Membrane Targeting of C2 Domains of Phospholipase C-δ Isoforms*

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The C2 domain is a Ca$^{2+}$-dependent membrane-targeting module found in many cellular proteins involved in signal transduction or membrane trafficking. To understand the mechanisms by which the C2 domain mediates the membrane targeting of PLC-δ isoforms, we measured the in vitro membrane binding of the C2 domains of PLC-δ1, -δ3, and -δ4 by surface plasmon resonance and monolayer techniques and their subcellular localization by time-lapse confocal microscopy. The membrane binding of the PLC-δ1-C2 is driven by nonspecific electrostatic interactions between the Ca$^{2+}$-induced cationic surface of protein and the anionic membrane and specific interactions involving Ca$^{2+}$, Asn$^{647}$, and phosphatidylserine (PS). The PS selectivity of PLC-δ1-C2 governs its specific Ca$^{2+}$-dependent subcellular targeting to the plasma membrane. The membrane binding of the PLC-δ3-C2 also involves Ca$^{2+}$-induced nonspecific electrostatic interactions and PS coordination, and the latter leads to specific subcellular targeting to the plasma membrane. In contrast to PLC-δ1-C2 and PLC-δ3-C2, PLC-δ4-C2 has significant Ca$^{2+}$-independent membrane affinity and no PS selectivity due to the presence of cationic residues in the Ca$^{2+}$-binding loops and the substitution of Ser for the Ca$^{2+}$-coordinating Asp in position 717. Consequently, PLC-δ4-C2 exhibits unique prelocalization to the plasma membrane prior to Ca$^{2+}$ import and non-selective Ca$^{2+}$-mediated targeting to various cellular membranes, suggesting that PLC-δ4 might have a novel regulatory mechanism. Together, these results establish the C2 domains of PLC-δ isoforms as Ca$^{2+}$-dependent membrane targeting domains that have distinct membrane binding properties that control their subcellular localization behaviors.

Mammalian phosphatidylinositol-specific phospholipases C (PLC)$^1$ are responsible for converting phosphatidylinositol 4,5-

bispahosphate (Ins(4,5)P$_2$) into diacylglycerol and inositol 1,4,5-trisphosphate (IP$_3$), which promote the activation of protein kinases C (PKC) and the release of Ca$^{2+}$ from intracellular stores, respectively (1, 2). The PLC family comprises eleven isoforms that can be subdivided into four types ($\beta$, $\gamma$, $\delta$, and $\epsilon$) based on their structural differences. All PLC isoforms except newly discovered PLC-ε possess three regulatory domains: PH, EF-hand, and C2 (2). Among PLC isoforms, Ca$^{2+}$-sensitive PLC-δ1 has been the subject of extensive structure-function studies due to the availability of tereply structural information. Crystallographic structures of PLC-δ1 lacking the aminoterminal PH domain (3) and of its isolated PH domain (4) revealed a four-module organization of the enzyme comprising the amino-terminal PH domain, the EF-hand domain, catalytic domain, and the carboxy-terminal C2 domain. Based on these structures, it was proposed that PH and C2 domains, both of which are well-characterized membrane-targeting domains, are involved in the membrane targeting of PLC-δ1 (3). The PH domain is a $\beta$-barrel-like structure that is present in many membrane-binding proteins (5–7). The essential role of the PH domain in the membrane targeting of PLC-δ1 has been experimentally demonstrated both in vitro and in vivo (8). The PH domain of PLC-δ1 is capable of anchoring the protein to the membrane by specifically binding to Ins(4,5)P$_2$ in the membrane, and the competitive binding of the PH domain to soluble IP$_3$ can induce the membrane dissociation of PLC-δ1. However, the role of the C2 domain in PLC-δ1 catalysis remains unclear.

The C2 domain has been identified in many cellular proteins involved in signal transduction or membrane trafficking (9–11). Many C2 domains bind Ca$^{2+}$ and mediate Ca$^{2+}$-dependent membrane targeting of proteins. Structural analyses of multiple Ca$^{2+}$-dependent membrane-binding C2 domains have shown that they share a common fold consisting of an eight-stranded antiparallel $\beta$-sandwich connected by variable loops, which form the binding sites for multiple Ca$^{2+}$ ions at one end of the domain (3, 12–17). The crystal structure of the PLC-δ1 C2 domain reveals three metal binding sites in the loop region (18, 19), which led to the proposal that the C2 domain is involved in the Ca$^{2+}$-dependent membrane targeting of the protein. However, a mutant of PLC-δ1 lacking the C2 domain Ca$^{2+}$ binding sites showed the same activity toward Ins(4,5)P$_2$ in phosphatidylycerol (PC)-containing vesicles and micelles as the wild type, suggesting the Ca$^{2+}$ requirement of PLC-δ1 largely reflects the binding of Ca$^{2+}$ to the active site (20). More recently, it was shown that, in the presence of phosphatidylserine (PS) in the assay mixture, the C2 domain plays a key role in the activation of PLC-δ1 through the formation of a C2-Ca$^{2+}$-PS ternary complex (21). To better understand the role

SPR, surface plasmon resonance; CHAPS, 3-[3-cholamidopropyl]dimethylammoniio]-1-propanesulfonic acid; Ni-NTA, nickel-nitrilotriacetic acid.

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‡These abbreviations are used: PLC, phospholipases C; BSA, bovine serum albumin; cPLA$_2$, cytosolic phospholipase A$_2$; DMEH, Dulbecco’s modified Eagle’s medium; EGFP, enhanced green fluorescence protein; FBS, fetal bovine serum; GST, glutathione S-transferase; IP$_3$, inositol 1,4,5-trisphosphate; PC, phosphatidylycerol; PKC, protein kinase C; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; PS, phosphatidylserine; Ins(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate;
of the C2 domain in the membrane targeting and activation of PLC-δ1 and other isoforms, we measured the binding of the C2 domains of PLC-δ1, -δ3, -δ4, and their mutants to model membranes, and analyzed the binding in terms of the electrostatic properties of the domains. We also measured the spatiotemporal dynamics of enhanced green fluorescence protein (EGFP)-tagged C2 domains and mutants in living cells. Results described herein establish the C2 domains of PLC-δ isoforms as Ca \(^{2+}\)-dependent membrane targeting domains that have distinct membrane binding properties, which in turn control their subcellular localization behaviors. Together, these studies shed new light on the roles of the C2 domains in the membrane targeting and activation of PLC-δ isoforms.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Ins \((4,5)P_2\) was from Calbiochem (San Diego, CA). Tritiated Ins \((4,5)P_2\) \(([^3]H)Ins(4,5)P_2\) was purchased from PerkinElmer Life Sciences (Boston, MA). Phospholipid concentrations were determined by phosphate analysis (22). The BSA fraction containing 100-mM polyethylene glycol was from Avastin (Ontario, Canada). Fatty acid-free bovine serum albumin (BSA) was from Bayer (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 Chemical Co. and Fisher Scientific, respectively. Pioneer L1 sensor chip was from BIACORE AB (Piscataway, NJ). Ionomycin was from Calbiochem (San Diego, CA). Zeocin and pmonasterone A were from Invitrogen (Carlsbad, CA).

**Construction of Expression Vectors and Mutagenesis**—To subclone the cDNA of PLC-δ1 into the pGEX-4T-1 vector (Amersham Biosciences, Inc., Piscataway, NJ) that contains the amino-terminal glutathione S-transferase (GST) sequence, the start codon was removed and new restriction sites \((Sma I, Sma I, and Xho I)\) were introduced in the gene by overlap extension PCR mutagenesis using \(Pfu\) polymerase (Stratagene, La Jolla, CA). Using \(Nde I\) and \(HindIII\) sites, the cDNAs of PLC-δ3 and PLC-δ4 were subcloned into the pET28a vector (Novagen, Madison, WI) that encodes the amino-terminal His tag and thrombin cleavage site \((M_{\text{His-tag}}=22000\text{Da})\). The GST fusion protein was finally cloned into the pGEX-4T-1 vector. The first major peak was collected from 100-mM urea and then against 50 mM Tris-HCl, pH 8.0. The refolded C2 domain was purified using a Ni-NTA column (Qiagen) according to the manufacturer’s instructions. Purity of all protein samples was higher than 90% electrophoretically. Aliquots of purified protein were stored at \(-20^\circ\). A solution of C2 domain in 50-mM Tris-HCl buffer, pH 8.0, containing 1 M NaCl. Insoluble matter was removed by centrifugation at 100,000 \(\times g\) for 15 min at 4 \(^\circ\). The supernatant was loaded onto a Sephadex G25 column \((2.5 \times 25\text{ cm})\) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 1 M NaCl and 5 mM EDTA. The major peak was collected (35 ml), analyzed against 50 mM Tris-HCl, pH 8.0, containing 1 M NaCl and then against 50 mM Tris-HCl, pH 8.0. The refolded C2 domain was purified using a Ni-NTA column (Qiagen) according to the manufacturer’s instructions. Purity of all protein samples was higher than 90% electrophoretically. Aliquots of purified protein were stored at \(-20^\circ\).

**Determination of PLC Activity**—Activity of PLC was assayed by measuring the initial rate of \(\text{Ins}(4,5)P_2,\), hydrolysis as described by Cifuentes et al. (23) with some modifications. Small unilamellar vesicles \((500 \mu\text{M})\) containing 1% \(\text{Ins}(4,5)P_2,\), a trace of \([{^3}H]\text{Ins}(4,5)P_2\) \((2 \times 10^4 \text{ dpm})\) and bulk phospholipids (POPC, POPS, or POPS; each 495 \(\mu\text{M}\)) were prepared in 10 ml HEPES buffer, pH 7.0, containing 0.1 M KCl, 500 \(\mu\text{g/ml}\) BSA, and 0.5 M Ca\(^{2+}\). Free calcium concentration was maintained by adding a mixture of EGTA and CaCl\(_2\), and a method of Bres (24). The reaction was initiated by adding the indicated amount of enzyme, continued for 5 min, and quenched by adding 0.25 ml of 10% ice-cold trichloroacetic acid and 25 \(\mu\text{g}\) of 10% Triton X-100 ml. Samples were kept on ice for 15 min, and the precipitate containing \([{^3}H]\text{Ins}(4,5)P_2\) was separated from the supernatant containing \([{^3}H]\text{IP}_3\) by centrifugation at 12,000 \(\times g\) for 2 min at 4 \(^\circ\). To the supernatant, 0.5 ml of CHCl\(_3/\text{MeOH}\) \((2:1)\) was added and the aqueous phase containing \([{^3}H]\text{IP}_3\) was extracted. Radioactivity of the hydrolyzed product was measured by liquid scintillation counting.

**Monolayer Measurements**—Surface pressure \((\pi)\) of solution in a circular Teflon trough was measured using a Wilhelmy plate attached to a computer-controlled tensiometer (25). The trough (4 cm diameter \(\times 1\) cm depth) has a 0.5-cm deep well for a magnetic stir bar and a small hole drilled at an angle through the wall to allow an addition of protein solution. A solution of ten milliliters of phospholipid solution in ethanol/hexane \((1:9, v/v)\) was spread onto 10 ml of subphase \((20 \text{ mM Tris-HCl, pH 7.5, containing 0.1 mM KCl and 0.5 mM free Ca}^{2+})\) to form a monolayer with a given initial surface pressure \((\pi_1)\). The subphase was continuously stirred at 60 rpm with a magnetic stir bar. Once the surface pressure \((\pi)\) had been stabilized about 5 min, \(50 \mu\text{l}\) of a protein solution \((\text{typically } 50 \mu\text{l})\) was injected into the subphase through the hole, and the change in surface pressure \((\Delta\pi)\) was measured as a function of time. Typically, the \(\Delta\pi\) value reached a maximum after 20 min. The maximal \(\Delta\pi\) depended on the protein concentration at the low concentration range and reached saturation when the protein concentration was higher than 5 \(\mu\text{g/ml}\). Protein concentrations in the subphase were then calculated from such values to ensure the observed \(\Delta\pi\) represented a maximal value.

**SPR Measurements**—The preparation of vesicle-coated Pioneer L1 sensor chip (BIACore) was described in detail elsewhere (26). The sensor surface was coated with POPC/POPS (7:3) or POPC/POPG (7:3) vesicles. In control experiments, the fluorescence intensity of the flow buffer after rinsing the sensor chip coated with vesicles incorporating 5-carboxyfluorescien (Molecular Probes) was monitored to confirm that the vesicles remained intact on the chip. All experiments were performed with a second sensor cell in which a second sensor surface was coated with POPC, because all C2 domains of PLC-δ isoforms showed negligible binding to POPC-coated chip. The drift in signal for both sample and control flow cells was allowed to stabilize below 0.3 resonance unit/min before any kinetic experiments were performed. All kinetic experiments were performed at 24 \(^\circ\), and a flow rate of 60 \(\mu\text{ml/min}\) in 10 ml HEPES, pH 7.4, containing 0.1 M NaCl and varying concentration of Ca\(^{2+}\). A high flow rate was used to circumvent mass transport effects. The association was monitored for 90 s and dissociation for 4 min. The immobilized vesicle surface was then regenerated for subsequent measurements using 10 ml of 50 mM NaOH. The generation signal was monitored for 4 min. 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 removed by injection of 25 \(\mu\text{M}\) of 40 mM CHAPS, followed by 25 \(\mu\text{M}\) of octyl glucoside at 5 \(\mu\text{M}\), and the sensor chip was re-coated with a fresh...
vesicle solution for the next set of measurements. All data were evaluated using BIAevaluation 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 verified, the fitted data were exported for further analysis. From the set of fitted data, the association and dissociation phases of all sensograms were globally fit to a 1:1 Langmuir binding model: [protein × vesicle] => protein + vesicle. The association phase was analyzed using an equation, \( R = \frac{R_\text{max}}{1 + \frac{K_\text{d}}{[\text{protein}]}} \) where \( R_\text{max} \) is the theoretical binding capacity, \( K_\text{d} \) is the dissociation constant, and \( [\text{protein}] \) is the time at start of fit data. The dissociation phase was analyzed using an equation, \( R = R_\text{eq} \cdot e^{-\frac{K_\text{d}}{[\text{protein}]}} \) where \( K_\text{d} \) is the dissociation rate constant and \( R_\text{eq} \) is the response at the start of fit data. The curve fitting efficiency was checked by residual plots and \( x^2 \). The dissociation constant (\( K_d \)) was then calculated from the equation, \( K_d = R_\text{eq} \cdot K_\text{d} \).

Construction of Gene Constructs of C2 Domains Fused with EGF-A

All constructs were ligated into the modified pLND vector (Invitrogen). An amino-terminal EGFP fusion was found to yield higher gene expression than the carboxy-terminal counterpart. The spacer sequence between EGF and the gene was AAA.

Cell Culture—A stable HEK293 cell line expressing the ecdysone receptor (Invitrogen) was used for all experiments. Briefly, cells were cultured and induced in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% \( CO_2 \) and 98% humidity until 90% confluent. Cells were passaged into eight wells of a Lab-Tech-chambered coverglass for later transfection and visualization. Before transfection, the transfected medium was removed, and the cells were washed once with FBS-supplemented DMEM containing 0.5 g of endotoxin-free DNA and 1 g 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 FBS-supplemented DMEM, and overlaid with FBS-supplemented DMEM containing Zeocin and 140 \( \mu \text{g} \text{ml}^{-1} \) palonosetron A to induce protein production.

Confocal Microscopy—Images were obtained using a four-channel Zeiss 510 laser scanning confocal microscope. 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. An LP 505 filter was used on channel 1 for all experiments. A \( \times 63 \) magnification, 1.2 numerical aperture water immersion objective was used for all experiments. Cells for imaging were selected based on their initial intensity, which needed to fall in the upper third of the photomultiplier tube’s range. The 510 imaging software provides an option for time series imaging and was used to control the time intervals for imaging. \( Ca^{2+} \)-dependent translocation of C2 domains was monitored as follows: Thirty minutes before imaging, the cells were treated with 2 g of Fura Red AM (Molecular Probes). Immediately before imaging, induction media were removed and the cells were washed with 150 g of Fura Red AM (Molecular Probes). Imaging was then started with imaging media and using the 488-nm line of an argon/krypton laser. All experiments were carried out in 0.1 M KCl. All homology models were built based on the alignment of the sequence being modeled to the PLC-\( \delta \)-C2 homology model. The curve fitting efficiency was checked by residual plots and \( x^2 \). The dissociation constant (\( K_d \)) was then calculated from the equation, \( K_d = R_\text{eq} \cdot K_\text{d} \).

RESULTS

Membrane Binding of PLC-\( \delta \)-C2 Domains—Four distinct PLC-\( \delta \) isoforms have been identified so far (2). Although all four isoforms are homologous in general, some sequence variations are noticed in the calcium-binding loops in the C2 domains (see Fig. 1). For instance, PLC-\( \delta \)-C2 has Ser in place of a calcium-ligating Asp residue (i.e. Asp2108 for PLC-\( \delta \)). To determine how these variations affect the membrane binding properties of the C2 domains, we measured the membrane binding of the C2 domains of PLC-\( \delta \)-1, -3, and -4. Calcium ligating aspartates and mutated residues are shown in boldface characters.

The program CHARMM (29). The structures with hydrogens were subjected to conjugate gradient minimization with a harmonic restraint force of 50 kcal/mol A\(^2\) applied to the heavy atoms located at the original crystallographic coordinates to minimize atomic clashes. Each model was evaluated using the program Verify 3D (30), which scores structures according to how well each residue fits into its structural environment based on criteria derived from statistical analyses of the Protein Data Bank; all models scored well relative to the PLC-\( \delta \)-1 C2 domain structure. Rather than build separate models for both the Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms of PLC-\( \delta \)-C2 and PLC-\( \delta \)-4C2 and to facilitate comparison of their electrostatic properties, we assumed that the structures in the absence of Ca\(^{2+}\) are similar to those in its presence, and the calcium-free forms of the C2 domains were derived from the Ca\(^{2+}\)-bound models by deleting the Ca\(^{2+}\) ions from the models’ coordinate files. This assumption is consistent with the studies of PLC-\( \delta \)-1 (31). Confirming this, our results for Ca\(^{2+}\)-free PLC-\( \delta \)-C2 are insensitive to whether we used the Ca\(^{2+}\)-free structure (1isd) or deleted the Ca\(^{2+}\) ions from the Ca\(^{2+}\)-bound form.

Membrane Targeting of PLC-\( \delta \)-C2 Domains—With 10% fetal bovine serum (FBS) at 37 °C.

Electrostatic equipotential contours in 0.1 M KCl. All homology models were built based on the alignment of the sequence being modeled to the PLC-\( \delta \)-C2 homology model. The curve fitting efficiency was checked by residual plots and \( x^2 \). The dissociation constant (\( K_d \)) was then calculated from the equation, \( K_d = R_\text{eq} \cdot K_\text{d} \).

Membrane Binding of PLC-\( \delta \)-C2 Domains—Four distinct PLC-\( \delta \) isoforms have been identified so far (2). Although all four isoforms are homologous in general, some sequence variations are noticed in the calcium-binding loops in the C2 domains (see Fig. 1). For instance, PLC-\( \delta \)-C2 has Ser in place of a calcium-ligating Asp residue (i.e. Asp2108 for PLC-\( \delta \)). To determine how these variations affect the membrane binding properties of the C2 domains, we measured the membrane binding of the C2 domains of PLC-\( \delta \)-1, -3, and -4 isoforms by SPR analysis. We have shown that the SPR analysis allows direct determination of membrane association (\( k_a \)) and dissociation (\( k_d \)) rate constants for peripheral proteins (25, 26). We first measured the binding of PLC-\( \delta \)-C2 to immobilized POPC/POPS (7:3) vesicles as a function of Ca\(^{2+}\) concentration (see Table I). In the absence of Ca\(^{2+}\) (i.e. 0.1 mM EGTA), no appreciable binding was detected with protein concentration up to 1 \( \mu \text{M} \), indicating that PLC-\( \delta \)-C2 has >\( \mu \text{M} \) affinity for POPC/POPS (7:3) under this condition. As illustrated in Fig. 2, an increase in Ca\(^{2+}\) from 0.5 \( \mu \text{M} \) to 0.5 mM resulted in a 50-fold increase in the affinity (\( K_d \)) of PLC-\( \delta \)-C2 for POPC/POPS (7:3), demonstrating that it is a Ca\(^{2+}\)-dependent membrane target domain. Interestingly, Ca\(^{2+}\) affected both \( k_a \) (−6-fold) and \( k_d \) (−9-fold) to a comparable degree. Our previous study indicated that nonspecific electrostatic interactions primarily accelerate the association of protein to anionic membrane surfaces, whereas hydrophobic interactions and specific interactions, whether electrostatic interactions or hydrogen bonding, mainly slow the membrane dissociation (26). Thus, it appears that Ca\(^{2+}\) ions are involved in both nonspecific electrostatic interactions and specific and/or hydrophobic interactions. As was the case with PLC-\( \delta \)-C2, PLC-\( \delta \)-C2 (up to 1 \( \mu \text{M} \)) showed no detectable affinity for immobilized POPC/POPS (7:3) vesicles in the absence of Ca\(^{2+}\). Again, the membrane affinity of PLC-\( \delta \)-C2 increased as a function of Ca\(^{2+}\). For this
C2 domain, increasing the Ca\(^{2+}\) concentration from 0.5 \(\mu M\) to 0.5 mM led to a more pronounced 280-fold increase in affinity. Unlike the case with PLC-δ1-C2, Ca\(^{2+}\) affected \(k_{d}\) (90-fold) much more significantly than \(k_{a}\) (3-fold) (see Fig. 3), suggesting that for PLC-δ3-C2 the primary role of Ca\(^{2+}\) is to enhance nonspecific electrostatic interactions with the anionic membrane surface. Lastly, we measured the Ca\(^{2+}\)-dependence of membrane binding of PLC-δ4-C2. In contrast to PLC-δ1-C2 and PLC-δ3-C2 that lack the Ca\(^{2+}\)-independent membrane affinity, PLC-δ4-C2 showed relatively high affinity for immobilized POPC/POPS (7:3) vesicles in the absence of Ca\(^{2+}\) (see Table 1). Also, raising the Ca\(^{2+}\) level to 0.5 mM resulted in an 18-fold increase in \(k_{a}\) and an 1.3-fold decrease in \(k_{d}\); hence, resulting in a 23-fold decrease in \(K_{d}\). These results indicate that, although PLC-δ4-C2 has high Ca\(^{2+}\)-independent membrane affinity, Ca\(^{2+}\) further promotes its membrane binding by enhancing the nonspecific electrostatic interactions with the anionic membrane surface.

We then measured the PS selectivity of the three C2 domains in the presence of 0.5 mM Ca\(^{2+}\). As shown in Table I, PLC-δ1-C2 clearly has PS selectivity: It binds POPC/POPG (7:3) membranes 10-fold more strongly than POPC/POPS (7:3) membranes. The difference in \(K_{d}\) originates from a 2-fold larger \(k_{a}\) and a 5-fold smaller \(k_{d}\) for PS-containing membranes. PLC-δ3-C2 also prefers PS to PG, albeit to a smaller degree (i.e., 5-fold smaller \(K_{d}\) for PS), which was exclusively due to smaller \(k_{d}\). The fact that the PS preferences of PLC-δ1-C2 and PLC-δ3-C2 derive primarily from \(k_{d}\) effects indicates that PS is involved in specific interactions with these C2 domains, which enhances their membrane affinity primarily by slowing the membrane dissociation step. Unlike these two C2 domains, PLC-δ4-C2 did not show PS selectivity. Given that PLC-δ4-C2

### Table I

| Lipid        | [Ca\(^{2+}\)] (M) | \(k_{a}\) \(s^{-1}\) | \(k_{d}\) \(s^{-1}\) | \(K_{d}\) \(\mu M\) |
|--------------|------------------|-----------------|-----------------|----------------|
| PLC-δ1-POPC/POPS 0 0.0005 | (1.3 ± 0.2) × 10⁶ | (1.5 ± 0.2) × 10⁻² | (1.2 ± 0.2) × 10⁻⁷ |
| PLC-δ1-POPC/POPS 0.001 | (1.6 ± 0.3) × 10⁶ | (5.5 ± 0.2) × 10⁻³ | (3.4 ± 0.6) × 10⁻⁸ |
| PLC-δ1-POPC/POPS 0.01 | (3.1 ± 1.5) × 10⁶ | (4.1 ± 1.8) × 10⁻³ | (1.3 ± 0.8) × 10⁻⁸ |
| PLC-δ1-POPC/POPS 0.1 | (5.9 ± 1.2) × 10⁶ | (1.4 ± 0.3) × 10⁻³ | (2.3 ± 0.7) × 10⁻⁸ |
| PLC-δ1-POPC/POPS 0.5 | (7.3 ± 2.9) × 10⁶ | (1.7 ± 0.6) × 10⁻³ | (2.3 ± 1.2) × 10⁻⁹ |
| PLC-δ1-POPC/POPS 0.5 | (7.3 ± 2.9) × 10⁶ | (1.7 ± 0.6) × 10⁻³ | (2.3 ± 1.2) × 10⁻⁹ |
| PLC-δ3-POPC/POPS 0 0.0005 | (5.5 ± 0.3) × 10⁶ | (4.0 ± 0.9) × 10⁻³ | (7.3 ± 1.5) × 10⁻⁷ |
| PLC-δ3-POPC/POPS 0.001 | (9.4 ± 0.4) × 10⁴ | (3.8 ± 0.6) × 10⁻³ | (4.0 ± 0.7) × 10⁻⁸ |
| PLC-δ3-POPC/POPS 0.01 | (2.7 ± 0.9) × 10⁵ | (2.3 ± 0.6) × 10⁻³ | (8.3 ± 3.5) × 10⁻⁹ |
| PLC-δ3-POPC/POPS 0.1 | (2.4 ± 0.4) × 10⁵ | (1.6 ± 0.8) × 10⁻³ | (6.7 ± 3.5) × 10⁻⁹ |
| PLC-δ3-POPC/POPS 0.5 | (5.0 ± 1.0) × 10⁶ | (1.3 ± 0.1) × 10⁻³ | (2.6 ± 0.5) × 10⁻⁸ |
| PLC-δ3-POPC/POPS 0.5 | (4.9 ± 0.4) × 10⁶ | (6.2 ± 0.2) × 10⁻³ | (1.3 ± 0.1) × 10⁻⁸ |
| PLC-δ4-POPC/POPS 0 0.001 | (2.8 ± 0.9) × 10⁴ | (2.5 ± 0.4) × 10⁻³ | (8.9 ± 4.1) × 10⁻⁸ |
| PLC-δ4-POPC/POPS 0.01 | (2.7 ± 0.4) × 10⁵ | (3.6 ± 1.1) × 10⁻³ | (1.4 ± 0.5) × 10⁻⁸ |
| PLC-δ4-POPC/POPS 0.1 | (4.0 ± 1.3) × 10⁵ | (9.8 ± 2.5) × 10⁻⁴ | (2.4 ± 0.9) × 10⁻⁹ |
| PLC-δ4-POPC/POPS 0.5 | (5.1 ± 0.9) × 10⁶ | (3.2 ± 0.3) × 10⁻³ | (6.2 ± 1.2) × 10⁻⁹ |
| PLC-δ4-POPC/POPS 0.5 | (3.9 ± 0.3) × 10⁵ | (3.5 ± 0.2) × 10⁻³ | (8.8 ± 0.9) × 10⁻⁸ |

\(\text{NM}\) not measurable.
has the lowest degree of Ca\(^{2+}\) dependence in PS binding, these data suggest that PLC-δ1-C2 and PLC-δ3-C2 specifically coordinate a PS molecule via Ca\(^{2+}\).

To further characterize the membrane binding properties of the three C2 domains, we measured their interactions with POPC/POPS (7:3) monolayers in the presence and absence of Ca\(^{2+}\). The monolayer technique has been shown to be a sensitive tool for assessing the relative membrane penetrating ability of peripheral proteins (25, 31). As shown in Fig. 4, all PLC-δ-C2 domains showed significantly lower monolayer penetration than the C2 domain of cytosolic phospholipase A\(_2\) (cPLA\(_2\)) that has been shown to penetrate into the membrane. Ca\(^{2+}\) and PS did not have much effect on the monolayer penetration of PLC-δ-C2 domains (data not shown). Thus, the membrane binding of PLC-δ-C2 domains does not seem to involve significant membrane penetration and hydrophobic interactions. The predominantly electrostatic nature of membrane binding of PLC-δ-C2 domains is further supported by the SPR binding data showing the lack of appreciable binding of the C2 domains to immobilized POPC/POPS (7:3) membranes in the presence of 0.5 mM NaCl (with 0.5 mM Ca\(^{2+}\)) (data not shown).

**PS-dependent Activities of PLC-δ Isoforms**—It was shown that PS enhances the enzymatic activity of PLC-δ1 via the formation of a C2-Ca\(^{2+}\)-PS complex (21). Differential PS selectivity of the PLC-δ-C2 domains seen in our membrane binding measurements predicts that the enzyme activities of PLC-δ1, -δ3, and -δ4 should also display different PS dependence. To test this notion, we measured the PS-dependent enzymatic activities of PLC-δ-C2 domains in the presence of POPC/POPS (7:3) membranes. As shown in Fig. 5, PLC-δ1 showed the most pronounced PS selectivity. The enzyme activity in the presence of POPC vesicles was \(10\) times higher than that in the presence of POPC or POPG vesicles. PLC-δ3 was activated \(\sim 7\) fold by POPGs, whereas PLC-δ4 showed no activation by POPGs. Thus, the increases in the enzyme activity of PLC-δ isozymes by POPG correlate with the increases in the membrane affinity of their C2 domains by POPGs. In general, PLC-δ3 and PLC-δ4 were much less active than PLC-δ1. Even in the presence of POPG vesicles, PLC-δ3 was \(\sim 10\) times less active than PLC-δ1. At present, little is known about the activities and specificities of PLC-δ isozymes for different phosphoinositides. It is thus possible that PLC-δ3 and PLC-δ4 are more active toward other phosphoinositides.

**Membrane Binding of PLC-δ-C2 Domain Mutants**—To better understand the mechanisms of Ca\(^{2+}\)-dependent membrane binding of PLC-δ-C2 domains, we prepared selected mutants of PLC-δ1-C2 and PLC-δ4-C2 and measured their membrane interactions by SPR analysis. The x-ray crystal structure of the C2 domain of PKC-α showed that a Ca\(^{2+}\) ion and an Asn residue (Asn\(^{647}\)) in the Ca\(^{2+}\)-binding loop are involved in specific PS coordination (15). Our recent study confirmed that Asn\(^{647}\) is the determinant of its PS specificity. The sequence alignment shows that PLC-δ1-C2 also contains an Asn residue (Asn\(^{647}\)) in the corresponding position (see Fig. 1). PLC-δ3-C2 and PLC-δ4-C2 have Glu and Thr, respectively, in the position. We thus mutated Asn\(^{647}\) of PLC-δ1-C2 to Ala (N647A) and measured the mutational effect on PS selectivity. As shown in Table II, N647A showed no PS selectivity; it actually binds PG slightly better. This demonstrates that Asn\(^{647}\) is directly involved in PS coordination and PS selectivity of PLC-δ1-C2. We then prepared two mutants of PLC-δ4-C2 to elucidate the origin of its unique membrane binding properties. In the x-ray structure of the PLC-δ1-C2, four Asp residues, Asp\(^{653}\), Asp\(^{708}\), Asp\(^{708}\), and Asp\(^{714}\), directly participate in metal coordination (18, 19). PLC-δ3-C2 has the corresponding four Asp residues. However, PLC-δ4-C2 contains Ser (Ser\(^{717}\)) in place of Asp\(^{708}\) and has a more conservative Asp to Ser substitution in position 714 (of PLC-δ1). Based on this structural information, we first generated S717D to see if the mutation can convert the PLC-δ4-C2 into a PLC-δ1-C2-like molecule. S717D showed no detectable activity (in terms of \(K_m\)) for POPC/POPS membranes in the absence of Ca\(^{2+}\) but at 0.5 mM Ca\(^{2+}\), S717D had 2-fold higher affinity for POPC/POPS membranes than wild type; as a result, it displayed much larger Ca\(^{2+}\) dependence than wild type (see Table II). Also, S717D had definite PS selectivity. These results thus indicate that the unique membrane binding properties of PLC-δ4-C2 derive in large part from the Asp to Ser substitution at the position 717. We then mutated two cationic residues (Lys\(^{714}\) and Arg\(^{718}\)) in the Ca\(^{2+}\)-binding loop of PLC-δ4-C2 to Glu to assess their contribution to membrane binding. These residues are predicted to be surface-exposed in a homology model structure (see Fig. 6). In the absence of Ca\(^{2+}\), K714E/R718E had no appreciable affinity for POPC/POPS (7:3) membranes (see Table II), indicating that these cationic residues are the main contributors to Ca\(^{2+}\)-independent mem-

\(^2\) R. V. Stahelin, J. D. Rafter, S. Das, and W. Cho, submitted for publication.
brane affinity of PLC-δ4-C2. Even at 0.5 mM Ca\(^{2+}\), the mutant had 7-fold lower affinity than the wild type, due largely to lower \(k_a\), again showing that the two residues are involved in non-specific electrostatic interactions with the anionic membrane surface, whether the binding is Ca\(^{2+}\)-dependent or not.

**Calculation of the Electrostatic Potential**—Our SPR and monolayer measurements described above indicated that the membrane binding of the three C2 domains is largely driven by electrostatic interactions. To account for the differential membrane binding properties of the C2 domains and mutants, we therefore compared the electrostatic equipotential profiles of these molecules in the absence and presence of Ca\(^{2+}\) ions. Results are illustrated in Fig. 6. In the absence of Ca\(^{2+}\), PLC-δ1-C2 and PLC-δ3-C2 had neutral to negative electrostatic potential in the Ca\(^{2+}\)-binding loops, respectively, consistent with their lack of Ca\(^{2+}\)-independent binding to anionic membranes. In contrast, PLC-δ4-C2 that has unique Ca\(^{2+}\)-independent membrane affinity possesses a significant degree of positive electrostatic potential in the Ca\(^{2+}\)-binding loops, particularly around Lys\(^{714}\) and Arg\(^{718}\), even in the absence of Ca\(^{2+}\). Under the same conditions, however, K714E/R718E has a highly negative electrostatic potential in the same region. Also, the S717D mutation generates a large negative electrostatic potential that overshadows the positive electrostatic potential around Lys\(^{714}\) and Arg\(^{718}\). This accounts for the extremely low Ca\(^{2+}\)-independent affinity of the two mutants for anionic membranes. The addition of three Ca\(^{2+}\) ions (see the “Experimental Procedures”) to the C2 domains leads to the development of highly positive electrostatic potentials for all wild type and mutant C2 domains, albeit to different extents. The largest change in electrostatic potential is seen with PLC-δ3-C2, primarily due to the highly negative electrostatic potential in the absence of Ca\(^{2+}\), which agrees with its most pronounced Ca\(^{2+}\)-dependence in binding to anionic membranes. Even with three Ca\(^{2+}\) ions, PLC-δ4-C2-K714E/R718E has a smaller cationic lobe in the Ca\(^{2+}\)-binding loop region than the wild type, again consistent with its lower affinity for anionic membranes than wild type. Taken together, our electrostatic potential calculation is in excellent agreement with the observed membrane binding properties of the PLC-δ-C2 domains and mutants.

**Subcellular Translocation of PLC-δ-C2 Domains**—To determine the role of PLC-δ-C2 domains in the subcellular localization of PLC-δ enzymes and also to assess the physiological relevance of our in vitro measurements, we transfected PLC-δ-C2 domains and mutants tagged with EGFP into HEK293 cells and measured their spatiotemporal dynamics by time-lapse confocal microscopy. EGFP was linked to the amino-terminal end of each protein, because the carboxyl-terminal attachment interfered with protein overexpression. As shown in Fig. 7, PLC-δ1-C2-EGFP and PLC-δ3-C2-EGFP were evenly dispersed in the cytoplasm in the resting state. When the cells were activated by the Ca\(^{2+}\) ionophore, ionomycin, these C2 domains rapidly translocated to the plasma membrane. The translocation was completed within 5 min. It has been shown that the inner plasma membrane of mammalian cells is rich in PS (32, 33). It thus appears that the subcellular localization is governed in large part by their membrane binding properties.

**DISCUSSION**

The role of the C2 domain in the membrane binding and activation of PLC-δ isoforms has been controversial. This work represents the first systematic in vitro and cell studies on the isolated C2 domains of PLC-δ isoforms. Our SPR and monolayer measurements, electrostatic potential calculation, and cell translocation studies show that PLC-δ-C2 domains are Ca\(^{2+}\)-dependent membrane targeting domains, the membrane binding of which is driven mainly by electrostatic interactions. The predominantly electrostatic nature of their membrane binding is supported by low monolayer penetration and high dependence on the ionic strength. At least three roles have been proposed for the C2-bound Ca\(^{2+}\) ions (11, 34): i.e. nega-
tive-to-positive electrostatic potential switch, formation of a protein-Ca\(^{2+}\)-anionic lipid complex, and inducing conformational changes. Previous studies have suggested that PLC-δ1-C2 binds three Ca\(^{2+}\) ions (18). Although we did not determine the stoichiometry of Ca\(^{2+}\) binding to other PLC-δ-C2 domains, it is reasonable to assume, based on the sequence homology, that PLC-δ3-C2 binds three Ca\(^{2+}\) ions. Due to the substitution of two of four Ca\(^{2+}\)-coordinating Asp residues, PLC-δ4-C2 might bind less Ca\(^{2+}\) ions. Our electrostatic potential calculation showed that binding of two Ca\(^{2+}\) ions to PLC-δ4-C2 results in only a slightly smaller positive electrostatic potential than that due to three ions (data not shown). Thus, we analyzed our membrane binding data of PLC-δ-C2 domains based on the assumption that all C2 domains bind three Ca\(^{2+}\) ions, albeit with different affinities.

Our recent studies on the C2 domains of PKC-α and cPLA\(_2\), both of which bind two Ca\(^{2+}\) ions, showed that the two Ca\(^{2+}\) ions play distinct roles (35–37). Likewise, these studies indicate that Ca\(^{2+}\) ions play two different roles for PLC-δ1-C2; i.e. bridging to PS and inducing electrostatic switch. For the C2 domain of PKC-α that has been shown to directly coordinate a PS molecule via Ca\(^{2+}\) and an Asn residue (37), Ca\(^{2+}\) has comparable effects on \(k_p\) and \(k_d\), and PS has an effect primarily on \(k_d\). As shown in Table 1, similar observations were made for PLC-δ1-C2 in terms of effects of Ca\(^{2+}\) and PS on \(k_p\) and \(k_d\), respectively. This, in conjunction with high PS selectivity of PLC-δ1-C2, suggests that at least one Ca\(^{2+}\) ion of PLC-δ1-C2 is directly involved in PS coordination. As was the case with the C2 domain of PKC-α, the putative PS coordination by PLC-δ1-C2 is also mediated by Asn\(^{647}\), the mutation of which drastically reduces the PS selectivity. In addition to the PS-coordinating role of Ca\(^{2+}\), the good agreement between our electrostatic potential calculations and SPR binding data points to the importance of nonspecific electrostatic interactions that are induced by Ca\(^{2+}\) binding. Ca\(^{2+}\) ions switch the predominantly negative electrostatic potential of the Ca\(^{2+}\) binding loops, due to the presence of anionic Ca\(^{2+}\) ligands, to a strongly positive electrostatic potential and thereby drive the membrane association. Although it was reported that the Ca\(^{2+}\) analogue Sm\(^{3+}\) induces the local conformational changes of PLC-δ1-C2 (19), the importance of this conformational effect is not evident from our studies. For PLC-δ3-C2, the main role of Ca\(^{2+}\) ions appears to be the electrostatic switch, because PLC-δ3-C2 shows the highest degree of Ca\(^{2+}\) dependence in membrane affinity (\(K_J\)) that is mainly due to a \(k_d\) effect. PLC-δ3-C2 has a significant degree of PS selectivity (−5-fold) even though it contains a Glu in place of the PS-binding Asn\(^{647}\) of PLC-δ1-C2 (see Fig. 1). It is unclear whether this residue or an alternative site is involved in binding to a PS molecule. Further studies are required to identify residues, if any, that are involved in putative PS coordination of PLC-δ3-C2. Lastly, it is clear that the primary role of Ca\(^{2+}\) ions in the membrane binding of PLC-δ4-C2 is the electrostatic switch. Ca\(^{2+}\)-enhanced affinity for anionic lipids is insensitive to the phospholipid headgroup and is ascribed exclusively to a \(k_d\) effect. Thus, Ca\(^{2+}\) ions shift the electrostatic potential of the calcium bind-

![Electrostatic potential of PLC-δ-C2 domains](http://www.jbc.org/)

**FIG. 6. Electrostatic potential of PLC-δ-C2 domains.** The electrostatic potentials for the PLC-δ1-C2 structure and the PLC-δ3-C2 and PLC-δ4-C2 homology models were calculated and visualized in the program GRASP (27). In each panel of the figure, the C2 domains are oriented similarly with the Ca\(^{2+}\)-binding loops pointed downward. The red and blue meshes represent, respectively, the −25 and +25 mV electrostatic equipotential contours in 0.1 M KCl.

![Subcellular localization of PLC-δ-C2 domains](http://www.jbc.org/)

**FIG. 7. Subcellular localization of PLC-δ-C2 domains.** HEK293 cells transiently transfected with EGFP-tagged PLC-δ-C2 domains were treated with ionomycin. The images were taken before (top row) and 5 min after (bottom row) the ionomycin treatment.
Membrane Targeting of PLC-δ-C2 Domains

The mechanisms by which PLC-δ isoforms are regulated in the cell and their coupling to membrane receptors remain unclear (2). PLC-δ isoforms show the highest Ca\(^{2+}\) sensitivity among all known PLC enzymes (2). Our studies indicate that differential Ca\(^{2+}\)-dependent membrane binding properties of the three C2 domains, different PS selectivity in particular, result in their different subcellular localization behaviors. Although the exact lipid composition of different subcellular membranes of HEK293 cells has not been determined yet, it is expected from the known lipid compositions of mammalian endoplasmic reticulum membranes (32, 33) that its inner plasma membrane is rich in PS and the perinuclear membranes, including the nuclear envelope, contains higher PC content and lower anionic lipids. In response to Ca\(^{2+}\) import, PLC-δ1-C2 and PLC-δ3-C2, which exhibit PS selectivity, translocate to the PS-rich plasma membrane. N647A of PLC-δ1-C2, which has dramatically reduced PS selectivity, is localized to both the plasma membrane and the nuclear envelope, supporting the notion that the specific targeting of PLC-δ1-C2 to plasma membrane is due to its PS selectivity. Similarly, the plasma membrane targeting of PLC-δ3-C2 appears to be due to its PS selectivity. It has been shown that the PH domain plays a critical role in the binding of PLC-δ1 to the membrane containing Ins(4,5)P\(_2\). Our results indicate that the favorable Ca\(^{2+}\)-dependent interactions between PLC-δ1-C2 (and PLC-δ3-C2) and PS molecules would augment the targeting of PLC-δ1 (and PLC-δ3) to the plasma membrane containing Ins(4,5)P\(_2\). Thus, PLC-δ1-C2 (and PLC-δ3-C2) in response to Ca\(^{2+}\) rise would not only accelerate the membrane binding of PLC-δ1 but also ensure its specific targeting to the plasma membrane. Lastly, the subcellular localization pattern of PLC-δ4-C2 is consistent with its membrane binding affinity: A definite degree of pre-localization to the plasma membrane is seen in the absence of Ca\(^{2+}\) import and the cytoplasmic population of PLC-δ4-C2 translocates nonspecifically to various subcellular membranes in response to Ca\(^{2+}\) import. In addition, the Ca\(^{2+}\)-independent pre-localization and the non-selective Ca\(^{2+}\)-dependent targeting were not observed for the S717D mutant that has greatly reduced Ca\(^{2+}\)-independent binding but definite PS selectivity. Although the physiological significance of this finding awaits further investigation that is beyond the scope of this work, the unique properties of PLC-δ4-C2 suggest the existence of a novel regulatory mechanism for PLC-δ. For instance, PLC-δ might be selectively activated at lower intracellular Ca\(^{2+}\) levels. Also, the specific targeting of PLC-δ might involve protein-protein interactions, giving the nonspecific nature of its membrane targeting.

In summary, this work establishes the C2 domains of PLC-δ isoforms as Ca\(^{2+}\)-dependent membrane targeting domains that have distinct membrane binding properties and play a major role in their subcellular localization behaviors. As such, these studies lay the foundation for further investigation of the complex mechanisms of membrane targeting and activation of PLC-δ isoforms, which involve the interplay among different domains and the interactions with calcium, phosphoinositides, and other factors.

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