Published in final edited form as:

J Mol Biol. 2010 September 17; 402(2): 301–310. doi:10.1016/j.jmb.2010.07.037.

Membrane Docking Geometry and Target Lipid Stoichiometry of Membrane-Bound PKC α C2 Domain: A Combined Molecular Dynamics and Experimental Study

Chun-Liang Lai^{1,n}, Kyle E. Landgraf^{2,n}, Gregory A. Voth^{1,*}, and Joseph J. Falke^{2,*}

¹Department of Chemistry, James Franck Institute, and Computation Institute, University of Chicago, 5735 S. Ellis Ave, Chicago, Illinois, 60637, USA

²Molecular Biophysics Program and Department of Chemistry & Biochemistry, University of Colorado, Boulder, CO 80309-0215, USA

Abstract

Protein kinase Ca (PKC α) possesses a conserved C2 domain (PKC α C2) that acts as a Ca²⁺-regulated membrane targeting element. Upon activation by Ca²⁺, PKC α C2 directs the kinase protein to the plasma membrane, thereby stimulating an array of cellular pathways. At sufficiently high Ca²⁺ concentrations the binding of the C2 domain to the target lipid phosphatidylserine (PS) is sufficient to drive membrane association, but at typical physiological Ca²⁺ concentrations binding both to PS and to phosphoinositidyl-4,5-bisphosphate (PIP₂) is required for specific plasma membrane targeting. Recent EPR studies have revealed the membrane docking geometries of PKC α C2 docked to (i) PS alone, and to (ii) both PS and PIP₂ simultaneously. These two EPR docking geometries exhibit significantly different tilt angles relative to the plane of the membrane, presumably induced by the large size of the PIP₂ headgroup. The present study utilizes the two EPR docking geometries as starting points for molecular dynamics simulations that investigate the atomic features of the protein-membrane interaction. The simulations yield approximately the same PIP₂-triggered change in tilt angle observed by EPR. Moreover, the simulations predict a PIP₂:C2 stoichiometry approaching 2:1 at high PIP₂ mole density. Direct binding measurements titrating the C2 domain with PIP₂ in lipid bilayers yield a 1:1 stoichiometry at moderate mole densities, and a saturating 2:1 stoichiometry at high PIP₂ mole densities. Thus, experiment confirms the target lipid stoichiometry predicted by EPR-guided molecular dynamics simulations. Potential biological implications of the observed docking geometries and PIP₂ stoichiometries are discussed.

Keywords

protein kinase C activation; phosphatidylinositol-(4,5)-bisphosphate regulation; conserved membrane targeting domain; positive cooperativity; molecular dynamics

© 2010 Elsevier Ltd. All rights reserved.

*Corresponding Authors: Gregory A. Voth, Department of Chemistry, James Franck Institute, and Computation Institute, University of Chicago, 5735 S. Ellis Ave, Chicago, Illinois, 60637, USA, gavoth@uchicago.edu and Joseph J. Falke, University of Colorado at Boulder, Department of Chemistry and Biochemistry, UCB 215, Boulder, CO 80309-0215, USA, falke@colorado.edu.

ⁿThese authors contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The C2 domain is a widely-distributed, conserved signaling motif often involved in Ca^{2+} -triggered membrane docking.¹⁻⁴ It was first discovered in the ubiquitous signaling enzyme protein kinase C, wherein it is the second conserved (C2) domain.⁵ It has long been known that Ca^{2+} binding can drive the docking of PKC α C2 domain to the plasma membrane via direct association of the Ca^{2+} binding site with its target PS headgroup.⁶

Recently, however, it was discovered that at physiological Ca^{2+} levels, the specific plasma membrane docking of PKC α C2 domain requires a second target lipid, PIP_2 , as well as PS.^{3,7,8} In cells, all internal membranes contain PS but only the plasma membrane possesses significant levels of both PS and free PIP_2 . Recognition of PIP_2 is essential for the plasma membrane targeting of both isolated PKC α C2 domain and for full length PKC α .^{3,7,8} The PIP_2 binding site is a conserved cluster of 4 lysine residues on the $\beta 3$ - $\beta 4$ hairpin of the C2 domain. Neutralization of two or more lysines reduces the C2 domain affinity for plasma membrane-like membranes containing PIP_2 *in vitro*, and reduces plasma membrane specificity in live cells.^{3,7,8}

Full length PKC α parent protein possesses another lipid binding motif, the C1 domain, as well as the C2 domain.¹ However, C1 domain binding to its target lipid diacylglycerol is not sufficient to drive the targeting reaction. By contrast, Ca^{2+} -stimulated binding of the C2 domain to PS and PIP_2 is sufficient to drive plasma membrane targeting of PKC α and is a central molecular event in signaling pathways controlling cell migration, growth, gene regulation, metabolism, and hormone release.^{1,3,7,8} An important subgroup of other C2 domains (including those of other conventional protein kinase C and synaptotagmin isoforms) also possess a lysine cluster that binds PIP_2 , demonstrating that PIP_2 recognition is a central feature of many C2 domain-controlled pathways.³

Recent EPR docking geometry studies have shown that when PKC α C2 domain is bound to membranes containing PS but lacking PIP_2 , the domain lays flat on the membrane surface with its long axis nearly parallel to the bilayer plane.⁹ By contrast, C2 domain bound to membranes containing both PS and PIP_2 exhibits a tilted geometry, such that the long axis of the domain rotates away from the membrane plane to yield a $40 \pm 10^\circ$ increase in tilt angle.⁹ The latter PS(+) PIP_2 lipid binding mode also exhibits significantly higher membrane binding affinity and bound state lifetime.⁷ It is likely that both binding modes are present under cellular conditions, raising the possibility that the conformation and activity of the PKC α enzyme could be regulated by variations in local PIP_2 density on the plasma membrane surface.⁹ Overall, an enhanced molecular understanding of the binding of PKC α C2 domain to its PS and PIP_2 target lipids will illuminate a fundamental class of regulatory events in cell signaling.

In this report, the binding of PKC α C2 domain to PS and PIP_2 molecules on a bilayer surface is investigated by EPR-guided molecular dynamics (MD) simulations *in silico* and by experimental binding measurements *in vitro*. The EPR-guided MD simulations yield new insights into the structure, stoichiometry and dynamics of the C2 domain-membrane complex. Surprisingly, the simulation predicts that at sufficiently high PIP_2 densities the lysine cluster can be simultaneously occupied by two adjacent PIP_2 molecules. Subsequent binding measurements demonstrate this predicted limiting PIP_2 stoichiometry is correct. The findings further illustrate the power of a combined computational and experimental approach.

Results

Molecular dynamics simulations

A detailed description of how the MD simulations were performed is provided in Methods. Briefly, simulations were begun using the two experimental EPR membrane docking geometries of PKC α C2 domain⁹ to define the initial state of C2 domain bound to a POPC/POPS (1:1) mixed bilayer. In one simulation, the initial state was the EPR docking geometry in which the Ca²⁺ binding pocket and lysine cluster both associate with PS molecules. In two other simulations, the initial state was the EPR docking geometry in which Ca²⁺ binding pocket associates with PS while the lysine cluster associates with PIP₂. The latter two simulations included one or two PIP₂ molecules in the bilayer, respectively. Thus, the three simulations effectively mimic a plasma membrane microenvironment of varying PIP₂ density, and are termed (–)PIP₂, (+)PIP_{2(one)} and (+)PIP_{2(two)}.

Figure 1a-c shows the equilibrated membrane docking geometries generated by the (–)PIP₂, (+)PIP_{2(one)} and (+)PIP_{2(two)} simulations and Figure 2 shows the temporal stability of the C2 domain docking geometry over the duration of simulations. In the (–)PIP₂ simulation, the equilibrated docking geometry exhibits a tilt angle of $7^\circ \pm 2^\circ$ with the β -strands of the C2 domain core nearly parallel to the membrane surface (Figure 1a and Figure 2). In the (+)PIP_{2(one)} simulation, the occupancy of the lysine cluster with one PIP₂ headgroup causes the long axis of the domain to rotate away from the membrane plane, yielding a significantly larger tilt angle of $37^\circ \pm 2^\circ$ (Figure 1b and Figure 2). In the (+)PIP_{2(two)} simulation, the occupancy of the lysine cluster with two PIP₂ headgroups (the latter shown as red and orange in the figure) also causes the β -strands to away from the membrane plane to yield an intermediate tilt angle ($30^\circ \pm 1^\circ$) (Figure 1c and Figure 2). The simulations reveal that the total number of lipids contacted by the C2 domain decreases with increasing tilt angle, ranging from 18 in the (–)PIP₂ state to 14 lipids in the (+)PIP_{2(one)} and (+)PIP_{2(two)} states, as illustrated in Figure 3. It should be noted that a simulation starting from the (+)PIP₂ EPR docking geometry of the C2 domain in the absence PIP₂ was also performed (data not shown). In this simulation, the initial tilt docking geometry of C2 domain relaxes to the flat docking geometry within 20 ns.

Several parameters observed in the simulations can be compared with measured parameters in the literature. The (–)PIP₂ simulation, like the EPR docking geometry analysis in the absence of PIP₂, yields an orientation with the domain long axis nearly parallel to the membrane plane.⁹ Moreover, the $30^\circ \pm 4^\circ$ tilt angle difference between the (–)PIP₂ and (+)PIP_{2(one)} simulations agrees, within error, with the experimental tilt angle difference of $40^\circ \pm 10^\circ$ measured by EPR⁹ wherein the dominant bound state for membranes containing PIP₂ was the C2 domain bound to a single PIP₂ molecule (in EPR studies the PIP level was 2 mole percent, which yields one bound PIP₂ molecule per C2 domain – see Discussion). Similarly, the 18 contact lipids predicted by the (–)PIP₂ simulation is consistent, within error, with the previously defined upper limit on the number of footprint lipids, 14 ± 4 , as measured for domain binding to membranes lacking PIP₂.¹⁰ Finally, the (–)PIP₂ simulation indicates that the C2 domain contacts primarily the charged/polar lipid headgroups, consistent with the electrostatic docking mechanism of previously observed for this state, in contrast to the hydrophobic docking mechanism of cytosolic phospholipase A2 C2 domain that penetrates into the bilayer hydrocarbon core and is not displaced by high ionic strength.^{10,11}

In addition to parallels with previous experimental observations, the simulations yield the novel prediction that the lysine cluster can spontaneously associate with either one or two PIP₂ molecules placed nearby, yielding a stable 1:1 or 2:1 PIP₂ binding stoichiometry, respectively. Recent experimental studies revealed that the lysine cluster binds PIP₂,^{3, 7, 8}

but it is generally assumed that the lysine cluster binds only one PIP₂ molecule and such 1:1 stoichiometry is observed in a crystal complex between the free C2 domain and the isolated PIP₂ headgroup IP₃.¹² Thus the apparent binding of two adjacent PIP₂ molecules is surprising.

PIP₂ stoichiometry measurements

To experimentally measure the PIP₂ stoichiometry, the binding of PKCα C2 domain to PIP₂ on membrane surfaces was quantitated using an established protein-to-membrane FRET assay (see Methods^{13,14}). To ensure that the experiment measured the saturating PIP₂ stoichiometry, conditions were chosen to drive the binding reaction to completion (10 mole % in sonicated unilamellar-membrane vesicles, SUVs). As a result, when membranes were titrated into the sample, the available PIP₂ molecules on the membrane surface were immediately occupied by protein and the protein-to-membrane FRET increased linearly until all protein molecules were bound to membrane, yielding a simple linear approach to a plateau (the signature of a successful stoichiometry determination) as illustrated in Figure 4a. The resulting stoichiometry measured for three replicates is 2.1 ± 0.1 PIP₂ molecules per C2 domain, confirming the ability of the C2 domain to bind two PIP₂ molecules as predicted by the MD simulation. To ensure that the stoichiometry was limited by PIP₂ binding and not by the size of the protein footprint, another stoichiometry determination was carried out for a 2-fold lower PIP₂ density (5 mole %). This titration also yielded a stoichiometry approaching two PIP₂ molecules per C2 domain (1.7 ± 0.1), indicating that the plateau was defined by saturating PIP₂ stoichiometry rather than close packing of protein molecules on the membrane surface.

Since the C2 domain can bind two PIP₂ molecules, the possibility that the PIP₂ binding reaction possesses positive cooperativity was examined. A second titration experiment was carried out under conditions more closely approximating a physiological Ca²⁺ signal. This titration fixed the concentrations of Ca²⁺ (1 μM), C2 domain (0.5 μM) and total lipid (60 μM) while increasing the density of PIP₂ in the SUVs from 0 to 20 mole %. As illustrated in Figure 4b, the resulting protein-to-membrane FRET binding curve exhibits a Hill coefficient of 6 ± 2 in three replicates. This Hill coefficient is consistent with the cooperative binding of multiple PIP₂ molecules. However, as noted in the Discussion below, the PIP₂ binding reaction is complex and several factors contribute to the apparent positive cooperativity, such that the observed Hill coefficient likely overestimates the number of cooperative PIP₂ binding sites.

Discussion and Summary

Together these findings provide molecular views of three different membrane docking states of PKCα C2 domain as the lysine cluster is titrated with its target ligand PIP₂. In all three states, the Ca²⁺ binding site associates with PS on the membrane surface, but the states differ in the lipid occupancies of the lysine cluster. In the absence of PIP₂ the lysine cluster associates with PS headgroups, yielding a membrane docking geometry in which the long axis of the core β-sandwich is nearly parallel to the membrane plane. The MD simulation reveals this parallel geometry, originally defined by EPR docking geometry analysis,⁹ is indeed stable (Figure 2) and exhibits a tilt angle of $7^\circ \pm 2^\circ$ relative to the membrane plane. At intermediate PIP₂ density the MD simulation describes the previously anticipated second state in which the lysine cluster binds to a single PIP₂ molecule, tilting the long axis $37^\circ \pm 2^\circ$ relative to the membrane plane due to the larger size of the PIP₂ headgroup. Finally, at higher PIP₂ density the MD simulation predicts a novel third state in which the lysine cluster stably binds two molecules of PIP₂, yielding a somewhat reduced tilt angle of $30^\circ \pm 1^\circ$ relative to the membrane plane. Direct binding measurements confirm this is the saturated binding state of the lysine cluster (Fig. 4a). Together, the present and previous studies

provide a detailed, self-consistent molecular view of the three docking states as defined by EPR docking geometry analysis, MD simulations, and direct binding measurements.

The saturating stoichiometry of two PIP₂ molecules per C2 domain, predicted by the MD simulations and confirmed by experiment, contrasts with the recent crystal structure of a complex between the C2 domain and a single molecule of a soluble PIP₂ analogue bound to the lysine cluster.¹² At least two explanations can be offered for this apparent contradiction: (i) in contrast to soluble PIP₂, the headgroup of membrane-bound PIP₂ possesses strong steric constraints that could lead to a different binding geometry for the inositol ring, or (ii) the environmental differences between aqueous PIP₂ and bilayer-imbedded PIP₂ may alter the binding reaction in a complex fashion. Consistent with the idea that soluble and membrane-bound PIP₂ possess different binding geometries, the crystal structure of the IP₃ complex reveals direct contacts between IP₃ and conserved residues Tyr 195 and Trp 245.¹² By contrast, the MD simulation detects no direct contacts between PIP₂ and these two residues. Instead, the (+)PIP_{2(one)} and (+)PIP_{2(two)} simulations reveals direct PIP₂ contacts with residues Lys 197, Lys 209 and Lys 211, and these contacts are also observed from the crystal structure. However, in the MD simulations the contacts with Lys 197, Lys 209 and Lys 211 are solely to the 4- and 5- phosphate groups of PIP₂ that are exposed on the bilayer surface, while in the crystal structure contacts are also observed to the 1-phosphate that would be significantly less accessible in the bilayer.

Under physiological conditions in the cell, it is likely that the membrane-bound C2 domain population includes all three states characterized herein: (–)PIP₂, (+)PIP_{2(one)} and (+)PIP_{2(two)}. This conclusion arises from the fact that the global plasma membrane PIP₂ density (2 mole %) is well below the level required to yield half maximal protein binding (Fig. 4b), while the local density of PIP₂ in the plasma membrane is believed to be heterogeneous due to lipid-protein microdomains possessing high PIP₂ levels.¹⁵ Thus, a C2 domain located in a region of low PIP₂ density may be occupied by zero or one PIP₂ molecules while a C2 domain located in a high PIP₂ density region may possess two bound PIP₂ molecules. Since these different PIP₂ stoichiometries yield different membrane tilt angles (Fig. 1), and the tilt angle in principle could modulate the activity of the kinase domain, such different stoichiometries may provide local regulation of PKC α kinase activity.⁹

The large Hill coefficient (6 ± 2) observed for the titration of PIP₂ mole density (Fig. 4b) is consistent with positive cooperativity in PIP₂ binding to the C2 domain but likely overestimates the number of cooperative PIP₂ binding sites. For a simple association reaction, the binding of two ligands to a pair of positively cooperative sites would yield a Hill coefficient no larger than 2.0. However, the Ca²⁺-triggered binding of a C2 domain to a membrane surface exhibits several complexities that complicate the cooperativity analysis. As the density, and concentration, of PIP₂ in the reaction increases the Ca²⁺-free protein is more likely to bind PIP₂ on the membrane surface, which will dramatically increase its affinity for Ca²⁺ and PS. This effect, termed Target Activated Messenger Affinity or TAMA,⁷ will increase the apparent Hill coefficient. The increasing PIP₂ density also increases the negative charge of the membrane surface and strengthens its nonspecific electrostatic interactions with basic regions of the C2 domain, including the lysine cluster, thereby further increasing the apparent Hill coefficient. Finally, any tendency for PIP₂ to cluster at high mole densities would facilitate the binding of the lysine cluster to adjacent PIP₂ molecules and again increase the apparent Hill coefficient. Thus, while the large Hill coefficient observed for the PIP₂ mole density titration is not directly related to the number of PIP₂ molecules bound to the C2 domain, it does further support the hypothesis that local variations in PIP₂ density on the plasma membrane could alter the docking of PKC α C2

domain at physiological Ca^{2+} concentrations and modulate the kinase activity of full length PKC α .

In summary, a MD simulation predicting that the lysine cluster of PKC α C2 domain is saturated by the binding of two PIP₂ molecules is confirmed by direct PIP₂ binding measurements on membrane surfaces. Physiological PIP₂ densities likely yield binding to a single PIP₂ molecule except in microdomains where the PIP₂ density is high and saturation with two PIP₂ molecules may occur. The lysine cluster is a conserved motif shared by an important subset of C2 domains, suggesting that an array of important signaling pathways may well be modulated by switching between different membrane-bound states occupied by zero, one or two PIP₂ molecules.

Methods

Molecular dynamics simulations

The MD simulations utilized a single C2 domain bound to the bilayer solvated with explicit TIP3P water¹⁶ and sufficient Na⁺ counterions to maintain overall charge neutrality. The original coordinates used for the C2 domain were those of the crystal structure from Gomez-Fernandez and colleagues (Protein Data Bank ID code 1DSY).⁶ For the lipid bilayer, an all-atom 1:1 palmitoyl-oleoyl phosphatidylcholine / palmitoyl-oleoyl phosphatidylserine (POPC/POPS) mixed bilayer was used to ensure that sufficient PS lipids are available in the simulation to satisfy the PS binding requirements of the Ca^{2+} binding pocket and the lysine cluster. The mixed bilayer was generated using an equilibrated pure POPC lipid bilayer from previous work.¹⁷ For the POPC/POPS lipid bilayer, 50% of the phosphatidylcholine (PC) head groups from the pure POPC lipid bilayer system were changed to phosphatidylserine (PS) headgroups to yield a nearly uniform distribution across the system. The procedure for replacing PC headgroups into PS headgroups was performed as previously described.¹⁸ The POPC/POPS/solvent membrane system was first equilibrated for 75 ns under constant NPT conditions. The C2 domain/lipid bilayer system was then built in the following way: the C2 domain was placed on the mixed POPC/POPS patch in the docking geometry defined by EPR analysis of the protein-membrane complex lacking PIP₂,⁹ yielding the initial (–)PIP₂ system. Then, one or two PIP₂ headgroups were introduced by substitution for PC headgroups near the lysine cluster (Lys197, Lys199, Lys209, and Lys211) while retaining existing tail groups yielding the two systems (+)PIP_{2(one)} and (+)PIP_{2(two)}, respectively. In both cases the initial geometry was adjusted to match the EPR docking geometry determined for the protein-membrane complex containing PIP₂.⁹ The resulting simulated systems possessed ~92,000 and ~98,000 atoms in the (–) PIP₂ and (+) PIP₂ states, respectively.

For each of the three systems, subsequent solvation, minimization, and MD equilibration protocols were carried out as follows. The protein and membrane system was equilibrated using a stepwise relaxation procedure for the lipid head and tail groups, sidechains, backbone, and calcium ions. After placing the C2 domain on the membrane surface, systems were solvated with TIP3P water and neutralized by Na⁺ counterions. Initially, all alpha carbons, calcium ions and phosphorous atoms were harmonically restrained with a 5 kcal/(mol Å²) force constant and a conjugate gradient minimization of 5,000 steps was applied followed by heating to 310 K followed by 100 ps constant NPT equilibration. To fully relax the interaction of the C2 domain and bilayer, four stages of 10 ns constant NPT equilibration were then performed. In the first stage, restraints were turned off for all phosphorous atoms except for the calcium-bound PS and for PIP₂ in order to relax the bilayer. For the second stage the restraints on PS and PIP₂ were released. The alpha carbons were released in the third stage to relax the protein. Finally, a 10 ns unrestrained simulation was performed to release the calcium ions. A production run of 30 ns was then performed for analysis.

All the molecular dynamics simulations employed CHARMM2219 and CHARMM2720 force field parameters to describe the protein and the lipid-protein interactions, respectively. The parameterization for PIP₂ head groups was as previously described²¹. Simulations were performed under isothermal, isobaric conditions (NPT) and the periodic boundary conditions is applied. A Langevin thermostat with a damping coefficient of 0.5 ps⁻¹ was used to maintain the system temperature at 310 K. The system pressure was maintained at 1 atm using a Langevin piston barostat.²² Short-range non-bonded interactions were truncated smoothly between 10 and 12 Å. The particle mesh Ewald algorithm²³ was used to compute long-range electrostatic interactions at every time-step. All covalent hydrogen bonds were constrained by the SHAKE algorithm (or SETTLE for water),²⁴ permitting an integration time step of 2 fs. System minimization, equilibration, and dynamics were performed using the NAMD 2.6 software package.²⁵ System construction and image generation were done by using the VMD 1.8.6 software package.²⁶

It should be noted that the net charge of PIP₂ under physiological conditions can vary depending on several factors such as local lipid composition, interaction with nearby proteins or local pH level.²⁷ Experiments show that the net charge of PIP₂ can range from -3 to -5.^{27,28,29} The original parameterization for the PIP₂ molecule adopted in current MD study assumes that PIP₂ has a charge of -5, which means that both the 4' and 5' phosphates are deprotonated.²¹ In order to examine the effect of the charge state of PIP₂ on the interaction between the C2 domain and the bilayer, four additional simulations were performed with the PIP₂ having a lower net charge (-3 or -4, data not shown). Using the same simulation protocols as described above, the stability of the C2 domain-membrane complex was not affected by changing the net charge on PIP₂. The Na⁺ counterions were often observed to locally compensate for the charge in the more highly negatively charged PIP₂ cases. It should also be noted that the orientation of the PIP₂ headgroup found in the (+)PIP₂ simulation systems exhibits a somewhat greater tilt than reported for PIP₂ simulations in the absence of protein.²¹ This is likely due to the association of the PIP₂ headgroup with the lysine cluster located in the β-strands of the C2 domain.

Reagents for experimental studies

All lipids were synthetic unless otherwise indicated. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine, POPC, PC); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (phosphatidylethanolamine, POPE, PE); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (phosphatidylserine, POPS, PS); L-α-phosphatidylinositol (PI) natural from bovine liver; L-α-phosphatidylinositol- 4,5-bisphosphate (PI(4,5)P₂, PIP₂) natural from porcine brain; and sphingomyelin (SM) natural from brain were all purchased from Avanti Polar Lipids. Cholesterol (CH) was from Sigma. N-[5-(Dimethylamino)naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-PE, dPE) was from Molecular Probes.

PKCα C2 domain expression and purification

The wild type C2 domain of human PKCα (residues 155-293) was expressed as a His-tagged fusion in *E. coli* BL21 cells as previously described⁹ with the following modifications. Transformed cells were grown at 37 °C to O.D. 0.6 and induced with 500 μM IPTG. After induction cells were grown at 30 °C for 8 hours. Protein was bound on a Ni-NTA agarose affinity column, washed with 50 mM imidazole, and eluted off the column by cleavage of the N-terminal His-tag with thrombin. Thrombin was affinity extracted from the protein sample using p-aminobenzamidine resin (Sigma). Protein was determined to be >95% pure by SDS-PAGE and protein concentration was determined using the BCA method (where concentration error is less than 10% as determined from multiple concentration measurements).

Preparation of plasma membrane-like phospholipid vesicles

Membrane vesicles mimicking the inner leaflet of the plasma membrane were prepared largely as previous described.⁹ All lipid components were mixed in solvent containing chloroform/methanol/water (1/2/0.8) to give the desired lipid ratios (below), dried under vacuum to remove all solvents, and then hydrated in assay buffer (25 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) at pH 7.4 with KOH, 140 mM KCl, 15 mM NaCl, and 0.5 mM MgCl₂) by rapid vortexing. Small unilamellar vesicles (SUV) were generated by sonication of the hydrated lipid mixture to clarity with a Misonix XL2020 probe sonicator. Vesicles used in the FRET assays were prepared at a total lipid concentration of 3 mM with varying mole percentages of the PIP₂ signaling lipid. The lipid mixture designed to mimic the plasma membrane inner leaflet in PIP₂ stoichiometry titrations was PE/PC/PS/PI/SM/CH/dPE/PIP₂ (23.8/9.1/18.1/4.5/4.5/25/5/10), where the PIP₂ density was zero for PM(-)PIP₂ and 10 mol % for PM(+)PIP₂ membranes, respectively. The lipid mixtures employed in the PIP₂ density titrations were the same composition, except the mole percent PIP₂ was varied while simultaneously adjusting the mole percentages of PE and PC to compensate for PIP₂ gain or loss, at the same time keeping the PE:PC mole ratio at 2.6:1. As a control, for all PIP₂-containing membranes the accessible PIP₂ concentration was independently verified using the high-affinity AKT E17K PH30 to titrate accessible PIP₂.

Protein-to-membrane FRET stoichiometry titration

The PIP₂ stoichiometry titration was carried out using an established protein-to-membrane fluorescence resonance energy transfer (FRET) assay to quantitate membrane binding.^{13; 14} Briefly, samples contained C2 domain (0.2 μM or 1 μM) and 10 μM free [Ca²⁺] in physiological buffer (25 mM HEPES at pH 7.4 with KOH, 140 mM KCl, 15 mM NaCl, 0.5 mM MgCl₂, and 15 mM EDTA). EDTA was included in these samples to reduce the levels of contaminating Ca²⁺ from low micromolar to nanomolar concentrations. Sufficient Ca²⁺ was added in excess of EDTA to achieve a concentration of 10 μM free Ca²⁺ where Ca²⁺ concentrations were determined using Maxchelator (www.stanford.edu/~cpatton/maxc.html). PM(+)PIP₂ (10% PIP₂) membranes were titrated into protein and the protein-to-membrane FRET was measured in a Photon Technologies International spectrofluorimeter (QM-2000-6SE) at 25 °C, with excitation and emission slits at 4 and 8 nm, respectively. Intrinsic donor tryptophan residues in the C2 domain were excited at 284 nm and emission at 522 nm from the dansyl-PE acceptor residues was quantitated. To correct for direct acceptor excitation, the background dansyl fluorescence of a control sample lacking protein was subtracted from experimental samples. Since C2 domain binding to membrane in these assays was in the stoichiometric binding regime the data was analyzed using linear least squares fitting of the rise phase and of the saturation phase, such that the stoichiometry was defined by the intersection of these two straight lines.

The PIP₂ mole density titration was carried out using the same protein-to-membrane FRET assay but now the total protein and lipid concentrations were both held constant while systematically increasing the mole percentage of PIP₂ in the vesicles. Samples were made as above with 0.5 μM C2 domain, 60 μM total lipid (where the density of PIP₂ varied 0-20 mole %), and a physiological free Ca²⁺ concentration of 1 μM to mimic a cytoplasmic Ca²⁺ signal. The resulting protein-to-membrane FRET titration curve was fit using the Hill equation.

Statistics

All averages ± 1 S.D. were calculated for 3 or more independent titrations.

Acknowledgments

This study was supported by grants from National Institutes of Health (R01-GM063796 to G.A.V., R01-GM063235 to J.J.F., and T32-GM065103 to K.E.L.). Computational resources were provided by the National Science Foundation through TeraGrid computing resources of the Texas Advanced Computing Center, the Pittsburgh Supercomputing Center, the San Diego Supercomputing Center, and the National Institute for Computational Sciences. The authors thank Dr. Gary Ayton for his valuable input. The contribution to this work by C.-L. Lai and G. A. Voth was initiated at the Center for Biophysical Modeling and Simulation, University of Utah.

References

1. Newton AC. Lipid activation of protein kinases. *J. Lipid Res.* 2009; 50(Suppl):S266–S271. [PubMed: 19033211]
2. Lemmon MA. Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell. Biol.* 2008; 9:99–111. [PubMed: 18216767]
3. Corbalan-Garcia S, Guerrero-Valero M, Marin-Vicente C, Gomez-Fernandez JC. The C2 domains of classical/conventional PKCs are specific PtdIns(4,5)P₂-sensing domains. *Biochem. Soc. Trans.* 2007; 35:1046–1048. [PubMed: 17956275]
4. Nalefski EA, Falke JJ. The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci.* 1996; 5:2375–2390. [PubMed: 8976547]
5. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* 1995; 9:484–496. [PubMed: 7737456]
6. Verdaguer N, Corbalan-Garcia S, Ochoa WF, Fita I, Gomez-Fernandez JC. Ca²⁺ bridges the C2 membrane-binding domain of protein kinase Ca directly to phosphatidylserine. *EMBO J.* 1999; 18:6329–6338. [PubMed: 10562545]
7. Corbin JA, Evans JH, Landgraf KE, Falke JJ. Mechanism of specific membrane targeting by C2 domains: localized pools of target lipids enhance Ca²⁺ affinity. *Biochemistry.* 2007; 46:4322–4336. [PubMed: 17367165]
8. Evans JH, Murray D, Leslie CC, Falke JJ. Specific translocation of protein kinase Calpha to the plasma membrane requires both Ca²⁺ and PIP₂ recognition by its C2 domain. *Mol. Biol. Cell.* 2006; 17:56–66. [PubMed: 16236797]
9. Landgraf KE, Malmberg NJ, Falke JJ. Effect of PIP₂ binding on the membrane docking geometry of PKCa C2 domain: an EPR site-directed spin-labeling and relaxation study. *Biochemistry.* 2008; 47:8301–8316. [PubMed: 18610985]
10. Kohout SC, Corbalan-Garcia S, Torrecillas A, Gomez-Fernandez JC, Falke JJ. C2 domains of protein kinase C isoforms α , β , and γ : activation parameters and calcium stoichiometries of the membrane-bound state. *Biochemistry.* 2002; 41:11411–11424. [PubMed: 12234184]
11. Malmberg NJ, Van Buskirk DR, Falke JJ. Membrane-docking loops of the cPLA2 C2 domain: detailed structural analysis of the protein-membrane interface via site-directed spin-labeling. *Biochemistry.* 2003; 42:13227–13240. [PubMed: 14609334]
12. Guerrero-Valero M, Ferrer-Orta C, Querol-Audi J, Marin-Vicente C, Fita I, Gomez-Fernandez JC, Verdaguer N, Corbalan-Garcia S. Structural and mechanistic insights into the association of PKCa-C2 domain to PtdIns(4,5)P₂. *Proc. Natl. Acad. Sci. USA.* 2009; 106:6603–6607. [PubMed: 19346474]
13. Nalefski EA, Slazas MM, Falke JJ. Ca²⁺-signaling cycle of a membrane-docking C2 domain. *Biochemistry.* 1997; 36:12011–12018. [PubMed: 9340010]
14. Nalefski EA, Falke JJ. Use of fluorescence resonance energy transfer to monitor Ca²⁺-triggered membrane docking of C2 domains. *Methods Mol. Biol.* 2002; 172:295–303. [PubMed: 11833355]
15. Day CA, Kenworthy AK. Tracking microdomain dynamics in cell membranes. *Biochim. Biophys. Acta.* 2009; 1788:245–253. [PubMed: 19041847]
16. Jorgensen WL, Chandrasekhar J, Madura JD. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 1983; 79:926–935.
17. Jaud S, Tobias DJ, Falke JJ, White SH. Self-induced docking site of a deeply embedded peripheral membrane protein. *Biophys. J.* 2007; 92:517–524. [PubMed: 17071664]

18. Blood PD, Voth GA. Direct observation of Bin/amphiphysin/Rvs (BAR) domain-induced membrane curvature by means of molecular dynamics simulations. *Proc. Natl. Acad. Sci. USA*. 2006; 103:15068–15072. [PubMed: 17008407]
19. MacKerell AD. All-atom empirical potential for molecular modeling and dynamics studies of proteins. *J. Phys. Chem. B*. 1998; 102:3586–3616. al., e.
20. Feller SE, MacKerell AD. An improved empirical potential energy function for molecular simulations of phospholipids. *J. Phys. Chem. B*. 2000; 104:7510–7515.
21. Lupyan D, Mezei M, Logothetis DE, Osman R. A molecular dynamics investigation of lipid bilayer perturbation by PIP₂. *Biophys. J*. 2010; 98:240–247. [PubMed: 20338845]
22. Feller SE, Zhang Y, Pastor RW, Brooks BR. Constant pressure molecular dynamics simulation: The Langevin piston method. *J. Chem. Phys.* 1995; 103:4613–4621.
23. Darden T, York D, Pedersen L. Particle mesh Ewald: An N-log(N) method for Ewald sums in large systems. *J. Chem. Phys.* 1993; 98:10089–10092.
24. Miyamoto S, Kollman PA. Settle: An analytical version of the SHAKE and RATTLE algorithm for rigid water models. *J. Comput. Chem.* 1992; 13:952–962.
25. Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, Chipot C, Skeel RD, Kale L, Schulten K. Scalable molecular dynamics with NAMD. *J. Comput. Chem.* 2005; 26:1781–1802. [PubMed: 16222654]
26. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J. Mol. Graph.* 1996; 14:33–8. [PubMed: 8744570]
27. McLaughlin S, Wang JY, Gambhir A, Murray D. PIP₂ and proteins: Interactions, organization, and information flow. *Annu. Rev. Biophys. Biomol. Struc.* 2002; 31:151–175.
28. McLaughlin S, Murray D. Plasma membrane phosphoinositide organization by protein electrostatics. *Nature*. 2005; 438:605–611. [PubMed: 16319880]
29. Kooijman EE, King KE, Gangoda M, Gericke A. Ionization Properties of Phosphatidylinositol Polyphosphates in Mixed Model Membranes. *Biochemistry*. 2009; 48:9360–9371. [PubMed: 19725516]
30. Landgraf KE, Pilling C, Falke JJ. Molecular mechanism of an oncogenic mutation that alters membrane targeting: Glu17Lys modifies the PIP lipid specificity of AKT1 PH domain. *Biochemistry*. 2008; 47:12260–12269. [PubMed: 18954143]

Fig. 1. MD simulations of the PKC α C2 domain on a lipid bilayer

Snapshots from MD simulations of the complex composed of a C2 domain and (a) a bilayer lacking PIP₂; (b) a bilayer containing one PIP₂ molecule; and (c) a bilayer containing two PIP₂ molecules. These simulations were termed (–)PIP₂, (+)PIP_{2(one)}, and (+)PIP_{2(two)} respectively. Lipids are shown in gray, PS headgroups are shown as small pink spheres, and the lysine cluster formed by the side chains of K197, K199, K209, and K211 are blue spheres. Nearby K205 is also highlighted by blue spheres. In (+)PIP_{2(one)} system, the PIP₂ headgroup is shown as red spheres; in (+)PIP_{2(two)} system, the two PIP₂ headgroups are colored differently as red and orange spheres. In each case the simulation was initiated by placing the C2 domain (PDB entry 1DSY,6 drawn as a ribbon) with two bound calcium ions (yellow spheres) on the surface of a 1:1 POPC/POPS mixed lipid bilayer. The C2 domain was positioned in the center of the bilayer patch with an initial penetration depth and angle relative to the bilayer surface that matched the docking geometry previously determined by EPR analysis⁹. As indicated, zero, one or two PIP₂ molecules were placed near the lysine cluster. This initial system was equilibrated as described in Methods, then a 30 ns unconstrained MD simulation was carried out during which a representative single frame snapshot was taken for the figure. The vector (orange arrow) operationally defines the long axis of the core β -sandwich (from Ca²⁺501 to the Ca atom of N206). The plane (dashed line) indicates the average location of the lipid backbone phosphate groups. In the absence of PIP₂ the long axis of the membrane-bound C2 domain is oriented nearly parallel to the bilayer surface with a tilt angle of $7^\circ \pm 2^\circ$. The binding of one PIP₂ to the lysine cluster tilts the domain away from the membrane plane, yielding a tilt angle of $37^\circ \pm 2^\circ$, while the binding of two PIP₂ yields an intermediate tilt angle of $30^\circ \pm 1^\circ$. Each tilt angle and its standard deviation was calculated by averaging the mean tilt angle of six time blocks obtained by breaking the 30 ns simulation into 5 ns sections.

Fig. 2. Temporal stability of the docking geometry

Shown are the time-varying tilt angles of the three different C2 domain-membrane complexes during each 30 ns simulation. Details as in Figure 1 legend. (-)PIP₂, black line; (+)PIP_{2(one)}, green line; and (+)PIP_{2(two)}, blue line.

Fig. 3. Lipids contacting the bound C2 domain

Snapshots from the $(-)\text{PIP}_2$ and $(+)\text{PIP}_{2(\text{two})}$ simulations, highlighting the 18 and 14 lipid headgroups contacting the C2 domain, respectively. The lipid footprint of the $(+)\text{PIP}_{2(\text{one})}$ simulation (not shown) is indistinguishable from that of the $(+)\text{PIP}_{2(\text{two})}$ simulation. The two PIP_2 headgroups are colored differently as red and orange spheres, other headgroups in contact with the C2 domain are grey spheres.

Fig. 4. Binding of PKCα C2 domain to PIP₂-containing membranes

A standard protein-to-membrane FRET assay was employed to quantitate membrane-bound C2 domain at 25° C13; 14. **(a)** Determination of PIP₂ stoichiometry. Target membranes containing a fixed PIP₂ mole fraction were titrated into a fixed concentration of C2 domain. Conditions were chosen to drive high-affinity PIP₂ binding, such that the titration yielded a linear increase in membrane-associated C2 domain until all protein was bound and a plateau was achieved. The intersection of best-fit straight lines for the linear increase and plateau regions represents the saturation point, yielding 2.1 PIP₂ molecules per C2 domain in this representative example. The target membranes employed were sonicated unilamellar vesicles containing 10 mole % PIP₂ in a lipid mixture mimicking plasma membrane inner leaflet. Samples contained 1.0 μM C2 domain and 10 μM free Ca²⁺ in a physiological buffer: 25 mM HEPES at pH 7.4 with KOH, 140 mM KCl, 15 mM NaCl, 0.5 mM MgCl₂, and sufficient EDTA to generate the desired free [Ca²⁺] (see Methods). **(b)** Analysis of PIP₂ cooperativity. Target membranes containing the indicated mole fractions of PIP₂ were added to yield a fixed total lipid concentration in a fixed concentration of C2 domain. Conditions were chosen to simulate a cytoplasmic Ca²⁺ signal. Nonlinear least squares best-fit of the resulting membrane binding curve with the Hill equation yields 5.1 ± 0.4 for this representative example, but the interpretation of this apparent positive cooperativity is complex (see Discussion). Samples contained 60 μM total lipid (see (a) for composition), 0.5 μM C2 domain and 1 μM free Ca²⁺ in physiological buffer (see (a)).