to these vesicles determined from SPR analysis.

PKC-α-C2 showed about 30-fold higher affinity for the plasma membrane mimic than for POPC/POPG (7:3) vesicles, despite lower PS content in the former. This suggests that the presence of PE and/or cholesterol enhances the membrane affinity of PKC-α-C2. Intriguingly, the mutation of Asn\textsuperscript{189} that is directly involved in PS headgroup interaction resulted in a large 34-fold drop in affinity, whereas the double mutation of Arg\textsuperscript{249} and Arg\textsuperscript{250} led to 5-fold decrease in affinity, which is comparable to the data obtained with POPC/POPG (7:3) vesicles. Thus, it would seem that specific PS coordination is critical for interaction of PKC-α-C2 to the plasma membrane, which was not fully expressed when binding assay was performed with POPC/POPG (7:3) vesicles. Most importantly, PKC-α-C2 and R249A/R252A greatly (i.e. 121-fold and 114-fold, see Table III) preferred the plasma membrane mimic to the nuclear envelope mimic, whereas N189A showed dramatically reduced 10-fold selectivity for the plasma membrane mimic. This suggests that N189A might be targeted to the nuclear envelope in addition to the plasma membrane under certain physiological conditions.

cPLA\textsubscript{2}-C2 also showed higher (i.e. 5-fold) affinity for the nuclear envelope mimic than for POPC vesicles. For the nuclear envelope mimic, L39A, Y96A, and V97A had 100-fold, 310-fold, and 43-fold lower affinity, respectively, than wild type. Importantly, cPLA\textsubscript{2}-C2 exhibited 33-fold selectivity for the nuclear envelope mimic, whereas all mutants of cPLA\textsubscript{2}-C2, including L39A, Y96A, and V97A, had much reduced 3- to 5-fold selectivity. This again suggests that these mutants might be targeted to both nuclear envelope and plasma membrane in the cell. Lastly, affinity of PKC-α-C2 for the plasma membrane mimic is 4.5-fold higher than that of cPLA\textsubscript{2}-C2 for the nuclear envelope mimic due in large part to larger \(k_s\) for PKC-α-C2. This suggests that PKC-α-C2 might respond faster than cPLA\textsubscript{2}-C2 in response to a rise in [Ca\textsuperscript{2+}], under the same conditions.

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TABLE I

**Binding parameters for PKC-α-C2 and mutants determined from SPR analysis**

| Proteins          | \(10^3 k_s\) \(s^{-1}\) | \(10^{-3} k_d\) \(s^{-1}\) | \(K_d\) \(mM\) | PS selectivity* |
|-------------------|--------------------------|----------------------------|----------------|----------------|
| POPC/POPS (70:30) |                          |                            |                |                |
| PKC-α-C2          | 3.2 ± 0.7                 | 0.46 ± 0.01                | 14 ± 3         | 11             |
| N189A             | 1.7 ± 0.4                 | 1.2 ± 0.2                  | 71 ± 2         | 2              |
| R216A             | 1.0 ± 0.3                 | 0.91 ± 0.08                | 91 ± 3         | 10             |
| R249A/R252A       | 0.50 ± 0.08               | 0.43 ± 0.06                | 86 ± 2         | 9              |
| T251A             | 0.90 ± 0.09               | 2.3 ± 0.4                  | 250 ± 50       | 10             |
| POPC/POPG (70:30) |                          |                            |                |                |
| PKC-α-C2          | 3.6 ± 0.6                 | 5.5 ± 0.2                  | 150 ± 30       |                |
| N189A             | 4.1 ± 0.6                 | 5.9 ± 0.5                  | 140 ± 20       |                |
| R216A             | 1.3 ± 0.3                 | 12 ± 2                     | 920 ± 260      |                |
| R249A/R252A       | 0.64 ± 0.07               | 5.1 ± 0.4                  | 800 ± 110      |                |
| T251A             | 0.98 ± 0.07               | 24 ± 5                     | 2400 ± 500     |                |

* PS selectivity is defined as the ratio of \((1/K_d)/K_d\) for POPC/POPG (7:3) to \((1/K_d)/K_d\) for POPC/POPS (7:3).
Membrane Targeting by C2 Domains

Values represent the mean ± S.D. deviation from five determinations. All measurements were performed in 10 mM HEPES, pH 7.4, containing 0.1 M NaCl and 0.1 mM Ca$^{2+}$.

| Proteins | $k_a$ | $k_d$ | $K_d$ | PC selectivity$^a$ |
|----------|-------|-------|-------|------------------|
| POPC     | $10^5$ M$^{-1}$ s$^{-1}$ | $10^{-2}$ s$^{-1}$ | nM    |
| cPLA$_2$-C2 | 3.0 ± 0.3 | 0.33 ± 0.04 | 11 ± 2 | 11 |
| cPLA$_2$-C2 F35A | 0.15 ± 0.04 | 5.9 ± 0.5 | 3900 ± 1100 | 0.7 |
| cPLA$_2$-C2 L39A | 0.57 ± 0.05 | 12 ± 2 | 2100 ± 400 | 0.6 |
| cPLA$_2$-C2 Y96A | 0.21 ± 0.05 | 4.9 ± 0.4 | 2300 ± 600 | 1.1 |
| cPLA$_2$-C2 V97A | 1.4 ± 0.3 | 12 ± 2 | 860 ± 230 | 1 |
| cPLA$_2$-C2 | 0.21 ± 0.04 | 0.25 ± 0.03 | 120 ± 30 | |
| POPS     | $10^5$ M$^{-1}$ s$^{-1}$ | $10^{-2}$ s$^{-1}$ | nM    |
| cPLA$_2$-C2 | 0.077 ± 0.005 | 2.0 ± 0.2 | 2600 ± 300 | 10 |
| cPLA$_2$-C2 L39A | 0.11 ± 0.02 | 1.3 ± 0.3 | 1200 ± 400 | 114 |
| cPLA$_2$-C2 Y96A | 0.042 ± 0.007 | 1.1 ± 0.2 | 2600 ± 600 | 1 |
| cPLA$_2$-C2 V97A | 0.18 ± 0.04 | 1.5 ± 0.3 | 830 ± 200 | 1 |

$^a$ PC selectivity is defined as the ratio of (1/$K_d$) for POPC to (1/$K_d$) for POPS.

| Proteins | $k_a$ | $k_d$ | $K_d$ | Increase in $K_d$$^a$ | PM/NE$^b$ |
|----------|-------|-------|-------|------------------|----------|
| POPC/POPC/POPS/POPC/cholesterol (12:35:22:9:22) | $10^5$ M$^{-1}$ s$^{-1}$ | $10^{-2}$ s$^{-1}$ | nM    | -fold |
| PKC-cC2 | 36 ± 5 | 0.17 ± 0.02 | 0.47 ± 0.09 | 1 | 121 |
| PKC-cC2/N189A | 7.5 ± 0.9 | 1.2 ± 0.2 | 10 ± 3 | 34 | 10 |
| PKC-cC2/R249A/R252A | 6.4 ± 0.8 | 0.14 ± 0.02 | 2.2 ± 0.4 | 5 | 114 |
| cPLA$_2$-C2 | 2.6 ± 0.4 | 1.6 ± 0.3 | 62 ± 15 | 1 |
| cPLA$_2$-C2/L39A | 1.0 ± 0.2 | 9.1 ± 1.1 | 910 ± 210 | 1 |
| cPLA$_2$-C2/Y96A | 0.45 ± 0.6 | 7.2 ± 0.8 | 1600 ± 300 | 1 |
| cPLA$_2$-C2/V97A | 1.8 ± 0.3 | 8.7 ± 0.8 | 540 ± 110 | 1 |
| Plasma membrane mimic: POPC/POPC/POPS/POPC/cholesterol (61:21:4:7:7) | $10^5$ M$^{-1}$ s$^{-1}$ | $10^{-2}$ s$^{-1}$ | nM    | -fold |
| PKC-cC2 | 2.1 ± 0.4 | 1.2 ± 0.2 | 57 ± 14 | 1 |
| PKC-cC2/N189A | 1.5 ± 0.6 | 2.5 ± 0.3 | 160 ± 50 | 1 |
| PKC-cC2/R249A/R252A | 0.55 ± 0.04 | 1.4 ± 0.3 | 250 ± 60 | 1 |
| cPLA$_2$-C2 | 9.8 ± 0.9 | 0.21 ± 0.03 | 21 ± 0.3 | 1 | 1/33 |
| cPLA$_2$-C2/L39A | 2.1 ± 0.4 | 4.5 ± 0.6 | 210 ± 50 | 1/4 |
| cPLA$_2$-C2/Y96A | 0.51 ± 0.06 | 3.3 ± 0.4 | 650 ± 110 | 1/3 |
| cPLA$_2$-C2/V97A | 4.5 ± 0.5 | 4.1 ± 0.4 | 91 ± 13 | 1/6 |

$^a$ The increase (-fold) in $K_d$ relative to the binding of PKC-cC2 to the plasma membrane mimic (for PKC-cC2 mutants) and cPLA$_2$-C2 to the nuclear envelope mimic (for cPLA$_2$-C2 mutants).

$^b$ Ratio of (1/$K_d$) for the plasma membrane mimic to (1/$K_d$) for the nuclear envelope mimic.

Subcellular Translocation of cPLA$_2$-C2 and PKC-cC2—It remains unknown whether specific subcellular localization of C2 domains are due to their different lipid specificities or the presence of specific adapter proteins for individual proteins. To address this question, we transiently transfected HEK293 cells with isolated C2 domains, mutants, full-length cPLA$_2$ and full-length PKC-c, all tagged with EGFP at their carboxyl termini, and determined their spatiotemporal dynamics by time-lapse confocal microscopy. In control experiments, all EGFP-tagged proteins showed the membrane binding affinities comparable to those of their non-tagged counterparts when assayed by SPR analysis under the same conditions (data not shown). Also, transfected cells expressed the corresponding proteins when tested by Western blotting analysis of lysed cells (data not shown). As shown in Fig. 2, both cPLA$_2$-C2-EGFP and PKC-c-C2-EGFP are evenly dispersed in the cytoplasm when cells were incubated in a Ca$^{2+}$-depleted medium. When cells were activated with 2 μM ionomycin that gave rise to ~0.5 μM [Ca$^{2+}$], wild type C2 domains, PKC-c-C2 in particular, showed rapid membrane translocation, but most of the mutants with reduced membrane affinity did not migrate even after 30 min (data not shown). We therefore activated the cells with 10 μM ionomycin and 1 mM CaCl$_2$, which resulted in sustained [Ca$^{2+}$], level at ~70 μM (Fig. 3). It should be noted that, although this

FIG. 2. Subcellular translocation of PKC-c-C2 and cPLA$_2$-C2 upon calcium activation. HEK293 cells treated with Magni-1 were washed with 2 mM EGTA and then overlaid with HEK buffer for PKC-c-C2 (A) or cPLA$_2$-C2 (B). Cells were then activated by adding 10 μM ionomycin and 1 mM calcium. Cell images were taken every 4 s for PKC-c-C2 and every 15 s for cPLA$_2$-C2, respectively.
does not reflect a physiological condition, it allowed quantitative determination of translocation rates of wild type and mutant proteins under the same experimental conditions. Upon calcium activation, PKC-α-C2-EGFP rapidly moved to the plasma membrane, which was synchronized with the \([\text{Ca}^{2+}]_i\) change as observed by mag-indo 1 imaging (Fig. 3). In contrast, cPLA2-C2 moved more slowly to the perinuclear region that includes the nuclear envelope and the endoplasmic reticulum, which is consistent with its lower affinity and smaller \(k_a\) than PKC-α-C2.

Also, full-length PKC-α and cPLA2 showed the same subcellular targeting patterns as their respective C2 domains (Fig. 4). Furthermore, the time dependences of their subcellular localization (data not shown) were reminiscent of those of respective isolated C2 domains shown in Fig. 2. This suggests that subcellular targeting of these proteins is driven by their C2 domains under the given conditions. To further verify this notion we measured the subcellular targeting behaviors of chimera proteins. As shown in Fig. 4, a chimera of PKC-α containing the C2 domain of cPLA2 migrated to the perinuclear region, whereas a chimera of cPLA2 containing the C2 domain of PKC-α translocated to the plasma membrane in response to calcium influx. As was the case with wild type PKC-α and cPLA2, the time dependences of their subcellular localization (data not shown) were similar to those of respective isolated C2 domains (Fig. 2).

We then measured the subcellular localization of C2 domain mutants. For PKC-α-C2, the R249A/R252A mutant translocated to the plasma membrane, whereas N189A with much reduced PS selectivity translocated to both plasma membrane and perinuclear region (Fig. 5). To demonstrate the dual targeting of N189A, we quantitatively determined the cellular distribution of EGFP intensity after \(\text{Ca}^{2+}\) stimulation (Fig. 6). Clearly, PKC-α-C2 is primarily localized to the plasma membrane, while N189A is localized both at the plasma membrane and at the perinuclear region, albeit more abundantly at the plasma membrane. This supports the notion that specific PS coordination by Asn189 plays a key role in the targeting of PKC-α-C2 to the plasma membrane. As was the case with PKC-α-C2, the cPLA2-C2 mutants showed expected cellular behaviors. In this case, all mutants showed dual targeting to the plasma membrane and the perinuclear region (see Fig. 5). Fig. 6 demonstrates the difference in cellular EGFP intensity distribution of cPLA2-C2 and Y96A upon \(\text{Ca}^{2+}\) stimulation.

![Image 1](image1.png)  ![Image 2](image2.png)

**Fig. 3.** Change in \([\text{Ca}^{2+}]_i\) and subcellular translocation of PKC-α-C2 and cPLA2-C2 upon calcium activation. Experimental conditions are the same as described for Fig. 2. A, the time-lapse changes in EGFP intensity ratio at the plasma membrane (= plasma membrane/plasma membrane + cytoplasm) for PKC-α-C2 (○) and R249A/R252A (□) and the change in \([\text{Ca}^{2+}]_i\) in the cytoplasm (●). B, the time-lapse changes in EGFP intensity ratio at the perinuclear region (= nuclear membrane/nuclear membrane + cytoplasm) for cPLA2-C2 (○) and the change in \([\text{Ca}^{2+}]_i\) in the cytoplasm (●).

**Fig. 4.** Subcellular translocation of PKC-α, cPLA2, and chimera proteins. Experimental conditions are the same as described for Fig. 2. Images represent cells expressing PKC-α (A), cPLA2 (B), a cPLA2 chimera harboring the PKC-α-C2 (C), and a PKC-α chimera containing the cPLA2-C2 (D) before (first column) and 10 min after (second column) the addition of 10 μM ionomycin and 1 mM calcium. Membrane translocation kinetic patterns of full-length proteins were similar to those of the respective isolated C2 domains shown in Fig. 2.
Lastly, we determined the membrane translocation rates for the proteins. Those mutants showing dual membrane targeting were excluded in this analysis because of ambiguity involved in determining their membrane targeting rates. As shown in Fig. 7, the R249A/R252A mutant of PKC-α-C2 translocated to the plasma membrane 4.4-fold slower than the wild type, which is consistent with its 5-fold lower affinity (and 6-fold smaller $k_a$) for the plasma membrane mimic than the wild type. Furthermore, cPLA$_2$-C2 migrated to the perinuclear region about 7 times slower than PKC-α-C2 translocated to the plasma membrane, which is in reasonable agreement with the finding that the former has 4.5-fold lower affinity (and 3.7-fold smaller $k_a$) for the nuclear envelope mimic than the latter has for the plasma membrane mimic. In conjunction with the altered in vitro lipid selectivity of cPLA$_2$-C2 and PKC-α-C2 mutants described above, these results support the notion that subcellular targeting by C2 domains is driven by the forces that govern their in vitro membrane binding.

**DISCUSSION**

The membrane binding of proteins involves different types of interactions that depend upon the physicochemical properties of both membrane and protein. Extensive structural and mutational studies of membrane binding proteins have shown that their membrane binding surfaces are composed of cationic, aliphatic, and aromatic residues that drive the membrane binding by electrostatic and non-electrostatic forces (46). Our recent study by surface plasmon resonance analysis indicated that cationic residues primarily accelerate the association of protein to anionic membrane surfaces, whereas aliphatic residues mainly slow the membrane dissociation by penetrating...
into the hydrophobic core of the membrane (29). Aromatic residues, particularly Trp, play a pivotal role in binding to zwitterionic PC membranes (46, 47) by affecting both membrane association and dissociation steps (29). The present study systematically and quantitatively addresses the question as to whether or not these physicochemical principles governing the in vitro membrane binding of membrane targeting domains also determine their subcellular targeting behaviors, using two C2 domains as a model.

Lipid Selectivity of cPLA2-C2 and PKC-α-C2—A large degree of structural variations have been found in the Ca$^{2+}$ binding loops of C2 domains in terms of both primary and tertiary structures (8–14, 48). Mutational (17, 19, 23, 24, 26, 28), labeling (49, 50), and structural (12–14) studies of C2 domains have identified the residues in the Ca$^{2+}$ binding loops that play a key role in membrane binding. The present study shows that cationic residues, Arg$^{249}$ and Arg$^{252}$, in Ca$^{2+}$ binding loops of PKC-α-C2 are involved in its binding to the anionic membrane surface, whereas Asn$^{189}$ plays a critical role in PS selectivity. Arg$^{216}$ and Thr$^{251}$ are also involved in PS binding but not specifically to the lipid headgroup. These results appear to be at odds with the report by Conesa-Zamora et al. (24) in which Thr$^{251}$ was shown to play a much more important role than Asn$^{189}$ in binding to PS-containing membranes as well as in PS-dependent PKC activity. It should be noted, however, that the study by Conesa-Zamora et al. (24) was performed only in the presence of PS-containing membranes: no comparison was made between PS and non-PS membranes. As shown in Table I, we also observed that T251A had the largest impact on the affinity for POPC/POPS (7:3) vesicles. Importantly, however, both T251A and R216A mutations reduced the affinity for POPC/POPS (7:3) and POPC/POPG (7:3) vesicles to comparable degrees. Only the N189A mutation selectively reduced the affinity for PS. This is consistent with the structure of a PKC-α-C2-PS complex, which shows that Arg$^{216}$ and Thr$^{251}$ primarily interact with sn-1 and sn-2 ester carbonyl moieties, respectively, whereas Asn$^{189}$ interacts directly with the serine headgroup (12). Also, a smaller effect of the N189A mutation on PS membrane affinity in the study by Conesa-Zamora et al. (24) might be due in part to the fact that not isolated C2 domains but full-length PKC-α proteins were used. Presumably, the rest of the PKC-α molecule, including the C1 domains, can compensate for the reduced PS affinity caused by the N189A mutation.

In the case of cPLA2-C2, both aliphatic and aromatic residues in Ca$^{2+}$ binding loops are essential for its membrane binding and PC selectivity. Aromatic side chains have been shown to be involved in PC interactions (46, 47); however, the involvement of aliphatic side chains in PC selectivity has not been reported. Further studies are required to understand the origin of this effect.

Subcellular Targeting of cPLA2-C2 and PKC-α-C2—It has been known that conventional PKCs and their C2 domains, including PKC-γ and its C2 domain (37), PKC-α (41), and PKC-α-C2 (20), translocate to plasma membrane in response to [Ca$^{2+}$]$_i$ spikes, whereas cPLA2-C2 and cPLA2 are targeted to the perinuclear region upon Ca$^{2+}$ import (21, 22, 43). However, the origin of these differential subcellular targeting behaviors of PKC C2 domains, cPLA2-C2, and their host proteins is relatively poorly understood. Intuitively, one can assume that the presence of site-specific adaptor proteins for these proteins (51, 52). The present study provides quantitative and structural evidence for an alternative view that holds that differential subcellular targeting behaviors of the C2 domains derive mainly from their different lipid selectivity.

It has been reported that mammalian cell membranes have different lipid compositions (44). In particular, the cytoplasmic plasma membrane contains a high PS content (44, 45, 53, 54), whereas the nuclear membrane is relatively rich in PC (44, 55). In this study, the cytoplasmic leaflets of plasma membrane and nuclear envelope were modeled based on the reported lipid compositions of rat liver cell membranes (44). Although the lipid compositions of these vesicles might not reflect the true lipid compositions of cytoplasmic plasma membrane and nuclear envelope of HEK293 cells, they should serve as good models, because kidney and liver cell membranes have similar lipid compositions (44). Consistent with their distinct lipid selectivity, PKC-α-C2 and cPLA2-C2 demonstrate pronounced specificity for the plasma membrane and nuclear envelope mimics, respectively (121-fold and 33-fold preference, respectively, for their favorite membranes). Agreement between this strict in vitro specificity and highly specific subcellular localization of the C2 domains suggests that their subcellular targeting is driven primarily by membrane-protein interactions. This notion is further supported by the good correlation between altered lipid specificities of PKC-α-C2 and cPLA2-C2 mutants and their subcellular localization. N189A of PKC-α-C2 with markedly reduced selectivity for the plasma membrane mimic showed dual targeting to the plasma membrane and the perinuclear region. Likewise, cPLA2-C2 mutants with lower selectivity for the nuclear envelope mimic exhibited the dual targeting behaviors. Similarly, it was shown that the subcellular targeting patterns of the C2 domains of phospholipase C-β (17) and 5-lipoxygenase (19) can be altered by changing their in vitro lipid selectivity. Lastly, for PKC-α-C2, its R249A/R252A mutant, and cPLA2-C2 that translocate to a single cellular destination, the relative affinity (100:22:22, see Table III) for their favorite membrane mimics agrees reasonably well with their relative membrane translocation rate (100:25:15, see Fig. 7).

In sum, these studies indicate that the specific subcellular targeting of PKC-α-C2 and cPLA2-C2 derives from their phospholipid headgroup specificities and that one can control the subcellular localization of a C2 domain by altering its lipid selectivity. The studies also demonstrate the semi-quantitative correlation between the in vitro membrane binding of C2 domains and their cellular membrane targeting. A more detailed study is underway to delineate the quantitative relation between in vitro membrane binding parameters of C2 domains and their cellular responses. Further studies on other membrane targeting domains will answer the question as to whether or not their subcellular targeting could be also explained by biophysical principles that govern their membrane binding.

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