Molecular Basis of the Specific Subcellular Localization of the C2-like Domain of 5-Lipoxygenase*

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The activation of 5-lipoxygenase (5-LO) involves its calcium-dependent translocation to the nuclear envelope, where it catalyzes the two-step transformation of arachidonic acid into leukotriene A₄, leading to the synthesis of various leukotrienes. To understand the mechanism by which 5-LO is specifically targeted to the nuclear envelope, we studied the membrane binding properties of the amino-terminal domain of 5-LO, which has been proposed to have a C2 domain-like structure. The model building, electrostatic potential calculation, and in vitro membrane binding studies of the isolated C2-like domain of 5-LO and selected mutants show that this Ca²⁺-dependent domain selectively binds zwitterionic phosphatidylcholine, which is conferred by tryptophan residues (Trp138, Trp75, and Trp102) located in the putative Ca²⁺-binding loops. The spatiotemporal dynamics of the enhanced green fluorescence protein-tagged C2-like domain of 5-LO and mutants in living cells also show that the phosphatidylcholine selectivity of the C2-like domain accounts for the specific targeting of 5-LO to the nuclear envelope. Together, these results show that the C2-like domain of 5-LO is a genuine Ca²⁺-dependent membrane-targeting domain and that the subcellular localization of the domain is governed in large part by its membrane binding properties.

Leukotrienes are potent lipid mediators of inflammation and allergic responses (1). 5-Lipoxygenase (5-LO) catalyzes the two-step transformation of arachidonic acid into leukotriene A₄, which then leads to the synthesis of all leukotrienes (2, 3). Because of its critical role in controlling leukotriene production and the potential to block the production of all leukotrienes by 5-LO, 5-LO is critically involved in the production of neutrophils, monocytes, and peritoneal macrophages, whereas it is predominantly located in the nuclei of rat basophilic leukemia cells and mouse bone marrow-derived mast cells and alveolar macrophages (4). However, cell activation leads to the translocation of 5-LO to the nuclear envelope, where 5-LO-activating protein is located (4). Group IV cytosolic phospholipase A₂ (cPLA₂), which is critically involved in the production of arachidonic acid, also translocates to the perinuclear region upon activation by calcium (5). Molecular modeling (6) predicted that the amino-terminal region of 5-LO (~130 amino acids) might form the structure similar to the C2 domain that has been found in many cellular proteins involved in signaling and membrane trafficking (7–10); hereafter, it will be referred to as the 5-LO C2-like domain.

C2 domains share a common fold consisting of an eight-strand antiparallel β-sandwich connected by variable loops, which at one end of the domain form the binding sites for multiple Ca²⁺ ions (7–10). A prototype C2 domain binds Ca²⁺ and mediates Ca²⁺-dependent membrane targeting of proteins. A recent study using the detergent-solubilized inclusion body of the glutathione S-transferase-tagged 5-LO C2-like domain and its mutants indicated that the 5-LO C2-like domain binds calcium ions via several ligands located in the putative calcium-binding loops (6). Also, a cell study using the green fluorescence protein-tagged 5-LO C2-like domain showed that the 5-LO C2-like domain drives the translocation of 5-LO to the nuclear envelope (11). Although these recent reports suggest that the 5-LO C2-like domain might function as a Ca²⁺-dependent membrane-targeting domain, Ca²⁺-dependent membrane binding properties of the isolated 5-LO C2-like domain have not been demonstrated. Furthermore, the mechanism by which the 5-LO C2-like domain specifically targets 5-LO to the nuclear envelope is unknown. This study was undertaken to fully characterize the membrane binding properties of the 5-LO C2-like domain and to identify the structural determinants of its specific nuclear envelope targeting. The model building, electrostatic potential calculation, and in vitro membrane binding studies of the isolated 5-LO C2-like domain and selected mutants establish that the 5-LO C2-like domain is a genuine Ca²⁺-dependent membrane-targeting domain with unique selectivity for zwitterionic phosphatidylcholine (PC), which is conferred by tryptophan residues located in the putative Ca²⁺-binding loops. The spatiotemporal dynamics of the enhanced green fluorescence protein (EGFP)-tagged 5-LO C2-like domain and mutants in living cells also indicate that the PC selectivity of the domain accounts for the specific targeting of 5-LO to the nuclear envelope.
EXPERIMENTAL PROCEDURES

**Materials**—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and N-5-dimethylaminophospholane-1-sulfonyl-1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Phospholipid concentrations were determined by phosphate analysis. The Liposofast (Alabaster, AL) and used without further purification. Phospholipid concentrations were determined by phosphate analysis. The Liposofast (Alabaster, AL) and used without further purification.

**Concentration**—The eluted protein fractions were pooled and dialyzed first against 25 mM HEPES (pH 7.5) for 1 h with 3 changes of dialysis buffer and then on ice for 15 h followed by centrifugation at 50,000 g for 15 min. The pellet was resuspended in the same buffer, centrifuged, and resuspended with stirring (∼2 h) in 50 ml of 50 mM Tris-HCl (pH 8.0) containing 8 μM urea at room temperature. The solubilized protein was purified using a Ni²⁺-nitrilotriacetic acid column (QIAGEN Inc.) according to the manufacturer’s instructions. Briefly, 25 μl of 25 μM 5-LO C2-like domain (final concentration) in 10 mM HEPES (pH 7.4) containing 0.1 mM NaCl in one chamber was equilibrated with 25 μl of the same buffer solution containing varying concentrations of 4CaCl₂ in the other chamber. Dialysis was performed in the presence and absence of POPC vesicles (1 μM). Free ([Ca²⁺]₀) and bound ([Ca²⁺]₀ bound) calcium concentrations were determined by scintillation counting of individual chambers, and the binding isotherms were analyzed by a nonlinear least-squares fit using a Langmuir-type equation, 

\[
[Ca^{2+}]_{\text{bound}} = (n + 1) \times K_d \times ([Ca^{2+}]_0 - [Ca^{2+}]_{\text{bound}})
\]

assuming the presence of n non-interacting calcium-binding sites with apparent dissociation constant K_d.

**Surface Plasmon Resonance (SPR) Measurements**—Preparation of the vesicle-coated Pioneer L1 sensor chip has been described in detail elsewhere (13). The sensor surface was coated with POPC, POPC/POPG (1:1), or POPC/POPS (1:1) vesicles. In control experiments, the fluorescently labeled flow buffer was added to the sensor chip to determine if the specific binding of the 5-LO C2-like domain with spacer sequence GGNSSG. Transfection and Protein Production—Cells (80–90% confluent) in Lab-Tech™ chambered cover glass were exposed to 150 μl of supplemented Dulbecco’s modified Eagle’s medium containing 0.5 μg of toxin-binding dnRNA and 1 μl of LipofectAMINE™ reagent (Invitrogen) for 7–8 h at 37 °C. After exposure, the transfection medium was removed, and the cells were washed with PBS and incubated with 5-LO C2-like domain with spacer sequence GGNSSG. Transfection and Protein Production—Cells (80–90% confluent) in Lab-Tech™ chambered cover glass were exposed to 150 μl of supplemented Dulbecco’s modified Eagle’s medium containing 0.5 μg of toxin-binding dnRNA and 1 μl of LipofectAMINE™ reagent (Invitrogen) for 7–8 h at 37 °C. After exposure, the transfection medium was removed, and the cells were washed with PBS and incubated with the appropriate ligand. The ligand was then washed over the immobilized vesicle surface until the SPR signal reached the initial background value before protein injection. For data acquisition, five or more different concentrations (typically within a 10-fold range above or below the K_d) of each protein were used. After each set of measurements, the entire immobilized vesicles were regenerated by passing over the immobilized vesicle surface until the SPR signal reached the initial background value before protein injection. For data acquisition, five or more different concentrations (typically within a 10-fold range above or below the K_d) of each protein were used. After each set of measurements, the entire immobilized vesicles were regenerated by passing 25 μl of 0.1 M KCl and 0.01 μM CaCl₂ at 5 μl/min, and the sensor chip was re-coated with a fresh vesicle solution for the next set of measurements. All data were evaluated using BIAevaluation Version 3.0 software (BIAcore). For each trial, the signal was corrected against the control surface response to eliminate any refractive index changes due to buffer change. Furthermore, the derivative plot was used to monitor potential mass transport effects. Once these factors were checked for each set of data, the association and dissociation phases of all sensorgrams were globally fit to a 1:1 Langmuir binding model: [protein-vesicle] ↔ protein + vesicle. The association phase was analyzed using the following equation: 

\[
R(t) = (k_d/K_d) \times \frac{[\text{protein}]}{[\text{vesicle}]} + K_d \times \frac{[\text{vesicle}]}{[\text{vesicle}][\text{protein}]} + RI
\]

where RI is the refractive index change, Rₘₐₓ is the theoretical binding capacity, C is analyte concentration, and k_d is the association rate constant. The dissociation phase was analyzed using the equation 

\[
R(t) = R_{\text{diss}} - k_d \cdot t
\]

where k_d is the dissociation rate constant and Rₜ is the initial response. The curve fitting efficiency was checked by residual plots and a. The dissociation constant (K_d) was then calculated from the equation 

\[
K_d = \frac{R_{\text{diss}}}{k_d}.
\]

**Cell Culture**—A stable HEK293 cell line expressing the edysone receptor (Invitrogen) was used for all experiments. Briefly, cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) streptomycin (Gibco, Invitrogen). The culture medium was changed every 2 days. 

**Transfection**—Cells (70–80% confluent) in Lab-Tech™ chambered cover glass were exposed to 150 μl of supplemented Dulbecco’s modified Eagle’s medium containing 0.5 μg of toxin-binding dnRNA and 1 μl of LipofectAMINE™ reagent (Invitrogen) for 7–8 h at 37 °C. After exposure, the transfection medium was removed, and the cells were washed once with fetal bovine serum-supplemented Dulbecco’s modified Eagle’s medium containing Zeocin™ and 140 μg/ml monastranol to induce protein production.

**Confocal Microscopy**—Cell imaging was performed using a four-channel Zeiss LSM 510 laser scanning confocal microscope. To trigger the membrane translocation of EGFP-tagged 5-LO C2-like domains, the

**5-LO C2 Domain**

**Construction of Expression Vectors and Mutagenesis**—For bacterial expression, the cDNA of the 5-LO C2-like domain (resides 1–115) was cloned into the modified pET22b vector with an amino-terminal His-tag. Site-directed mutagenesis was carried out by the overlap extension PCR method. **Bacterial Expression of the 5-LO C2-like Domain**—E. coli strain BL21(DE3) was used as the host for protein expression. One liter of Luria broth supplemented with 100 μM ampicillin was inoculated with 5 ml of overnight culture grown at 37 °C. Cells were grown at 37 °C until the OD₆₀₀ nm reached 0.6 and then protein expression was induced with 0.5 mM isopropyl-β-D-galactopyranoside (Research Products International Corp., Mount Prospect, IL). After overnight incubation at room temperature, cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C. Cells were resuspended in 50 ml of 50 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, 1% (v/v) Triton X-100, 0.1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was treated with 10 cycles of 15 s of sonication, followed by 45 s of incubation on ice. After the suspension was sonicated, the inclusion body pellet was obtained by centrifugation at 50,000 × g for 15 min at 4 °C. The pellet was resuspended in the same buffer, centrifuged, and resuspended with stirring (∼2 h) in 50 ml of 50 mM Tris-HCl (pH 8.0) containing 8 μM urea at room temperature. The solubilized protein was purified using a Ni²⁺-nitrilotriacetic acid column (QIAGEN Inc.) according to the manufacturer’s instructions. The eluted protein fractions were pooled and dialyzed first against 25 mM Tris-HCl (pH 8.0) containing 2 μM urea, followed by the same buffer containing 0.5 μM urea and no urea, respectively, and finally against deionized water. The refolded protein was lyophilized and stored at −20 °C. The lyophilized protein was resuspended in 10 mM HEPES (pH 7.4) for analysis. The protein concentration was measured using the enhanced BCA protein assay (Pierce). The purity of all protein samples was >90% electrophoretically. **Equilibrium Dialysis**—Equilibrium dialysis was performed using a two-chamber Micro-Equilibrium Dialyzer (Harvard Bioscience, Holliston, MA) according to the manufacturer’s instructions. Briefly, 25 μl of 25 μM 5-LO C2-like domain (final concentration) in 10 mM HEPES (pH 7.4) containing 0.1 mM NaCl in one chamber was equilibrated with 25 μl of the same buffer solution containing varying concentrations of 4CaCl₂ in the other chamber. Dialysis was performed in the presence and absence of POPC vesicles (1 μM). Free ([Ca²⁺]₀) and bound ([Ca²⁺]₀ bound) calcium concentrations were determined by scintillation counting of individual chambers, and the binding isotherms were analyzed by a nonlinear least-squares fit using a Langmuir-type equation, 

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cell medium was removed, and the cells were washed with 150 μl of 2 mM EGTA and overlaid with 300 μl of HEK buffer (1 mM HEPES (pH 7.4) containing 2.5 mM MgCl_2, 1 mM NaCl, 0.67 mM KCl, 0.67 mM glucose, and 6.4 mM sucrose) containing 10 μM ionomycin and 2 mM CaCl_2. Control experiments were done with dimethyl sulfoxide in place of ionomycin. EGFP was excited using the 488-nm line of an argon/krypton laser. All experiments were carried out at the same laser power, which was found to induce minimal photobleaching over 30 scans, and at the same gain and offset settings on the photomultiplier tube.

**RESULTS**

**Molecular Modeling and Calcium Binding of the 5-LO C2-like Domain**—Among various C2 and C2-like domains with known tertiary structures, we found two good potential structural templates for the 5-LO C2-like domain: 15-lipoxygenase (Protein Data Bank code 1lox) and the C2-like domain of *C. perfringens* α-toxin (code 1qmd) as structural template was used for the subsequent electrostatic potential calculations. In the homology modeling for the 5-LO C2-like domain was performed using an α-toxin as a structural template. The model is shown as a ribbon diagram, with the three mutated Trp residues and other putative membrane-binding residues shown as green and red stick models, respectively. *Magenta* space-filling models illustrate calcium ions.

The electrostatic properties of the 5-LO C2-like domain homology model were calculated and visualized in the program GRASP (24). In Fig. 7 (A and B), the *red and blue meshes* represent the −25 and +25 mV electrostatic equipotential contours, respectively, in 0.1 M KCl.

Fig. 2. **Homology model of the 5-LO C2-like domain.** The homology modeling for the 5-LO C2-like domain was performed using an α-toxin from *C. perfringens* (Protein Data Bank code 1qmd) as a structural template. The model is shown as a ribbon diagram, with the three mutated Trp residues and other putative membrane-binding residues shown as *green* and *red* stick models, respectively. *Magenta* space-filling models illustrate calcium ions.
with different compositions at 0.1 mM Ca\(^{2+}\) the binding of the 5-LO C2-like domain to immobilized vesicles constants for peripheral proteins (13, 15). We first measured the binding of the His\(^{6}\)-tagged 5-LO C2-like domain was expressed in E. coli as an inclusion body, which was solubilized, refolded, and purified to homogeneity. As shown in Fig. 3, the isolated 5-LO C2-like domain bound about two Ca\(^{2+}\) ions both in the presence and absence of POPC vesicles under our experimental conditions. Although it has been shown that calcium ions bind to some C2 domains in a cooperative manner (27, 28), the calcium binding properties of the isolated 5-LO C2-like domain originate from the 5-LO C2-like domain.

Membrane Binding Properties of the 5-LO C2-like Domain—To establish that the 5-LO C2-like domain is a genuine calcium-dependent membrane-targeting domain, we measured the membrane binding properties of the isolated 5-LO C2-like domain by SPR analysis. SPR analysis allows direct determination of membrane association (ka) and dissociation (kd) rate constants for peripheral proteins (13, 15). We first measured the binding of the 5-LO C2-like domain to immobilized vesicles with different compositions at 0.1 mM Ca\(^{2+}\). As shown in Table I, the 5-LO C2-like domain had higher affinity (in terms of Ka) for zwitterionic PC vesicles than for anionic PS and phosphatidyglycerol vesicles. In this regard, the 5-LO C2-like domain is similar to the cPLA\(_2\) C2 domain, which also has unique PC selectivity (10, 29). This is also consistent with the previous finding that 5-LO binds both membrane protein concentration up to 1 \(\mu\)M, indicating that the 5-LO C2-like domain has a greater than \(\mu\)M affinity for POPC under this condition. The increase in calcium concentration from 1 \(\mu\)M to 0.1 mM resulted in a 500-fold increase in affinity (\(K_d\)), demonstrating its Ca\(^{2+}\)-dependent membrane binding property. As summarized in Table I, the rise in Ca\(^{2+}\) increased \(k_a\) (−34-fold) and decreased \(k_d\) (−16-fold) to a comparable degree.

To further characterize the membrane binding properties of the 5-LO C2-like domain, we measured its interactions with phospholipid monolayers. The monolayer technique has been shown to be a sensitive tool for assessing the relative membrane penetrating ability of peripheral proteins (15, 31). We previously showed that Ca\(^{2+}\) drastically increases the monolayer penetration of the cPLA\(_2\) C2 domain by exposing aliphatic and aromatic residues in the Ca\(^{2+}\)-binding loops (12). As illustrated in Fig. 4, Ca\(^{2+}\) also enhanced the monolayer penetration of the 5-LO C2-like domain, albeit not as drastically as seen with the cPLA\(_2\) C2 domain. This suggests that a role of Ca\(^{2+}\) in the membrane binding of the 5-LO C2-like domain is to induce a local conformational change in the Ca\(^{2+}\)-binding loops (12). In agreement with the PC selectivity seen in the SPR binding data, the 5-LO C2-like domain displayed significantly reduced penetration into anionic phospholipid monolayers in the presence of 0.1 mM Ca\(^{2+}\).

Membrane Binding of 5-LO C2-like Domain Mutants—The model structure of the 5-LO C2-like domain suggests the presence of three surface-exposed tryptophan residues (Trp\(_{13}\), Trp\(_{75}\), and Trp\(_{102}\)) in the Ca\(^{2+}\)-binding loops. Our recent study on the cPLA\(_2\) C2 domain showed that aromatic residues in the Ca\(^{2+}\)-binding loops are involved in its PC selectivity (2). We thus mutated the three tryptophan residues to Ala and measured the membrane interactions of mutants by SPR and monolayer analyses to determine whether or not these residues are involved in the PC selectivity of the 5-LO C2-like domain. As shown in Table I, all mutations reduced the PC affinity of the domain, albeit to different degrees. W13A, W75A, and W102A had 4.5-, 9-, and 20-fold lower affinities for PC in terms of \(K_d\) respectively. For W13A and W75A, the mutations affected \(k_a\) and \(k_d\) to a comparable degree (2-fold), whereas for W102A, the mutation changed \(k_a\) (10-fold) much more significantly than \(k_d\) (2-fold). We previously reported that the mutations of interfacial tryptophan residues influence both \(k_a\) and \(k_d\) values, although changes in \(k_a\) are in general larger than those in \(k_d\) (32). Thus, our mutation data indicate that the three Trp residues (Trp\(_{102}\) in particular) are involved in the membrane binding of the 5-LO C2-like domain, playing a dual role of accelerating the membrane association and elongating the membrane residence time. For anionic POPC/POPS (1:1) membranes, the mutations of the three Trp residues had modest effects, with a <2-fold change in affinity. As a result, three mutants (W13A, W75A, and W102A) showed essentially no preference for PC. The PC selectivity expressed in terms of the ratio of \(K_d\) for PC to \(K_d\) for PS (1:1) varied from 0.5 to 1 for the mutants, whereas it was 4.5 for the wild-type 5-LO C2-like domain. Thus, it is evident that the three Trp residues are involved in the PC selectivity of the 5-LO C2-like domain.

We then measured the monolayer penetration of the mutants. As shown in Fig. 6, W75A and W102A exhibited significantly reduced penetration into the POPC monolayer, whereas

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\(^1\) R. V. Stahelin, J. D. Rafter, S. Das, and W. Cho, submitted for publication.

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**Fig. 3.** Equilibrium calcium binding isotherms of the 5-LO C2-like domain. All measurements were performed in 10 mM HEPES (pH 7.4) containing 0.1 mM NaCl, 25 \(\mu\)M 5-LO C2-like domain, and varying concentrations of Ca\(^{2+}\) in the presence (○) and absence (●) of POPC vesicles (1 \(\mu\)M). Each point represents the average of duplicate measurements. Nonlinear least-squares curve fitting using a Langmuir-type equation yielded \(K_{ca}\) and \(n\) values as follows: \(K_{ca}\) = 8.8 ± 0.4 \(\mu\)M and \(n\) = 2.3 ± 0.1 without PC and \(K_{ca}\) = 6.8 ± 0.5 \(\mu\)M and \(n\) = 2.2 ± 0.1 with 1 \(\mu\)M PC.
Trp residues are involved in specific PC binding. Mutants did not have PC selectivity in monolayer penetration wild-type 5-LO C2-like domain, which clearly preferred the involved in partial penetration into the membrane. Unlike the seven Trp residues (Trp75 and Trp102 in particular) are decrease in monolayer penetration. These data thus indicate the three Trp residues (Trp75 and Trp102 in particular) are associated with bringing the domain close to the membrane.

For binding to anionic membranes, electrostatic repulsion between the negative potential of the domain and anionic lipid head groups would also disfavor the binding. Fig. 7B illustrates that the negative potential in the Ca\(^{2+}\)-binding region is significantly neutralized by the Ca\(^{2+}\) ions so that the desolvation penalty associated with bringing the domain close to the membrane surface is decreased. In particular, the negative potential surrounding the region encompassing Trp13, Trp75, and Trp 102 is dramatically reduced. This would allow Trp residues to penetrate more easily into the membrane in.

Calculation of the Electrostatic Potential—Our SPR and monolayer measurements described above indicated that Ca\(^{2+}\)-induced interactions of surface Trp residues with PC play a major role in the membrane binding of the 5-LO C2-like domain. To account for the origin of this Ca\(^{2+}\)-induced effect, we calculated the electrostatic equipotential profiles of calcium-free and calcium-bound 5-LO C2-like domains. As shown Fig. 7A, the domain has a net charge of −9 and is thus highly negatively charged in the absence of Ca\(^{2+}\). This highly negative potential would provide a high energetic barrier for the domain to bind to membranes due to the high desolvation penalty associated with bringing the domain close to the membrane surface. For binding to anionic membranes, electrostatic repulsion between the negative potential of the domain and anionic lipid head groups would also disfavor the binding. Fig. 7B illustrates that the negative potential in the Ca\(^{2+}\)-binding region is significantly neutralized by the Ca\(^{2+}\) ions so that the desolvation penalty associated with bringing the domain close to the membrane surface is decreased. In particular, the negative potential surrounding the region encompassing Trp13, Trp75, and Trp 102 is dramatically reduced. This would allow Trp residues to penetrate more easily into the membrane interface, and because of the electrostatic considerations described above, the penetration is expected to be more significant for PC than for anionic membranes. As is the case with the cPLA\(_2\) C2 domain (12), the Ca\(^{2+}\) binding can also induce the conformational changes in these surface-exposed Trp side chains to orient themselves for more productive membrane insertion. Last, the 5-LO C2-like domain is still negatively charged even in the calcium-bound state. Thus, it is expected that there will be significant repulsion from anionic membranes, and this could partially contribute to the observed PC selectivity of the domain.

Subcellular Translocation of the 5-LO C2-like Domain and Mutants—To determine the role of the 5-LO C2-like domain in

### Table I

| 5-LO C2-like domain | Lipid | [Ca\(^{2+}\)] | \(k_a\) | \(k_d\) | \(K_d\) |
|---------------------|-------|--------------|--------|--------|--------|
| Wild-type           | PC    | 0.001        | \((5.0 \pm 0.7) \times 10^3\) | \((1.3 \pm 0.2) \times 10^{-2}\) | \((2.5 \pm 0.4) \times 10^{-6}\) |
| Mutants             |       |              |        |        |        |
| Wild-type           | PC    | 0.1          | \((6.7 \pm 0.1) \times 10^5\) | \((4.0 \pm 0.2) \times 10^{-4}\) | \((6.9 \pm 1.7) \times 10^{-10}\) |
| Wild-type           | PC/PS (1:1) | 0.1  | \((3.1 \pm 0.1) \times 10^5\) | \((8.3 \pm 0.2) \times 10^{-4}\) | \((2.7 \pm 0.1) \times 10^{-9}\) |
| Wild-type           | PC/PG (1:1) | 0.1  | \((2.5 \pm 0.1) \times 10^5\) | \((6.3 \pm 0.5) \times 10^{-4}\) | \((2.5 \pm 0.1) \times 10^{-9}\) |
| W13A                | PC    | 0.1          | \((4.2 \pm 0.1) \times 10^5\) | \((1.1 \pm 0.5) \times 10^{-3}\) | \((2.7 \pm 1.3) \times 10^{-9}\) |
| W13A                | PC/PS (1:1) | 0.1  | \((2.5 \pm 0.7) \times 10^5\) | \((6.5 \pm 0.3) \times 10^{-4}\) | \((2.6 \pm 0.7) \times 10^{-9}\) |
| W75A                | PC    | 0.1          | \((2.7 \pm 0.8) \times 10^5\) | \((1.5 \pm 0.1) \times 10^{-3}\) | \((5.6 \pm 1.7) \times 10^{-9}\) |
| W75A                | PC/PS (1:1) | 0.1  | \((1.6 \pm 0.6) \times 10^5\) | \((6.4 \pm 0.2) \times 10^{-4}\) | \((3.3 \pm 1.0) \times 10^{-9}\) |
| W102A               | PC    | 0.1          | \((6.5 \pm 1.5) \times 10^4\) | \((7.8 \pm 0.2) \times 10^{-4}\) | \((1.2 \pm 0.3) \times 10^{-8}\) |
| W102A               | PC/PS (1:1) | 0.1  | \((2.4 \pm 0.2) \times 10^5\) | \((1.3 \pm 0.2) \times 10^{-3}\) | \((5.5 \pm 1.0) \times 10^{-9}\) |
The subcellular localization of 5-LO and also to assess the physiological relevance of our in vitro measurements, we transfected the 5-LO C2-like domain and mutants tagged with EGFP into HEK293 cells and measured their spatiotemporal dynamics by time-lapse confocal microscopy. As shown in Fig. 8, the EGFP-tagged 5-LO C2-like domain was dispersed in the cytoplasm and the nucleus in the resting state. When the cells were activated by the Ca\textsuperscript{2+} ionophore ionomycin, the 5-LO C2-like domain rapidly translocated to the nuclear envelope; the translocation was completed within 5 min. Some residual EGFP-tagged protein signal in the nucleus appears to be due to the saturation of the nuclear envelope by the overexpressed protein. It has been shown that PS is rich in the inner plasma membranes of mammalian cells (33, 34), whereas PC is abundantly present in the nuclear membranes (35). We and others have also shown that PS-selective C2 domains of protein kinase C-\(\alpha\) and phospholipase C-\(\delta\) (36) translocate to the plasma membrane, whereas the PC-selective cPLA\textsubscript{2} C2 domain translocates to the perinuclear region (37, 38). Thus, it would seem that the subcellular localization pattern of the 5-LO C2-like domain is consistent with its PC selectivity. This notion is corroborated by the altered subcellular localization patterns of mutants W75A and W102A, which have no PC selectivity. In contrast to the wild-type 5-LO C2-like domain, these mutants translocate to both the plasma membrane and nuclear envelope in response to Ca\textsuperscript{2+} import. Together, these cell data show that the 5-LO C2-like domain is a genuine Ca\textsuperscript{2+}-dependent membrane-targeting domain, the subcellular localization of which is governed in large part by its membrane binding properties.

**DISCUSSION**

This work represents the systematic in vitro and cell studies on the isolated C2-like domain of 5-LO. Our SPR and monolayer measurements, electrostatic potential calculation, and cell translocation studies show that the 5-LO C2-like domain is a genuine Ca\textsuperscript{2+}-dependent membrane-targeting domain that binds two calcium ions and has PC selectivity. In this regard, the 5-LO C2-like domain is very similar to the cPLA\textsubscript{2} C2 domain. The cPLA\textsubscript{2} C2 domain cooperatively binds two Ca\textsuperscript{2+} ions with an apparent dissociation constant of \(\sim 10 \mu\text{M}\) (27, 28). Our homology modeling suggests the presence of two high affinity Ca\textsuperscript{2+}-binding sites in the 5-LO C2-like domain. It was previously reported that 5-LO binds two Ca\textsuperscript{2+} ions with an apparent dissociation constant of \(\sim 6 \mu\text{M}\) in the presence and absence of PC (26). Our equilibrium dialysis measurements show that the isolated 5-LO C2-like domain also binds two Ca\textsuperscript{2+} ions with comparable affinity (\(K_{\text{D}} = 7-9 \mu\text{M}\) under similar conditions. Furthermore, our results indicate that Ca\textsuperscript{2+} ions play similar roles in the membrane binding of the cPLA\textsubscript{2} C2 and 5-LO C2-like domains. At least three roles have been proposed for the C2 domain-bound Ca\textsuperscript{2+} ions (9, 10): i.e. negative-to-positive electrostatic potential switch, formation of a protein-Ca\textsuperscript{2+}-anionic lipid complex, and induction of conformational changes. For the cPLA\textsubscript{2} C2 domain, Ca\textsuperscript{2+} has been shown to change the side chain orientations of aliphatic and aromatic residues in the Ca\textsuperscript{2+}-binding loops, thereby leading to their membrane insertion and hydrophobic interactions (12, 39, 40). The electrostatic potential calculation also suggested that Ca\textsuperscript{2+} might enhance the binding of the cPLA\textsubscript{2} C2 domain to the PC membrane by charge neutralization, which reduces the desolvation costs associated with bringing the anionic and hence highly solvated domain to the PC membrane surface (41). The present study indicates that Ca\textsuperscript{2+} ions play the same dual role for the 5-LO C2-like domain. Because the 5-LO C2-like domain is highly negatively charged in the absence of Ca\textsuperscript{2+}, the charge neutralization by the Ca\textsuperscript{2+} ions is essential for reducing the desolvation penalty associated with bringing the domain close to the membrane surface. Moreover, our monolayer measurements indicate that Ca\textsuperscript{2+} binding induces the side conformation of the surface-exposed Trp side chains to orient themselves for more productive membrane insertion. A main difference between the two C2 domains is that Ca\textsuperscript{2+} is absolutely required for the monolayer penetration of the cPLA\textsubscript{2} C2 domain, whereas the 5-LO C2-like domain has lower but definite monolayer penetrating power in the absence of Ca\textsuperscript{2+} (see Fig. 4). Thus, it appears that interfacial aliphatic and aromatic residues in the 5-LO C2-like domain are in a semi-productive orientation in the calcium-free state and that the calcium binding causes a smaller scale reorientation. Our previous study showed that interfacial aliphatic residues and Phe largely slow the membrane dissociation, whereas interfacial Tyr and Trp residues affect membrane association more than membrane dissociation (13). In the case of the cPLA\textsubscript{2} C2 domain, calcium largely decreases \(k_{\text{off}}\) as it primarily promotes the membrane penetration of aliphatic residues and Phe in the calcium-binding loops (32). In contrast, calcium affects \(k_{\text{off}}\) more than \(k_{\text{on}}\) presumably because it mainly affects the membrane binding of three Trp residues.

A majority of Ca\textsuperscript{2+}-dependent membrane-binding C2 domains prefer anionic phospholipids to zwitterionic ones because calcium generates positive electrostatic potential on the membrane-binding surface (36, 41), or calcium itself coordinates to an anionic phospholipid(s) (42). To our knowledge, the cPLA\textsubscript{2} C2 and 5-LO C2-like domains are the only C2 domains with PC selectivity. Our recent structure-function study showed that aliphatic and aromatic side chains located in the Ca\textsuperscript{2+}-binding loops are critically involved in the PC selectivity of the cPLA\textsubscript{2} C2 domain. A recent electrostatic potential calculation also showed that the PC selectivity of the cPLA\textsubscript{2} C2 domain can be explained by a significantly higher electrostatic repulsion of anionic cPLA\textsubscript{2} C2 domain molecules at the surface of anionic membranes than at the surface of PC membranes (41). Our results indicate that the PC selectivity of the 5-LO C2-like domain originates from similar factors. The complete
loss of PC selectivity by the mutation of Trp^{13}, Trp^{75}, or Trp^{102} points to their direct interactions with the PC head group. It is also possible, however, that the PC selectivity derives from the fact that it is easier for Trp residues to partially insert their side chains into PC than into anionic phospholipids. Because the bulkier zwitterionic PC head groups are less hydrated and not tethered by intermolecular hydrogen bonds, it is likely that the penetration of the Trp residues into the PC membrane is easier than that into an anionic membrane. As with the cPLA_{2} C2 domain, the 5-LO C2-like domain is anionic even in the calcium-bound state, and it would be repelled from the anionic membrane surface; this should also contribute to the PC selectivity of the domain.

Taken together, the results described herein show great similarities between the cPLA_{2} C2 and 5-LO C2-like domains. We therefore propose that the 5-LO C2-like domain binds to the membrane in such an orientation to optimize the partial membrane insertion of Trp residues, as proposed for the cPLA_{2} C2 domain (10, 12). Although this investigation focuses on three Trp residues, our model structure suggests the presence of other hydrophobic residues, including Phe^{14}, Leu^{76}, and Tyr^{74}, on the putative membrane-binding surface (see Fig. 2) that might also play an important role in membrane binding of the domain. Further structure-function studies are in progress to assess the contributions of these residues to the energetics of membrane binding of the 5-LO C2-like domain.

The mechanisms by which 5-LO is regulated in the cell remain unclear. This study indicates that Ca^{2+}-dependent membrane binding properties of the 5-LO C2-like domain (PC selectivity in particular) govern its subcellular localization behaviors. Although the exact lipid composition of different cellular membranes of HEK293 cells has not been determined yet, it is expected from the known lipid compositions of mammalian subcellular membranes that the inner plasma membranes of HEK293 cells are rich in PS and that the perinuclear membranes, including the nuclear envelope, contain higher PC content and lower anionic lipids (33–35). In response to Ca^{2+} import, the 5-LO C2-like domain, with PC selectivity, translocates to the PC-abundant nuclear envelope. W75A and W102A, which have little PC selectivity, are localized to both the plasma membrane and the nuclear envelope, supporting the notion that the specific targeting of the 5-LO C2-like domain to the nuclear envelope is due to its PC selectivity.

In summary, this work establishes the 5-LO C2-like domain as a genuine Ca^{2+}-dependent membrane-targeting module that has distinct membrane binding properties and that plays a major role in the subcellular localization behaviors of 5-LO. As such, this study lays the foundation for further investigation of the complex mechanism of membrane targeting and activation of 5-LO, which involve calcium, ATP, protein phosphorylation, and presumably protein-protein interactions.

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