Specific Translocation of Protein Kinase C α to the Plasma Membrane Requires Both Ca²⁺ and PIP₂ Recognition by Its C2 Domain^[D] ^[V]

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The C2 domain of protein kinase C α (PKC α) controls the translocation of this kinase from the cytoplasm to the plasma membrane during cytoplasmic Ca²⁺ signals. The present study uses intracellular coimaging of fluorescent fusion proteins and an in vitro FRET membrane-binding assay to further investigate the nature of this translocation. We find that Ca²⁺-activated PKC α and its isolated C2 domain localize exclusively to the plasma membrane in vivo and that a plasma membrane lipid, phosphatidylinositol-4,5-bisphosphate (PIP₂), dramatically enhances the Ca²⁺-triggered binding of the C2 domain to membranes in vitro. Similarly, a hybrid construct substituting the PKC α Ca²⁺-binding loops (CBLs) and PIP₂ binding site (β -strands 3–4) into a different C2 domain exhibits native Ca²⁺-triggered targeting to plasma membrane and recognizes PIP₂. Conversely, a hybrid containing the CBLs but lacking the PIP₂ site translocates primarily to *trans*-Golgi network (TGN) and fails to recognize PIP₂. Similarly, PKC α C2 domains possessing mutations in the PIP₂ site target primarily to TGN and fail to recognize PIP₂. Overall, these findings demonstrate that the CBLs are essential for Ca²⁺-triggered membrane binding but are not sufficient for specific plasma membrane targeting. Instead, targeting specificity is provided by basic residues on β -strands 3–4, which bind to plasma membrane PIP₂.

INTRODUCTION

Recruitment of signaling proteins to specific membranes using modular targeting domains is a central theme in signal transduction (Rizo and Sudhof, 1998; Hurley and Misra, 2000; Teruel and Meyer, 2000; Itoh and Takenawa, 2002; Lemmon, 2003). Many targeting domains, such as pleckstrin homology (PH) domains, specifically recognize phospholipid components of their target membranes, thus conferring intracellular targeting specificity to the host protein. Another widely distributed targeting domain, the C2 domain, responds to intracellular calcium signals and mediates the transient and reversible translocation of its host protein to intracellular membranes (Coussens *et al.*, 1986; Rizo and Sudhof, 1998). Recent evidence suggests that specific interactions between membrane phospholipids and C2 domains may also account for the ability of some C2 domains to translocate specifically to a single type of intracellular membrane (Bai *et al.*, 2004).

Protein kinase C α (PKC α) is a member of the conventional PKC subgroup of serine/threonine protein kinases (cPKC; α ,

β I, β II, and γ isoforms), which, with the atypical (aPKC) and new (nPKC) subgroups, comprise the PKC family (Nishizuka, 1995). The PKC α C2 domain is structurally characterized by eight antiparallel β strands connected by interstrand loops to form a beta-sandwich structure (see Figure 1A; Verdaguer *et al.*, 1999). After increases in intracellular Ca²⁺ concentrations, the PKC α C2 domain binds 2 Ca²⁺ ions in a pocket defined by three interstrand loops (Ca²⁺-binding loops or CBLs), leading to a favorable electrostatic interaction with the isolated headgroup of the anionic lipid phosphatidylserine (PS) (Verdaguer *et al.*, 1999) and with PS-containing membranes (Murray and Honig, 2002; Evans *et al.*, 2004). Previous studies have concluded that the CBLs are primarily or fully responsible for specific targeting to the plasma membrane in preference to other intracellular membranes (Stahelin *et al.*, 2003; Marin-Vicente *et al.*, 2005). In addition to the CBLs, a second membrane-interacting region consisting of basic residues in the β -hairpin formed by β strands 3 and 4 has been identified and shown to interact with PS or another membrane lipid, PIP₂ (Verdaguer *et al.*, 1999; Ochoa *et al.*, 2002; Corbalan-Garcia *et al.*, 2003). Mutations at key positions in the CBLs or in the β 3–4 hairpin interfere with plasma membrane interaction in vivo and reduce the affinity of the PKC α C2 domain for PS or PIP₂ in vitro (Medkova and Cho, 1998; Ochoa *et al.*, 2002; Bolsover *et al.*, 2003; Stahelin *et al.*, 2003; Rodriguez-Alfaro *et al.*, 2004; Marin-Vicente *et al.*, 2005).

A significant and unresolved question concerns how the two membrane-interacting regions in the C2 domain together direct specific translocation of PKC α to the plasma

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membrane. In particular, the relative contributions of these two regions to the driving forces underlying 1) general, nonspecific docking to anionic membranes and 2) specific docking to the plasma membrane remain unknown. Using time-lapse epifluorescence microscopy of fluorescent proteins in vivo and protein-to-membrane FRET studies of membrane docking in vitro, we have investigated the roles of the CBLs and the β 3–4 hairpin in targeting the PKC α C2 domain from the cytoplasm to intracellular membranes, as well as their contributions to specific plasma membrane docking.

MATERIALS AND METHODS

Materials

Ionomycin was from Calbiochem (La Jolla, CA). DiD and FM4-64 were from Molecular Probes (Eugene, OR).

Fusion Protein Constructs

Construction of the plasmids encoding the human PKC α and PKC α C2 domain (a gift from J.-W. Soh, Inha University, Incheon, Korea) fused to cyan fluorescent protein (CFP) (pCFP-PKC α and pCFP-PKC α C2) were previously described (Evans *et al.*, 2004). Mutant plasmids (pCFP-PKC α C2/K209A/K211A and pCFP-PKC α /K209A/K211A) were produced by site-directed mutagenesis (Stratagene, La Jolla, CA). Plasmids encoding the human cytosolic phospholipase A₂ (cPLA₂) C2 domain (accession M72393) containing CBLs 1, 2, and 3 from PKC α C2 (pCFP-cPLA₂/PKC_CBLs) or both the PKC α C2 CBLs and β 3–4 strands (pCFP-cPLA₂/PKC_CBLs+ β 3–4) were assembled from overlapping PCR fragments obtained by amplification of pCFP-cPLA₂C2 with cPLA₂C2fwd and cPLA₂C2rev primers and primers containing the PKC α C2 CBL sequences and β 3–4 strand sequences. The fragments were assembled by PCR and cloned into the *HindIII*/*SalI* site of ECFP(C3). The resulting amino acid sequence of the cPLA₂/PKC_CBLs hybrid contained cPLA₂C2 sequences (S17-V30, D43-H62, P69-D93, T101-M148) interrupted by insertion of PKC α C2 loop sequences (I184-S192, I215-N220, W247-D254). The resulting amino acid sequence of the cPLA₂/PKC_CBLs+ β 3–4 hybrid contained cPLA₂C2 sequences (S17-V30, P69-D93, T101-M148) interrupted by insertion of PKC α C2 loop and β 3–4 strand sequences (I184-N220, W247-D254). Because of differing topologies, the PKC α C2 β 3–4 strands are homologous to the cPLA₂C2 β 2–3 strands.

Plasmids containing the N-terminal 20 residues of GAP43 fused to CFP or YFP (yellow fluorescent protein) (mGAP43-CFP and -YFP, respectively) were purchased from BD Biosciences Clontech (Palo Alto, CA). TGN38-CFP and TGN38-YFP plasmids were gifts from K. Simons (Max Planck Institute, Dresden, Germany). EGFP-PLC δ , PH domain plasmid was a gift from M. Katan (Cancer Research UK Centre for Cell and Molecular Biology, London, United Kingdom).

The pYFP plasmid used in this study was constructed from pEYFP purchased from BD Biosciences Clontech by introducing a Q70M mutation by site directed mutagenesis (Stratagene) to improve its qualities (Griesbeck *et al.*, 2001). Interchanging the *NheI*/*BsrGI* fragments encoding the fluorescent protein produced different color fluorescent protein plasmids.

For in vitro lipid-binding studies, PKC α C2, cPLA₂/PKC_CBLs, and cPLA₂/PKC_CBLs+ β 3–4 sequences were amplified using primers PKC α C2-GSTfwd and PKC α C2-GSTrev, for PKC α C2, and cPLA₂C2-GSTfwd and cPLA₂C2-GSTrev, for the hybrids, and cloned into the *EagI*/*EcoRI* site of a glutathione S-transferase (GST) fusion vector. Proteins expressed in *Escherichia coli* strain BL12D were isolated on a glutathione affinity column before cleavage with thrombin and elution. Protein purity was determined by SDS-PAGE (Laemmli, 1970), and protein concentration was determined by the tyrosine difference spectral method (Copeland, 1994). All constructs were confirmed by DNA sequencing.

Cell Culture

Madin-Darby canine kidney (MDCK) cells obtained from American Type Culture Collection (Manassas, VA) were plated on 35-mm glass-bottomed dishes (MatTek, Ashland, MA) at a density of 1×10^4 cells/cm² and cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.292 mg/ml glutamine in 5% CO₂ at 37°C. On the second day, cells were transfected with 1–2.5 μ g each of the relevant plasmids using FuGENE-6 (Roche Diagnostics, Indianapolis, IN) or Lipofectamine 2000 (Invitrogen) in DMEM containing 0.2% BSA, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.292 mg/ml glutamine following the manufacturer's protocol.

Microscopy of Fluorescent Proteins

Cotransfected MDCK cells were washed with and incubated in Hanks' balanced salt solution additionally buffered with 25 mM HEPES, pH 7.4 (HH-

BSS). Cells were imaged using an Olympus inverted microscope (Melville, NY) equipped with a 60 $\times$, 1.25 NA oil immersion objective, CFP and YFP emission filters (Chroma Technology, Brattleboro, VT) in a Sutter filter wheel, a CFP/YFP dichroic mirror, and a TILL Imago CCD camera (TILL Photonics, Grafelfing, Germany). Excitation light of 430 and 510 nm for CFP and YFP, respectively, was provided using a Polychrome IV monochromator (TILL Photonics). Images in Figures 3 and 4 were acquired using a Nikon inverted microscope (Melville, NY) equipped with a 60 $\times$ 1.4 NA oil immersion objective, CFP/YFP/RFP dichroic mirror and corresponding single band excitation and emission filters (Chroma Technology), and a CoolSNAP ES camera (Photometrics, Tucson AZ). Excitation light was provided by a mercury lamp. In all experiments, cells were stimulated with 10 μ M ionomycin between acquisition of the first and second CFP/YFP image sets. Intervals between image sets were 5–10 s. TILLvisION software was used for image acquisition and analysis. Final images were produced using Adobe Photoshop (Adobe, San Jose, CA) and ImageJ (NIH, <http://rsb.info.nih.gov/ij/>). Surface plots and videos were produced with ImageJ.

Lipid-binding Studies

The lipids used were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine, PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (phosphatidylserine, PS), and L- α -phosphatidylinositol-4,5-bisphosphate (PIP₂) from Avanti Polar Lipids (Alabaster, AL). The fluor used was N-[5-dimethylamino-naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-PE, dPE) from Molecular Probes. Lipids were dissolved in chloroform/methanol/water (1/2/0.8) to give a ratio of 71.25:23.75:5 PC:PS:dPE or 66.75:22.25:6.5 PC:PS:PIP₂:dPE, dried under vacuum at 45°C until all solvents were removed, and then hydrated with buffer A (25 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4, with KOH, 140 mM KCl and 15 mM NaCl, and 1 mM MgCl₂) by rapid vortexing. Sonicated small unilamellar vesicles (SUVs) were prepared by sonication of the hydrated lipids to clarity with a Misonix XL2020 probe sonicator (Misonix, Farmingdale, NY). After sonication, insoluble material was removed by centrifugation.

To measure proteins binding to lipids, equilibrium fluorescence experiments were carried out on a Photon Technology International QM-2000–6SE fluorescence spectrometer (Lawrenceville, NJ) at 25°C in buffer A essentially as described (Nalefski *et al.*, 1997). The excitation and emission slit widths were 2 and 8 nm, respectively, for all equilibrium fluorescence experiments. FRET was measured between donor intrinsic tryptophans in the proteins and SUVs composed of PC/PS/dPE or PC:PS:PIP₂:dPE containing the acceptor, dansyl-PE. CaCl₂ was titrated in mixtures of wild-type or hybrid C2 domains (1 μ M) and SUVs (100 μ M total lipid concentration) in buffer A, and the protein-to-membrane FRET was monitored by the increase in dPE emission using excitation and emission wavelengths of λ_{ex} = 284 nm and λ_{em} = 520 nm, respectively. The fluorescence due to the direct excitation of the dPE was subtracted from the protein data. Averages $\pm$ SD for three experiments are presented in the graph. Curves represent best-fit of data by the Hill equation using KaleidaGraph software (Synergy Software, Reading, PA).

Structural Models

The Protein Data Bank (Berman *et al.*, 2000) identifiers for the experimentally determined C2 domain models in Figure 1 are 1RLW (cPLA₂C2; Perisic *et al.*, 1998), and 1DSY (PKC α C2; Verdaguer *et al.*, 1999). The hybrid C2 domain models illustrated in Figure 1A were constructed by placing the respective CBL and β 3–4 regions from PKC α C2 onto the structural core of cPLA₂C2. The structure of cPLA₂C2 was superimposed onto the PKC α C2 structure using the program CE (Combinatorial Extension; Shindyalov and Bourne, 1998). Once structurally aligned, the appropriate portions of the PKC α C2 PDB file replaced regions of the cPLA₂C2 PDB file as dictated by the sequences given in Figure 1B. The hybrid C2 domain models were energy minimized in the program Modeller (Accelrys, San Diego, CA) (Sali and Blundell, 1993) to fix any mismatches between the various structural segments. Because the calcium coordination schemes of the hybrid C2 models are closest to that of PKC α C2 as judged by the multiple sequence alignment of the various constructs, it was assumed that the hybrid C2 models bind two calcium ions in the same manner as PKC α C2.

RESULTS

Intracellular Targeting of Native PKC α and Its C2 Domain

To monitor the intracellular targeting of PKC α in response to Ca²⁺ mobilization, we fused PKC α to CFP, expressed the fusion protein in MDCK cells, and imaged its cellular localization during increases in the cytoplasmic Ca²⁺ concentration produced by treatment of cells with the Ca²⁺ ionophore ionomycin. In unstimulated cells, both CFP-PKC α and the isolated C2 domain from PKC α fused to yellow fluorescent

Figure 1. Structure and sequence of wild-type PKC α and cPLA₂ α C2 domains and of hybrid C2 domains. (A) Ribbon diagrams of the PKC α C2 domain, showing CBLs 1–3 and β strands 3–4 (green, PDB entry 1DSY) of the cPLA₂ α C2 domain (blue, PDB entry 1RLW), of the cPLA₂/PKC_CBLs C2 domain (PKC α C2 CBLs in green, cPLA₂C2 scaffold in blue), and of the cPLA₂/PKC_CBLs+ β 3–4 C2 domain (PKC α C2 CBLs and β 3–4 strands in green, cPLA₂C2 scaffold in blue). (B) Alignment of the amino acid sequences of the PKC α C2 and cPLA₂ α C2 domains used to generate the hybrid domains. Conserved residues in black boxes, and similar residues in gray. CBL residues included in hybrids are identified by a single line. Residues of β strands 3 and 4 included in hybrids are identified by a double line. Mutated lysine residues in β strands 3 and 4 of PKC α C2 are indicated by asterisks.

protein (YFP-PKC α C2) were diffusely distributed in the cytosol (Figure 2, A and B). Because of its small molecular mass, YFP-PKC α C2 was also observed in the nucleus, as are many small domains fused to fluorescent proteins (Balla and Varnai, 2002). In response to an increase in the cytoplasmic Ca²⁺ concentration, CFP-PKC α translocated from the cytosol to the cell periphery and to small puncta on the apical surface of the plasma membrane, where it colocalized with YFP-PKC α C2, (Figure 2, A and B, and Supplementary Figure 2video). Because the fluorescence signals from CFP-PKC α and YFP-PKC α C2 colocalized completely (Figure 2C), it follows that the C2 domain not only provides Ca²⁺ regulation to PKC α , but all requisite targeting information as well.

To investigate the nature of the puncta, we first tested the hypothesis that they lie on the plasma membrane using two different plasma membrane markers, the phospholipase C δ_1 PH domain fused to YFP (YFP-PLC δ_1 PH), which is widely used as a marker for PIP₂ enriched in the plasma membrane (Stauffer *et al.*, 1998; Varnai and Balla, 1998), and the 20-residue doubly palmitoylated targeting region of growth associated protein 43 (GAP43) fused to YFP (mGAP43-YFP), which is known to specifically target plasma and Golgi membranes (Arni *et al.*, 1998). Cells coexpressing CFP-PKC α C2 and YFP-PLC δ_1 PH were treated with ionomycin to induce Ca²⁺ mobilization, and the fluorescence from each construct was imaged at apical surface of the cell. For completeness, images were also collected for the basal and medial planes of the cell. The fluorescence signals of CFP-PKC α C2 and YFP-PLC δ_1 PH overlapped completely (Figure 3A). CFP-PKC α C2 and YFP-PLC δ_1 PH fluorescence appeared in bright puncta at the apical surface and in bright patches at the basal surface of the cell. Images of medial sections showed fluorescence at the cell periphery, similar to those images reported by confocal microscopy (Sakai *et al.*, 1997; Oancea and Meyer, 1998; Stauffer *et al.*, 1998). In cells expressing CFP-PKC α C2 and mGAP43-YFP and treated with ionomycin, overlap of the fluorescent signals was identical, except where mGAP43-YFP associates with Golgi, and bright apical puncta and basal patches were again observed (Figure 3B). As expected, the plasma membrane fluorescence

of YFP-PLC δ_1 PH and mGAP43-YFP overlapped in unstimulated cells (Supplementary Figure S1).

In principle, the observed puncta and patches could represent large clusters of lipids and proteins containing high local densities of PIP₂ on the cytoplasmic surface of the plasma membrane, such as clusters of lipid rafts (Laux *et al.*, 2000; Raucher *et al.*, 2000; Tall *et al.*, 2000; Brown *et al.*, 2001; Kwik *et al.*, 2003; Huang *et al.*, 2004; Golub and Caroni, 2005). Alternatively, the puncta and patches could represent geometric features arising from high local densities of membrane surface area (Colarusso and Spring, 2002; van Rheenen and Jalink, 2002). To resolve these possibilities, we first used the lipophilic membrane dyes DiD and FM4-64 to provide uniform membrane staining in cells expressing mGAP43-YFP (Raucher *et al.*, 2000; van Rheenen and Jalink, 2002; Golub and Caroni, 2005). Cells expressing low levels of mGAP43-YFP were treated with DiD at 37°C for 5 min before being cooled to 4°C to slow the movement of dye to interior cell membranes. Cells expressing low levels of mGAP43-YFP were selected for analysis because cells expressing high levels of mGAP43-YFP had a considerable fraction of the fluorescence in the cytosol. As seen in Figure 4A, the punctate pattern of mGAP43-YFP at the apical surface overlapped with the DiD staining (top panels). Surface plots of the insets areas were pseudocolored to show fold changes in mGAP43-YFP and DiD intensity over the background membrane (Figure 4A, bottom panel). Both the mGAP43-YFP and DiD puncta were found to be ~1.6-fold brighter than the surrounding apical membrane, and the areas of brightness overlapped completely (Figure 4A, bottom panel). The brightness and distribution of the DiD-stained apical puncta were similar to those described in a similar study in MDCK cells (Colarusso and Spring, 2002). Analysis of the basal surface of cells expressing mGAP43-YFP and stained with DiD yielded similar results, except that the basal mGAP43-YFP and DiD patches were both found to be three- to fourfold brighter than the surrounding membrane (Figure 4B). Similar experiments using FM4-64 on mGAP43-YFP-expressing cells yielded similar results (unpublished data). These results indicate that the bright apical puncta and basal patches arise from local folding of

Figure 2. PKC α and the isolated PKC α C2 domain target identical puncta in the plasma membrane of MDCK cells. CFP and YFP image pairs of MDCK cells coexpressing (A) CFP-PKC α and (B) YFP-PKC α C2 before (left panels) and 140 s after (right panels) stimulation of a cytoplasmic Ca²⁺ signal by treatment of cells with 10 μ M ionomycin. Focus is on the apical surface of the cell. Insets, enlargements of white-boxed areas and are shown merged in C. Scale bars, 10 μ m. Images are representative of multiple cells observed in each of five or more independent experiments.

the plasma membrane rather than regions of lipid and protein heterogeneity (lipid rafts).

To further investigate whether the bright puncta and patches represent high local densities of plasma membrane or PIP₂-containing lipid rafts, we imaged the PIP₂-specific CFP-PLC δ_1 PH domain together with the plasma membrane marker FM4-64. CFP-PLC δ_1 PH-expressing cells were treated with FM4-64 for 5 min at 4°C. The resulting bright apical puncta and basal patches of CFP-PLC δ_1 PH were observed to colocalize with FM4-64 (Figure 4C, top panels). Because of the large fraction of CFP-PLC δ_1 PH in the cytosol not associated with membrane, analysis of brightness differences between FM4-64 and CFP-PLC δ_1 PH puncta and patches were precluded; however, the fold increase in fluorescence of CFP-PLC δ_1 PH was consistently lower than that for FM4-64. Again, these results indicate that the observed local accumulations of mGAP43 and PLC δ_1 PH domain were caused by local increases in membrane density.

Together, our comparisons of the cellular localizations of lipophilic probes and protein domains indicate that mGAP43, PLC δ_1 PH, and PKC α C2 are uniformly distributed throughout the plasma membrane within the limit of our optical resolution ($\sim$ 250 nm). The bright puncta and patches observed on the apical and basal cell surfaces, respectively, represent local regions of high plasma membrane

density arising from complex geometric features rather than heterogeneities within the plane of the membrane such as lipid rafts. These findings do not rule out the possibility that heterogeneities or rafts smaller than the limit of our resolution may exist and recruit proteins such as GAP43, PLC δ_1 , and PKC α .

Overall, the present cellular localization results confirm that Ca²⁺ activation causes the translocation of full-length PKC α and its isolated C2 domain from the cytoplasm exclusively to the inner leaflet of the plasma membrane in preference to other intracellular membranes, as reported in previous studies (Sakai *et al.*, 1997; Oancea and Meyer, 1998; Stauffer *et al.*, 1998). To understand how exclusive plasma membrane targeting is achieved, we next investigated the phospholipid preference of the isolated PKC α C2 protein in an *in vitro* system.

In Vitro Association of Native PKC α C2 Domain with Membrane-bound PIP₂

Previous studies have implicated PS and PIP₂ as regulators of PKC α C2 domain docking to artificial membranes (Corbalan-Garcia *et al.*, 2003; Stahelin *et al.*, 2003; Marin-Vicente *et al.*, 2005). As stated above, the membrane-bound target of the PLC δ_1 PH domain, PIP₂, is specifically enriched in the inner leaflet of the plasma membrane (Roth, 2004), whereas

Figure 3. The PKC α C2 domain colocalizes with the PLC δ_1 PH domain and mGAP43 at different focal planes after translocation. Images of cells coexpressing (A) YFP-PKC α C2 (left) and CFP-PLC δ_1 PH (right) after treatment with ionomycin at apical (top), medial (middle), and basal (bottom) planes of each cell. The same cell is imaged at a given focal plane. Insets, enlarged views of the indicated cell regions. The intracellular distribution of CFP-PLC δ_1 PH did not change significantly before or after ionomycin treatment. (B) Images of three cells coexpressing YFP-PKC α C2 (left) and mGAP43-CFP (right) after treatment with ionomycin at apical (top), medial (middle), and basal (bottom) planes of each cell. Insets, enlarged views of the indicated cell regions. The same cell is imaged at a given focal plane. Perinuclear targeting of mGAP43-CFP to Golgi is designated by an asterisk (right images). All results are representative of a minimum of five experiments.

PS is a constituent of all cellular membranes (van Meer, 1998). Many independent fluorescence studies have observed exclusive targeting of PLC δ_1 PH domain to plasma membrane, indicating that other intracellular membranes do not contain detectable levels of PIP $_2$ (Roth, 2004). Thus, a specific interaction between the PKC α C2 domain and plasma membrane PIP $_2$ could account for the observed plasma membrane-targeting specificity. To test this hypothesis, we used an established protein-to-membrane FRET assay to quantitate the effect of PIP $_2$ on Ca $^{2+}$ -dependent binding to phospholipid vesicles *in vitro* (Nalefski *et al.*, 1997). Specifically, FRET was monitored between four in-

trinsic tryptophan donors in the PKC α C2 domain and phospholipid vesicles containing 3:1 PC:PS and 5 mol percent dansyl-PE, the latter serving as the FRET acceptor. The vesicles also contained or lacked 6 mol percent PIP $_2$. The presence of PIP $_2$ significantly altered the Ca $^{2+}$ -dependence of C2 domain docking to vesicles, yielding an 18-fold decrease in the [Ca $^{2+}$] $_{1/2}$ (corresponding to an 18-fold affinity increase) for C2 domain docking relative to vesicles lacking PIP $_2$ (Figure 5A). In contrast, PIP $_2$ had no effect on the Ca $^{2+}$ -dependent membrane docking of the C2 domain from cytosolic phospholipase A 2α (cPLA 2α C2; Figure 5B). Together these findings show that PIP $_2$ significantly enhances

Figure 4. Colocalization of mGAP43 and PLC δ_1 PH domain with DiD. (A) Apical surface of a cell expressing mGAP43-YFP (left) stained with the lipophilic membrane dye DiD (right). Cells were incubated with 25 μ M DiD for 5 min at 37°C, rinsed with ice-cold HHBSS, and kept on ice until imaged to prevent the dye from staining internal membranes. Focus is on the apical surface. Insets, white-boxed areas. Surface plots (bottom) of the areas in the mGAP43 (left) and DiD (right) insets. Scale bar reflects fold increases in fluorescence intensities with the intensity of the dim surrounding membrane set to 1. (B) Basal surface of a cell expressing mGAP43-YFP (left) and stained with DiD (right). Surface plots (bottom) of the areas in the mGAP43 (left) and DiD (right) insets. Scale bar reflects fold increases in fluorescence intensities. (C) Apical (top) and basal (bottom) surfaces of a cell expressing CFP-PLC δ_1 PH (left) stained with the lipophilic membrane dye FM4-64 (right). Cells were incubated with 5 μ g/ml FM4-64 for 5 min in ice-cold HHBSS, rinsed, and kept on ice until imaged to prevent the dye from staining internal membranes.

Figure 5. PIP $_2$ increases the binding affinity of the PKC α C2 domain for phospholipid vesicles. Protein-to-membrane FRET analysis of (A) PKC α C2 domain and (B) cPLA $_2\alpha$ C2 domain binding to phospholipid vesicles containing 3:1 PC:PS and 5 mol percent dansyl-PE, either with (●) or without (○) 6 mol percent PIP $_2$. Samples contained 1 μM C2 domain, 100 μM total lipid, 1 mM MgCl $_2$, 140 mM KCl, 15 mM NaCl, 25 mM HEPES, pH 7.4, 25°C. Data points represent the average $\pm$ SD of three independent experiments. Best-fit analysis using the Hill equation (solid lines) yields $[Ca^{2+}]_{1/2}$ values of 2.9 ± 0.05 μM (+PIP $_2$) and 51 ± 1.5 μM (−PIP $_2$) for PKC α C2 and 15 ± 0.28 μM (+PIP $_2$) and 13 ± 0.29 μM (−PIP $_2$) for cPLA $_2\alpha$ C2. Hill coefficients ranged from 1.5 to 1.9 because of the activation of each C2 domain by multiple Ca $^{2+}$ ions (Kohout *et al.*, 2002).

the Ca $^{2+}$ -mediated recruitment of the PKC α C2 domain to a membrane surface by a Ca $^{2+}$ signal and suggests that PIP $_2$ binding drives plasma membrane targeting.

Intracellular Targeting and Lipid Binding of Hybrid C2 Domains Containing the PKC α C2 Ca $^{2+}$ -binding Loops

Our next aim was to define the structural elements of the C2 domain underlying specific plasma membrane targeting. One possibility is that the CBLs control targeting specificity. For example, our earlier work has shown that the CBLs of a related C2 domain from cPLA $_2$ were sufficient to promote the native intracellular targeting of this C2 domain, which docks to Golgi and ER membranes in vivo (Evans *et al.*, 2004). Furthermore, other studies have suggested that residues located in the PKC α C2 domain CBLs are important in targeting of the isolated C2 domain to the plasma membrane (Conesa-Zamora *et al.*, 2001; Stahelin *et al.*, 2003; Marin-Vicente *et al.*, 2005), and biophysical studies of PKC α C2 docking have demonstrated that the CBLs directly contact the membrane surface (Kohout *et al.*, 2003; Figure 1A).

To assess the role of the PKC α C2 CBLs in targeting and PIP $_2$ recognition, we fused the PKC α C2 CBLs onto a cPLA $_2\alpha$ C2 domain scaffold to produce a hybrid domain (cPLA $_2\alpha$ /PKC_CBLs; Figure 1, A and B). Because the crystal structures for cPLA $_2\alpha$ C2 and PKC α C2 are known (Perisic *et al.*, 1998; Verdaguer *et al.*, 1999), it was possible to align homologous structures to produce the hybrids (Figure 1B). Direct comparison of targeting between the cPLA $_2\alpha$ /PKC_CBLs hybrid fused to CFP (CFP-cPLA $_2\alpha$ /PKC_CBLs) and YFP-PKC α C2 revealed Ca $^{2+}$ -dependent translocation of the hybrid from the cytoplasm to a juxtanuclear location, with some binding to the plasma membrane still detectable (Figure 6A and Supplementary Figure 6A video). By imaging CFP-PKC α C2_CBLs and TGN38-YFP, a marker for the TGN (Toomre *et al.*, 1999), we established TGN membranes as the target of the juxtanuclear binding (Figure 4B). By contrast,

analogous hybrids containing PKC α C2 CBL 1 or CBLs 1 and 3 fused to a cPLA $_2\alpha$ C2 domain scaffold failed to exhibit translocation to any membrane in response to Ca $^{2+}$ mobilization (unpublished data). These findings demonstrate that the isolated PKC α C2 domain CBLs can direct Ca $^{2+}$ -dependent targeting to a limited set of anionic cellular membranes, but are not sufficient to confer PKC α C2-like exclusive targeting to the plasma membrane. Overall, these findings suggest that another structural element required for the native plasma membrane-targeting specificity of the PKC α C2 domain must lie outside the CBLs.

The hybrid C2 domain possessing the three PKC α C2 CBLs was also examined for its ability to recognize PIP $_2$ in the in vitro protein-to-membrane FRET assay. Even in the absence of PIP $_2$, the hybrid required higher Ca $^{2+}$ levels to drive membrane docking than wild-type PKC α C2 domain (compare Figures 5A and 6D), most likely because of sub-optimum orientation of the Ca $^{2+}$ coordinating residues as previously suggested for other C2 domain hybrids (Gerber *et al.*, 2001). Notably, PIP $_2$ had no effect on the Ca $^{2+}$ -dependence of membrane docking by this hybrid C2 domain, indicating that the CBLs alone are insufficient for PIP $_2$ recognition and suggesting that another region of PKC α C2 domain is involved.

Intracellular Targeting and Lipid Binding of a Hybrid C2 Domain-containing the PKC α C2 Ca $^{2+}$ -binding Loops and the β 3–4 Hairpin

A basic region formed by lysine residues in the β -hairpin formed by β strands 3 and 4 has been postulated to coordinate PS or PIP $_2$ in the membrane (Ochoa *et al.*, 2002; Corbalan-Garcia *et al.*, 2003; Rodriguez-Alfaro *et al.*, 2004; Marin-Vicente *et al.*, 2005), and an electron paramagnetic resonance study has demonstrated that this β 3–4 hairpin lies in close proximity to the membrane surface (Kohout *et al.*, 2003). To investigate the contribution of the β 3–4 hairpin to specific

Figure 6. PKCαC2 β strands 3 and 4 are required for plasma membrane targeting and for Ca²⁺-dependent binding to PIP₂-containing phospholipid vesicles. CFP and YFP image pairs of cells coexpressing (A) CFP-cPLA₂/PKC_CBLs and YFP-PKCαC2, (B) CFP-cPLA₂/PKC_CBLs and TGN38-YFP, or (C) CFP-cPLA₂/PKC_CBLs+β3-4 and YFP-PKCαC2 at 1–2.5 min after stimulation of a cytoplasmic Ca²⁺ signal by treatment with ionomycin. Insets, enlargements of white-boxed areas and are shown merged on the right of each panel. Scale bars, 10 μm. Results are representative of a minimum of five experiments. Protein-to-membrane FRET analysis of (D) cPLA₂/PKC_CBLs and (E) cPLA₂/PKC_CBLs+β3-4 protein binding to phospholipid vesicles with (m) or without (l) 6% PIP₂ (see Figure 5 legend for details). Best-fit analysis using the Hill equation (solid lines) yields [Ca²⁺]_{1/2} values of 647 ± 11 mM (+PIP₂) and 620 ± 5.5 μM (-PIP₂) for cPLA₂/PKC_CBLs and values of 91.6 ± 1.1 mM (+PIP₂) and 459 ± 12 mM (-PIP₂) for PKCαC2_CBLs+β3-4.

plasma membrane targeting, we constructed another hybrid C2 domain containing the PKCαC2 CBLs and β3-4 hairpin fused to a cPLA₂C2 scaffold and coupled to CFP (CFP-cPLA₂/PKC_CBLs+β3-4; Figure 1A). In response to Ca²⁺ increase, CFP-cPLA₂/PKC_CBLs+β3-4 moved from the cytosol and colocalized completely with YFP-PKCαC2 at apical puncta (Figure 6C and Supplementary Figure 6Cvideo). Imaging the translocation of both the CFP-cPLA₂/PKC_CBLs+β3-4 and YFP-cPLA₂/PKC_CBLs hybrid domains in the same cell clearly revealed the essential role of the β3-4 hairpin for specific targeting to the plasma membrane (Supplementary Figure S2 and Supplementary FigureS2video). These results indicate that the β3-4 hairpin of the PKCα C2 domain drives the plasma membrane-targeting specificity of the native domain.

To directly test the role of the β3-4 hairpin in PIP₂ recognition, we evaluated the *in vitro* binding of the cPLA₂/PKC_CBLs+β3-4 hybrid protein to vesicles with and without PIP₂ in the protein-to-membrane FRET assay. In contrast to the cPLA₂/PKC_CBLs hybrid protein, for which PIP₂ had no effect on Ca²⁺-dependent membrane binding (Figure 6D), PIP₂ significantly altered the Ca²⁺-dependent binding of the cPLA₂/PKC_CBLs+β3-4 protein to phospholipid vesicles, yielding a fivefold decrease in the [Ca²⁺]_{1/2} for binding relative to vesicles lacking PIP₂ (Figure 6E). In sum, the data presented here support the view that Ca²⁺ dependent translocation of PKCα to PIP₂-enriched plasma membrane is governed by two membrane-interacting regions of the PKCα C2 domain: the CBL region, which specifically binds Ca²⁺ and drives membrane association with only

Figure 7. Lysine residues in β strands 3 and 4 of PKC α C2 domain are required for plasma membrane targeting and for Ca $^{2+}$ -dependent binding to PIP $_2$ -containing phospholipid vesicles. CFP and YFP image pairs of cells coexpressing (A) YFP-PKC α C2/K209A/K211A and CFP-PKC α C2, (B) YFP-PKC α C2/K209A/K211A and CFP-cPLA $_2$ /PKC_CBLs, or (C) YFP-PKC α C2/K209A/K211A and CFP-PKC α C2/K209A/K211A at 55 s after stimulation of a cytoplasmic Ca $^{2+}$ signal by addition of ionomycin. Insets, enlargements of white-boxed areas and are shown merged on the right of each panel. Scale bars, 10 μ m. Results are representative of a minimum of five experiments. Protein-to-membrane FRET analysis of (D) PKC α C2/K197A/K199A protein and (E) PKC α C2/K209A/K211A protein binding to phospholipid vesicles with (●) or without (○) 6% PIP $_2$ (see Figure 5 legend for details). Best-fit analysis using the Hill equation (solid lines) yields [Ca $^{2+}$] $_{1/2}$ values of 51 ± 1.3 mM (+PIP $_2$) and 94 ± 4.1 mM (−PIP $_2$) for PKC α C2/K197A/K199A and 89 ± 1.7 mM (+PIP $_2$) and 122 ± 3.2 mM (−PIP $_2$) for PKC α C2/K209A/K211A. Dashed lines for comparison are Hill curves for wild-type PKC α C2 binding from Figure 5A.

limited specificity, and the β 3–4 hairpin, which is required for full plasma membrane specificity and PIP $_2$ recognition.

Intracellular Targeting and Lipid Binding of Mutant PKC α C2 Domains

We next turned our attention to four lysine residues located in the β 3–4 hairpin (Figure 1B). Earlier studies have suggested that lysines 197 and 199 in the β 3 strand and lysines 209 and 211 in the β 4 strand of PKC α C2 are important in Ca $^{2+}$ -independent, PIP $_2$ -dependent binding to vesicles, PS- and PIP $_2$ -dependent regulation of PKC α activity in vitro, and membrane residence (Ochoa *et al.*, 2002; Corbalan-Garcia *et al.*, 2003; Rodriguez-Alfaro *et al.*, 2004; Marin-Vicente *et al.*, 2005). The hypothesis that these lysine residues are important in PIP $_2$ recognition predicts that mutations at these positions would weaken membrane-targeting specificity in vivo and lipid-binding affinity in vitro. To test this hypoth-

esis, two mutants were created each containing a pair of substitutions at the critical lysine positions: K197A/K199A and K209A/K211A.

A previous study has shown that the K209A/K211A double mutation has a significantly larger effect than K197A/K199A on PIP $_2$ -dependent PKC α kinase activity in vitro and membrane association in vivo (Rodriguez-Alfaro *et al.*, 2004); thus K209A/K211A was selected for fluorescence imaging studies in vivo. First, the mutant PKC α C2 domain-containing K209A/K211A was fused to YFP (YFP-PKC α C2/K209A/K211A). The resulting mutant targeted primarily to juxtanuclear membranes, with some binding to the plasma membrane still detectable, in response to Ca $^{2+}$ mobilization. The observed targeting pattern differed significantly from that of CFP-PKC α C2 expressed in the same cell, which exclusively targeted to plasma membrane puncta and patches (Figure 7A and Supplementary Figure 7A video). Coimaging

YFP-PKC α C2/K209A/K211A with TGN38-CFP confirmed that the primary target of translocation was the TGN (Supplementary Figure S3A), as previously observed for the cPLA $_2$ /PKC_CBLs hybrid lacking the PKC α β 3–4 region (Figure 6B). These results suggest that the loss of essential lysines from the β 3–4 hairpin inactivates the PIP $_2$ binding site. In this case, targeting is controlled solely by the CBLs and exhibits diminished specificity. This is confirmed by the direct colocalization of YFP-PKC α C2/K209A/K211A with CFP-cPLA $_2$ /PKC_CBLs (Figure 7B and Supplementary Figure 7Bvideo).

To ensure that the targeting results observed for the isolated C2 domain were indicative of full-length PKC α , we also evaluated targeting of a full-length PKC α harboring the K209A/K211A double mutation fused to CFP (CFP-PKC α /K209A/K211A). Targeting of the full-length mutant CFP-PKC α /K209A/K211A to the juxtanuclear region and the plasma membrane in response to Ca $^{2+}$ increase differed greatly from the exclusively plasma membrane targeting observed for the wild-type, full-length YFP-PKC α (Figure 7C and Supplementary Figure 7Cvideo). As expected, the targeting of the full-length mutant CFP-PKC α /K209A/K211A was coincident with the corresponding mutant C2 domain YFP-PKC α C2/K209A/K211A (Figure S3B and Supplementary Figure S3Bvideo). The full-length mutant, however, exhibited more targeting to the plasma membrane than the C2 domain mutant, suggesting that other membrane-interacting regions of the full-length protein, most likely the C1 domain and/or the pseudosubstrate region, also play roles in targeting when the C2 domain is defective. Nonetheless, our results clearly demonstrate that, as for the isolated C2 domain, mutation of lysine residues 209 and 211 diminishes the targeting specificity of the full-length enzyme.

The hypothesis also predicts that mutation of the essential lysines in the β 3–4 hairpin will reduce the effect of PIP $_2$ on Ca $^{2+}$ -dependent vesicle binding in vitro. This prediction is confirmed by the observation that PIP $_2$ has little or no effect on the Ca $^{2+}$ -dependent membrane binding of the PKC α C2/K197A/K199A and PKC α C2/K209A/K211A double mutant C2 domains in the protein-to-membrane FRET assay (Figure 7, D and E). As previously suggested by kinase activity studies (Rodriguez-Alfaro *et al.*, 2004), the K209A/K211A double mutation has a greater effect than K197A/K199A on PIP $_2$ -dependent membrane binding. Altogether, these data highlight the importance of basic residues in the β 3–4 hairpin for both the specific plasma membrane targeting observed in vivo and the PIP $_2$ binding observed in vitro for the PKC α C2 domain.

DISCUSSION

In this study we examine the contributions of the two membrane-interacting regions of the PKC α C2 domain to 1) Ca $^{2+}$ -dependent, plasma membrane targeting in vivo and 2) Ca $^{2+}$ -dependent, PIP $_2$ binding in vitro. By functionally transplanting the membrane-interacting regions from the PKC α C2 domain to the body of a different C2 domain, we are able to clearly demonstrate the importance of the CBL to general membrane affinity, as well as the importance of the β 3–4 hairpin to plasma membrane specificity and PIP $_2$ recognition.

Targeting of PKC α and the Isolated PKC α C2 Domain to Puncta and Patches

Our initial PKC α translocation studies revealed apparent local targeting to apical puncta and to basal patches. Because

a number of reports have also observed apical puncta and basal patches with GFP-tagged PLC δ_1 PH domain and interpret them as focal accumulations of PIP $_2$ (as mentioned above), we explored further the origin of these regions. Using two chemically distinct dyes to uniformly stain the plasma membrane, we find that the puncta and patches are areas containing increased membrane surface area (folds and microvilli), consistent with two other reports (Colarusso and Spring, 2002; van Rheenen and Jalink, 2002) and are not sites of PIP $_2$ and/or lipid raft enrichment. Our observations, however, may be cell-type specific, because other groups using similar membrane dyes have concluded that PLC δ_1 PH domain and mGAP43 accumulation in localized regions of the plasma membrane is not caused by locally increased membrane area but instead is attributed to rafts (Huang *et al.*, 2004; Golub and Caroni, 2005). Recently, PKC α has been demonstrated to associate with detergent-insoluble fractions in a Ca $^{2+}$ -dependent manner attributed to raft binding (Rucci *et al.*, 2005). However, the correlation between lipid rafts in cells and detergent-insoluble fractions derived from cells continues to be challenged (van Rheenen *et al.*, 2005). Although the plasma membrane puncta and patches observed herein using traditional raft markers are clearly not rafts, we cannot rule out the existence of membrane inhomogeneities such as rafts, which could be too small to be resolved by light microscopy.

Role of CBLs in Targeting to Cell Membranes

Ca $^{2+}$ binding in the pocket defined by the three CBLs of the PKC α C2 domain has long been recognized as critical for the translocation of PKC α and the isolated PKC α C2 domain to the plasma membrane in cells and for lipid bilayer binding in vitro (Medkova and Cho, 1998; Corbalan-Garcia *et al.*, 1999; Verdaguer *et al.*, 1999; Conesa-Zamora *et al.*, 2001; Stahelin and Cho, 2001; Kohout *et al.*, 2002, 2003; Murray and Honig, 2002). In vitro, and presumably in vivo as well, this translocation involves binding of the Ca $^{2+}$ -loaded protein to anionic PS headgroups on the membrane surface (Verdaguer *et al.*, 1999; (Evans *et al.*, 2004). The results presented here further demonstrate that the PKC α C2 CBLs, fused to another C2 domain, are sufficient for Ca $^{2+}$ -dependent translocation to anionic cellular membranes and phospholipid vesicles. However, although previous studies have concluded that the CBLs are responsible for exclusive plasma membrane targeting (Stahelin *et al.*, 2003; Marin-Vicente *et al.*, 2005), the present findings indicate that the intracellular targeting mediated by the PKC α C2 CBLs alone is different, and less specific, than the plasma membrane targeting of the native C2 domain. Thus, in the absence of the β 3–4 hairpin, the CBLs target primarily to the TGN, with minor binding to the plasma membrane still detectable. It follows that the CBLs alone are not sufficient for specific plasma membrane targeting. This contrasts greatly with the targeting mechanism of another C2 domain, that of cPLA $_2\alpha$, for which the transplanted CBLs were sufficient to confer the native membrane-targeting specificity of cPLA $_2\alpha$ (Golgi and ER membranes) onto a hybrid domain (Evans *et al.*, 2004).

Although the PKC α C2 CBLs alone are not sufficient to confer plasma membrane specificity onto a hybrid C2 domain, it should be emphasized that this hybrid domain does exhibit Ca $^{2+}$ -dependent membrane binding and does translocate to a limited set of membrane targets in vivo. Thus, the CBLs of this hybrid domain retain their functional, Ca $^{2+}$ -activated structure and their binding to anionic membranes. Both the TGN and plasma membrane targets of the hybrid domain contain high levels of PS, because the TGN cytoplasmic leaflet is the precursor to the plasma membrane

cytoplasmic leaflet. The simplest explanation for the observed targeting of the hybrid domain to these membranes is that the transplanted CBLs retain their native interaction with membrane-bound PS headgroups, thereby ensuring translocation primarily to cellular membranes possessing high PS densities. Clearly, however, this weakened specificity is insufficient to ensure exclusive translocation to the plasma membrane.

Role of the Basic Region in the β 3–4 Hairpin in Targeting to Cell Membranes

Biophysical studies have shown that the β -hairpin formed by β strands 3 and 4 of the PKC α C2 domain lies close to the plasma membrane because of a nearly parallel orientation of the domain relative to the membrane (Kohout *et al.*, 2003), similar to earlier predictions (Verdaguer *et al.*, 1999). Further structural studies of PKC α C2 complexed with Ca²⁺ and soluble PS have revealed a basic region comprised of four lysine residues in the β 3–4 hairpin that endow the region with a strong positive charge (Ochoa *et al.*, 2002). Specific interactions between K209 and K211 and the soluble PS have been observed in a crystal structure (Ochoa *et al.*, 2002). Moreover, K209 and K211 have been implicated in studies of PIP₂ binding (Corbalan-Garcia *et al.*, 2003), whereas both PS- and PIP₂-dependent enzyme activation is reduced by mutation of basic region lysines (Corbalan-Garcia *et al.*, 2003; Rodriguez-Alfaro *et al.*, 2004). These studies point to the basic region of the β 3–4 hairpin as a second functional lipid-binding site distinct from the CBLs. The imaging experiments presented herein directly demonstrate that the β 3–4 hairpin and its interaction with PIP₂ are critical for targeting to the plasma membrane. Thus, addition of the PKC α β 3–4 hairpin to the cPLA₂/PKC_CBLs hybrid yields native PKC α C2-like targeting to the PIP₂-rich plasma membrane inner leaflet. Furthermore, protein-to-membrane FRET studies conclusively demonstrate that the β 3–4 hairpin is critical for recognition of PIP₂ by the hybrid domains. The importance of this second membrane-interacting site in plasma membrane targeting and PIP₂ recognition is further corroborated by our experiments where mutation of two lysine residues in the basic region, K209 and K211, promoted targeting to primarily the TGN rather than to plasma membrane. Notably, mutation of the basic region in the β 3–4 hairpin of the PKC α C2 domain gives the same pattern of translocation as the hybrid C2 domain possessing the PKC α C2 CBLs but lacking the β 3–4 hairpin. Moreover, our in vitro FRET studies show that PIP₂ binding is greatly affected by mutation of lysines in either the β 3 or β 4 strands. Overall, the results clearly implicate the basic region in the β 3–4 hairpin as being a second site necessary for proper targeting to the plasma membrane and recognition of PIP₂.

The CBLs and Basic Regions Serve a Coincidence Detection Function

Here we have provided evidence that two membrane-interacting sites of the PKC α C2 domain, the CBLs and the basic region of the β 3–4 hairpin, are both involved in Ca²⁺-mediated targeting to PS and PIP₂ on the cytoplasmic surface of the plasma membrane, respectively. The present findings show that Ca²⁺ binding to the CBLs is both necessary and sufficient to drive membrane binding in vivo and in vitro, but is not sufficient for specific plasma membrane targeting. By contrast, the interaction of the basic region of the β 3–4 hairpin with PIP₂, although not sufficient to drive translocation in the absence of Ca²⁺ binding to the CBLs, is required for specific targeting via its interaction with the PIP₂-rich plasma membrane. Ca²⁺ binding to the CBLs is

needed to provide an electrostatic driving force for anionic membrane docking (Nalefski *et al.*, 1997; Evans *et al.*, 2004) and could also be needed to rearrange the β 3–4 hairpin to yield a conformation suitable for PIP₂ binding.

The presence of these two functional regions is proposed to generate a Ca²⁺-activated, PS/PIP₂ coincidence detection function to the PKC α C2 domain, and, by extension, to the enzyme as a whole. Ca²⁺-triggered targeting to PS and PIP₂ in plasma membrane could play an important physiological role by directing membrane docking to regions of the plasma membrane containing high densities of diacylglycerol created by phospholipase C-mediated hydrolysis of PIP₂, which binds to the C1 domain of PKC α and is required for its full enzymatic activity. For example, it has been proposed that PIP₂ and certain proteins, some of which are known PKC α effector proteins, are enriched in lipid rafts that are too small or closely spaced to be detected by fluorescence microscopy (Hope and Pike, 1996; Laux *et al.*, 2000; Uchino *et al.*, 2004). If such PIP₂-enriched rafts exist they could serve as organizing centers for signaling complexes in which PKC α lies in close proximity to its substrate proteins. Moreover, modulation of the PIP₂ spatio-temporal distribution could play an important role in PKC α regulation. Further in vivo studies at higher spatial resolution or biochemical studies of isolated rafts are needed to investigate these possibilities.

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