Increased Membrane Affinity of the C1 Domain of Protein Kinase Cδ Compensates for the Lack of Involvement of Its C2 Domain in Membrane Recruitment

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Protein kinase C (PKC) family members are allosterically activated following membrane recruitment by specific membrane-targeting modules. Conventional PKC isozymes are recruited to membranes by two such modules: a C1 domain, which binds diacylglycerol (DAG), and a C2 domain, which is a Ca\(^{2+}\)-triggered phospholipid-binding module. In contrast, novel PKC isozymes respond only to DAG, despite the presence of a C2 domain. Here, we address the molecular mechanism of membrane recruitment of the novel isozyme PKCδ. We show that PKCδ and a conventional isozyme, PKCβII, bind membranes with comparable affinities. However, disruption of the contribution of individual domains to this binding revealed that, although the C2 domain is a major determinant in driving the interaction of PKCβII with membranes, the C2 domain of PKCδ does not bind membranes. Instead, the C1B domain is the determinant that drives the interaction of PKCδ with membranes. The C2 domain also does not play any detectable role in the activity or subcellular location of PKCδ in cells; in vivo imaging studies revealed that deletion of the C2 domain does not affect the stimulus-dependent translocation or activity of PKCδ. Thus, the increased affinity of the C1 domain of PKCδ allows this isozyme to respond to DAG alone, whereas conventional PKC isozymes require the coordinated action of Ca\(^{2+}\) binding to the C2 domain and DAG binding to the C1 domain for activation.

Many signaling proteins translocate from the cytosol to the membrane to become activated or to reach their substrates. Reversible binding to the membrane is mediated by modules such as the C1, C2, pleckstrin homology, and FYVE domains, with each recognizing specific lipid determinants or membrane properties. The protein kinase C (PKC) family is a classic example of an enzyme whose function is regulated by membrane translocation. For this family of signaling proteins, the C1 and C2 domains direct the enzyme to the membrane following generation of diacylglycerol (DAG) and, for some isoforms, Ca\(^{2+}\) (reviewed in Ref. 1).

The C1 domain is a 5-kDa globular domain composed primarily of two β-sheets that are pulled apart to expose a binding pocket for DAG and phorbol. Found in a number of other proteins in addition to PKC (notably, protein kinase D, DAG kinase, and Raf), this domain is an effective DAG sensor when expressed alone (reviewed in Refs. 2 and 3). Moreover, the C1 domain selectively recognizes phosphatidylinerine (PS) (4, 5). The C2 domain is another small (16 kDa) globular domain that is primarily composed of β-strands; in this case, three loops in the β-strands at one end of the domain form a Ca\(^{2+}\)-binding site. Conventional C2 domains such as those found in phospholipase C, phospholipase A\(_2\), phosphatidylinositol 3-kinase, syntaptoagmin, and Munc13 bind Ca\(^{2+}\) in this pocket, and the binding drives the membrane interaction (reviewed in Refs. 6 and 7). Novel C2 domains such as that found in PTEN do not bind Ca\(^{2+}\), yet can still bind phospholipids (8). The coordinated use of a C1 and C2 domain allows sensitivity in the regulation of Ca\(^{2+}\)/DAG-dependent PKC family members.

The PKC family is divided into three groups based on the composition of membrane-targeting modules: N-terminal to the kinase domain, which in turn dictates the second messenger dependence (reviewed in Ref. 9). Conventional isozymes (α, βI, βII, and γ) contain a C1 and C2 domain and bind the ligands DAG and Ca\(^{2+}\), respectively. Novel isozymes (δ, ε, η, and θ) contain a C1 domain that mediates recognition of DAG and a “novel” C2 domain that lacks determinants required for Ca\(^{2+}\) binding. Atypical isoforms (ζ and ι/α) contain only one membrane-targeting domain, an atypical C1 domain that has an impaired ligand-binding pocket and that does not bind DAG or phorbol ester. Thus, the composition of membrane-targeting modules allows selective activation of isoforms by stimuli that generate only DAG or that elevate both DAG and Ca\(^{2+}\) levels.

The mechanism of membrane recruitment and activation of conventional PKC isozymes has been well studied. In the absence of signals, PKC exists in an inactive state in the cytosol. The enzyme is recruited to membranes following receptor-mediated production of DAG and release of intracellular Ca\(^{2+}\). PKC first binds Ca\(^{2+}\) in the cytosol via its C2 domain, an event that engages the domain on the membrane via a low affinity interaction (10). The enzyme then diffuses in the two-dimensional plane of the membrane to find DAG, which binds the C1 domain. Although each domain binds with relatively low affinity to the membrane, the coordinated binding of both provides the energy to release an autoinhibitory pseudo-substrate sequence from the substrate-binding site of PKC, allowing binding of substrate and phosphorylation (5). Thus, conventional PKC uses a domain that binds a soluble ligand, the C2 domain, to pre-target it to membranes, thereby allowing the C1 domain to more efficiently find its ligand, which is restricted to the membrane (10). Although conventional isoforms have the benefit of pre-targeting by a Ca\(^{2+}\)-sensitive C2 domain, novel PKC isozymes lack a Ca\(^{2+}\)-interacting domain, raising questions about membrane recruitment.

Here, we address the molecular mechanism driving the membrane recruitment of PKCδ, a novel isozyme. We show that PKCδ relies exclusively on the C1 domain for membrane recruitment and that the intrin-
sic affinity of this module for membranes is 2 orders of magnitude higher in PKCδ compared with PKCβI, resulting in comparable membrane binding of both isozymes in the presence of saturating ligands. In vivo imaging studies of wild-type PKCδ and a C2-deleted construct revealed that, even in the context of the cell, the C2 domain does not affect the location or activity of PKCδ. Thus, this isozyme relies on one membrane-targeting domain (tuned to have an appropriately high affinity for membranes) for function.

**EXPERIMENTAL PROCEDURES**

**Materials**

1-Palmitoyl-2-oleoylphosphatidyl-1-serine, 1-palmitoyl-2-oleoylphosphatidylcholine, 1-palmitoyl-2-oleoylphosphatidylglycerol, ceramide, and cardiolipin were purchased from Avanti Polar Lipids. Glutathione, phorbol 12-myristate 13-acetate (PMA), bovine serum albumin, ATP, and protamine sulfate were purchased from Sigma. [γ-32P]ATP and [3H]dipalmitoylphosphatidylcholine were from PerkinElmer Life Sciences. Restriction enzymes were from New England Biolabs Inc. PCR primers were prepared by Genset Oligos. Thrombin and glutathione-Sepharose 4B were from Amersham Biosciences. Electrophoresis reagents were from Calbiochem and Bio-Rad. Isopropyl β-D-thiogalactopyranoside and leupeptin were from Calbiochem. Antibody to PKCδ was from Cell Signaling Technology. All other chemicals were reagent-grade.

**Preparation of Lipid Vesicles**

Mixtures of phospholipids in chloroform/methanol (2:1) were dried under a stream of nitrogen following volatility and then resuspended in buffer containing 170 mM sucrose, 5 mM MgCl₂, and 20 mM HEPES (pH 7.5). They were subjected to five freeze-thaw cycles. Vesicles were extruded through two 0.1-μm polycarbonate filters in an extruder (Avestin Inc.). The vesicles were suspended in 100 mM KCl and 20 mM HEPES (pH 7.5) to dilute the sucrose and centrifuged at 100,000 g for 30 min. The pellet was resuspended in KCl buffer to a final concentration of 1.0 mM lipid as monitored by trace amounts of [3H]dipalmitoylphosphatidylcholine.

**Incorporation of PMA into Vesicles**

PMA was dissolved in Me₂SO and added to a solution of vesicles while vortexing. The vesicles were incubated for 30 min prior to use to ensure incorporation of all of the PMA.

** Constructs**

C2-deleted PKCδ was made by removing the first 126 residues of full-length PKCδ. Full-length PKCδ and C2-deleted PKCδ were cloned into pFastBac (Invitrogen). C2-deleted PKCδ was made by removing the first 100 residues of full-length PKCδ. Full-length and C2-deleted PKCε constructs were cloned into pcDNA3.1(−) using the XhoI and HindIII sites. The isolated C1 domain of PKCβII was obtained by cloning residues 91–161 of PKCβII into pGEX-KG using the BamHI and HindIII sites. The isolated C2 domains of PKCβII and PKCδ were obtained by cloning residues 157–298 of PKCβII and residues 1–133 of PKCδ into the same sites. The P169G (C1A domain) mutant of PKCδ was kindly provided by Peter Blumberg. It was cloned into the XhoI and HindIII sites of pcDNA3.1(−).

** Purification of PKC**

PKCβII was purified to homogeneity from the baculovirus expression system as described previously (11). His-tagged wild-type PKCδ or C2-deleted PKCδ was expressed in SF21 cells. Cells were infected at 80% confluency for 1.5 h with virus containing the PKC constructs. The virus was then diluted in EX-CELL (10 ml of EX-CELL with 1% streptomycin for each T75 dish). Cells were grown for 72 h and then pelleted at low speed. 10 ml of lysis buffer (50 mM Tris (pH 8.2), 100 mM KCl, 1% Nonidet P-40, β-mercaptoethanol, microcystin, phenylmethylsulfonyl fluoride, benzamidine, and leupeptin) was added. Cells were lysed using a French press and spun at 9000 rpm for 15 min. The supernatant was added to 1.5 ml of nickel-agarose column. Washes were with 11 ml of Buffer A (20 mM Tris (pH 8.2), 500 mM KCl, 20 mM imidazole, 5 mM β-mercaptoethanol, and 10% glycerol), 1 ml of Buffer B (20 mM Tris (pH 8.2), 1 mM KCl, 5 mM β-mercaptoethanol, and 10% glycerol), and 3 ml of Buffer A. Elution was with 7 ml of Buffer C (20 mM Tris (pH 8.2), 100 mM KCl, 1 mM imidazole, 5 mM β-mercaptoethanol, and 10% glycerol). Fractions were assayed by kinase assay and Western blotting. The His tag was cleaved off with enterokinase. The isolated C1 and C2 domains from PKCβII and PKCδ were expressed as glutathione S-transferase fusion proteins in BL21-pLyS cells and purified using glutathione-Sepharose beads as described previously (5). PKCε constructs were transfected into human embryonic kidney cells using the Superfect transfection system (Qiagen Inc.). Cells were lysed in phosphate-buffered saline containing 0.1% Triton after 48 h. Lysates were spun at 4 °C for 30 min at 100,000 × g to separate the soluble material from the insoluble pellet. The supernatants were used in PKC binding assays. Binding curves obtained with purified PKC and with PKC from the supernatant of a high speed spin were shown previously to be similar (12).

**Sucrose-loaded Vesicle Binding Assay for Full-length PKCβII, Full-length PKCδ, and C2-deleted PKCδ**

Binding of PKC to sucrose-loaded vesicles was measured as described previously (13). PKC was incubated for 10 min with 100 μM sucrose-loaded vesicles in buffer containing 100 mM KCl, 5 mM MgCl₂, 20 mM HEPES (pH 7.5), 200 μM CaCl₂, and 0.3 mg/ml bovine serum albumin, followed by centrifugation at 100,000 × g for 30 min at 25 °C to separate free PKC from vesicle-bound PKC. The percentage of PKC bound to vesicles in the pellet was determined by assaying both the supernatant and pellet fractions for PKC activity under identical conditions using protamine sulfate as a substrate. Vesicles were added to the supernatant fraction to keep the total amount of lipid in both fractions equal.

The percentage of membrane-bound enzyme was determined using Equations 1 and 2,

\[
A_v = \frac{(\beta A_b + (\beta - 1) A_s)}{(\alpha + \beta - 1)} \quad (Eq. 1)
\]

\[
\% \text{ bound} = \frac{A_s}{A_b + A_s} \quad (Eq. 2)
\]

where \(A_s\) is the activity of the top fraction (in cpm), \(A_b\) is the activity of the bottom fraction (in cpm), \(\alpha\) is the fraction of sedimented vesicles, and \(\beta\) is the fraction of kinase activity found in the bottom fraction in the absence of vesicles.

Binding of isolated domains was determined similarly, but the top and bottom fractions were run on a gel and silver-stained, and bands were quantified by densitometry. Densitometry results were used in place of activity in Equations 1 and 2. Binding of PKCε was assayed using a Western blot in place of silver-stained gels.

The data were fit to Equation 3,

\[
\% \text{ bound} = \left(\frac{[L]^n}{[L]^n + K_d}\right) \quad (Eq. 3)
\]

where \([L]\) is the lipid concentration and \(n\) is the Hill coefficient.
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PKC Translocation in Whole Cells

Cell Culture—COS-7 cells were plated onto glass coverslips in 35-mm dishes and grown to 60–80% confluency. Cells were transfected with FuGENE 6 (Roche Applied Science) and grown 12 h post-transfection.

Imaging—Cells were imaged in the dark at room temperature with phorbol 12,13-dibutyrate (PDBu) or peroxide added as noted. Images were acquired on a Zeiss Axiosvert microscope (Carl Zeiss MicroImaging, Inc.) using a MicroMax digital camera (Roper Princeton Instruments) controlled by MetaFluor Version 3.0 software (Universal Imaging, West Chester, PA). Data were collected through a 10% neutral density filter. Yellow fluorescent protein images were acquired through a 495/10-nm excitation filter, a 505-nm dichroic mirror, and a 535/25-nm emission filter. Red fluorescent protein (monomeric; RFP) images were obtained through a 568/25-nm excitation filter, a 600-nm dichroic mirror, and a 653/95-nm emission filter. Cyan fluorescent protein (CFP) images were obtained through a 420/10-nm excitation filter, a 450-nm dichroic mirror, and a 475/40-nm emission filter. Fluorescence resonance energy transfer (FRET) images were obtained through the same excitation filter and dichroic mirror as CFP images, but the emission filter was 535/25 nm. Integration times were varied between 50 and 200 ms to optimize signals and to minimize photobleaching.

RESULTS

The C2 Domain of PKC\(\alpha\) Does Not Contribute to Membrane Binding—Fig. 1A shows that PKC\(\alpha\) bound to model membranes in a manner that was sigmoidally dependent on the mol fraction of PS; analysis of binding data revealed half-maximal binding with 26.0 \(\pm\) 0.8 mol % PS and a Hill coefficient of 4.2 \(\pm\) 0.5. Deletion of the C2 domain resulted in a modest enhancement of membrane binding, with half-maximal binding occurring at 20 \(\pm\) 2 mol % PS and a Hill coefficient of 4 \(\pm\) 1. This modest enhancement of membrane binding of the C2-deleted construct was even less apparent when binding was measured in the presence of Ca\(^{2+}\) (Fig. 1B); the construct lacking the C2 domain was half-maximally bound to membranes containing 21 \(\pm\) 2 mol % PS compared with 21.6 \(\pm\) 0.3 mol % for the full-length protein. Activity curves were similar to binding curves in that the C2-deleted construct reached half-maximal activation at 2–3% lower PS compared with full-length PKC\(\alpha\) (data not shown), whereas the Hill coefficient remained unchanged. Deletion of the C2 domain of PKC\(\alpha\) did not decrease the enzyme-specific activity. These data reveal that deletion of the C2 domain slightly increases the degree of apparent cooperativity. Thus, the C2 domain of PKC\(\alpha\) does not contribute to the PS dependence for membrane binding or activation of PKC\(\alpha\) or to the apparent cooperativity in these processes.

In contrast to PKC\(\alpha\), the binding of PKC\(\beta\)II to membranes was strongly dependent on Ca\(^{2+}\). Fig. 1C shows that the binding of PKC\(\beta\)II to membranes displayed a steep sigmoidal dependence on PS in the presence of Ca\(^{2+}\) (closed circles); half-maximal binding at 14.52 \(\pm\) 0.06 mol % PS, \(n = 12 \pm 1\) and was barely measurable in the absence of Ca\(^{2+}\) (open squares; only 20% maximal binding to vesicles containing 60 mol % PS). In the absence of Ca\(^{2+}\), the C2 domain of PKC\(\beta\)II was not engaged on the membrane, and membrane binding was driven by the C1 domain alone. Thus, the relative contributions of the C1 domains to membrane binding of PKC\(\beta\)II and PKC\(\alpha\) can be compared by determin-
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...ing the membrane affinity of PKCβII in the absence of Ca²⁺ with that of PKCα. From Fig. 1 (A and C), calculation of apparent binding affinities by quantifying the amount of bound and free PKCs at a fixed PS concentration (25 mol %) reveals that the affinity of PKCα for membranes was 100-fold greater than the affinity of PKCβII under conditions in which binding was mediated only by the C1 domain. These data reveal that PKCα compensates for its non-membrane-binding C2 domain by having a C1 domain that binds membranes with 2 orders of magnitude higher affinity compared with the C1 domain of a conventional PKC.

To confirm that the C2 domain of PKCα does not contribute to membrane binding, we tested the ability of the isolated C2 domain to bind anionic membranes. Fig. 2 shows that the C2 domain of PKCα did not bind membranes even at 0.5 mM total lipid. In contrast, the C2 domain of the conventional isozyme PKCβII bound vesicles containing 40 mol % PS with an apparent $K_a$ of $3.4 \times 10^3$ M$^{-1}$, consistent with previously published data (5). Similarly, the PMA dependence for membrane binding and activity were similar for both full-length PKCα and C2-deleted PKCα, with the C2-deleted construct again binding to membranes slightly better than the full-length enzyme (data not shown). Deletion of the C2 domain also did not affect the binding of PKCβ to vesicles containing phosphatidylglycerol, cardiolipin, or ceramide (data not shown). These data reveal that the C2 domain does not contribute to membrane binding regardless of the mechanism used to tether PKCα to membranes.

The C2 Domain of PKCα Is Predicted to Repel Anionic Membranes, whereas That of PKCβII Is Predicted to Bind Membranes—To probe the physical basis for the inability of the PKCβ C2 domain to bind membranes, we performed potential of mean force calculations to determine the free energy change as the C2 domain approaches anionic membranes. The construction of the 1-palmitoyl-2-oleoylphosphatidyl-L-serine/1-palmitoyl-2-oleoylphosphatidylcholine vesicle model with atomic details has been described elsewhere (14). The electrostatic solvation free energies were calculated by solving the Poisson-Boltzmann equation at an ionic strength of 0.15 M using the adaptive Poisson-Boltzmann solver (15). The Protein Data Bank codes for the atomic structures of the PKCβII C2 domain and the PKCα C2 domain are 1A25 and 1BDY, respectively. The orientation of the PKCβII C2 domain was determined according to Monte Carlo simulations with the above free energy calculation, whereas the orientation of the PKCα C2 domain was determined by structural alignment with the PKCβII C2 domain at its optimal orientation with the lowest binding free energy. Fig. 3 shows that the C2 domain of PKCβII is predicted to be attracted to membranes containing 28.8 mol % PS in phosphatidylcholine (PC) as its center of mass approaches a radial distance of 135 Å from the vesicle membrane. At this distance, where the PKCβII C2 domain is actually anchored right on top of the PS lipid, the free energy of binding is on the order of $-10$ kcal/mol. In striking contrast, the free energy of binding is predicted to increase dramatically as the C2 domain of PKCα approaches anionic membranes, with a free energy on the order of $+150$ kcal/mol describing the interaction of the PKCα C2 domain with membranes at a distance of 135 Å. It should be noted that this implies that the PKCα C2 domain will not approach this location in reality. This result suggests that the C2 domain of PKCα is strongly repelled from anionic membranes if it is forced to very close proximity and therefore will not contribute to the binding of PKCα to the anionic membrane.
Figure 4. A, membrane translocation of full-length PKCα fused to RFP (full length RFP) and C2-deleted PKCα fused to CFP (C2-del CFP) in COS-7 cells in response to 200 nM PDBu; B, translocation of full-length PKCα and C2-deleted (C1A domain) mutant PKCα fused to RFP and CFP, respectively, in COS-7 cells in response to 200 nM PDBu; C, translocation of full-length PKCα fused to yellow fluorescent protein (delta-YFP) in response to PDBu in cells stained with the Golgi marker BODIPY TR ceramide; D, phosphorylation of CKAR by full-length PKCα (closed circles) and C2-deleted PKCα (open squares) after addition of 200 nM PDBu; E, phosphorylation of CKAR by full-length PKCα (closed circles) and C2-deleted PKCα (open squares) after addition of 250 nM peroxide. Cells were plated on 35-mm dishes with glass coverslips, transfected with FuGENE 6 at ~50–60% confluency, and then grown an additional 12 h. FRET data were corrected for base-line drift by subtracting (initial slope of the curve × time) from each FRET measurement.
The C2 Domain of PKCδ Does Not Affect the Translocation of PKC in COS-7 Cells—We next addressed whether the C2 domain regulates the membrane translocation of PKCδ in the context of the cell. COS-7 cells were transfected either with the cDNA encoding full-length PKCδ tagged with RFP or with the cDNA encoding C2-deleted PKCδ tagged with CFP. Cells were imaged before and after addition of 200 nM PDBu. Deletion of the C2 domain of PKCδ did not significantly alter the fluorescence distribution pattern of tagged PKCδ or the kinetics of redistribution (Fig. 4A). Specifically, PKCδ redistributed from the cytosol to the Golgi following addition of PDBu. The rate of translocation of C2-deleted PKC to the Golgi was not significantly different from that of wild-type PKCδ (compare images at 30 s), indicating that the C2 domain does not affect the redistribution of PKCδ. Slightly more C2-deleted construct was observed in the nucleus before translocation and at the nuclear membrane after translocation. A fluorescently tagged construct of the isolated C2 domain of PKCδ was primarily cytosolic, and this distribution was unaffected by PDBu (data not shown). Taken together, these data suggest that the C2 domain in isolation or within the context of the full-length PKC protein does not contain determinants required for targeting or translocation of PKC to membranes. To test the effect of the C1B domain on translocation, full-length and C2-deleted constructs with a mutation in the C1A domain that impairs function were used. Fig. 4B shows that both constructs translocated to the Golgi, indicating that the C1B domain drives the interaction of the full-length protein. Cells stained with the Golgi marker BODIPY TR ceramide (Molecular Probes) displayed fluorescence patterns identical to those displayed by cells transfected with PKCδ and treated with PDBu (Fig. 4C), indicating that PKCδ does in fact translocate to the Golgi. Translocation of PKCβII to the plasma membrane has been shown previously (16).

The C2 Domain of PKCδ Does Not Affect the Activation of PKC in COS-7 Cells—We used the genetically encoded PKC activity reporter CKAR (C-kinase activity reporter) to measure the activation of PKCδ in live cells (19). Phosphorylation of CKAR by PKC leads to a decrease in FRET (19). CKAR and either full-length or C2-deleted PKCδ fused to RFP were transfected into COS-7 cells, and the change in the FRET ratio (plotted as CFP emission/FRET emission) was measured after addition of stimulus. The rate and magnitude of activation in response to PDBu were similar for both constructs (Fig. 4D). The initial activation rates were 0.12 ± 0.03 FRET units/min for full-length PKCδ and 0.11 ± 0.05 FRET units/min for C2-deleted PKCδ. More than 80% of the PKC activity was from the transfected constructs as assessed by measuring the PKC activity in lysates of control and transfected cells. The average rates for endogenous PKC were only 0.0140 ± 0.0008 FRET units/min (data not shown). The RFP tag did not interfere with CKAR emission properties; experiments performed with glutathione S-transferase-tagged PKCδ instead of the RFP-tagged construct yielded similar results. Similarly, both full-length and C2-deleted constructs were activated by H2O2 (Fig. 4E), which has been shown to activate PKCδ indirectly through phosphorylation of Tyr311 (possibly by Src family kinases) (17) and which has also been shown to activate PKCδ directly by oxidation of cysteines in the C1 domain (reviewed in Ref. 18). There was some cell-to-cell variation in activation rates and magnitudes with peroxide, but both constructs were activated to similar extents. Representative curves are shown. Control experiments using the non-phosphorylatable mutant of CKAR revealed no FRET change upon either PDBu or peroxide treatment in cells transfected with PKCδ constructs or in cells without PKC transfection (19) (data not shown).

PKCδ Compensates for Lack of Membrane Binding of the C2 Domain by Using the C1 Domain to a Greater Extent—Fig. 5 shows that full-length PKCβII reached half-maximal binding at lower PS compared with full-length PKCδ. Specifically, half-maximal binding to membranes was achieved with 22.2 ± 0.2 mol % PS. The remainder of this lipid was PC. Binding was measured as described under “Experimental Procedures.” Curves were fit to Equation 3 under “Experimental Procedures.” Data represent the average of two independent experiments performed in triplicate. Midpoints and Hill coefficients were 22.2 ± 0.3 mol % PS and 6.0 ± 0.4 for PKCβII in Ca2+, 45 ± 4 mol % PS and 4.6 ± 1.7 for PKCδ in Ca2+, and 48 ± 3 mol % PS and 4.2 ± 1.0 for PKCδ in EGTA.

FIGURE 5. Binding of full-length PKCβII and PKCδ to membranes containing 0.03% PMA and the indicated amounts of PS. The remainder of the lipid was PC. Binding was measured as described under “Experimental Procedures.” Curves were fit to Equation 3 under “Experimental Procedures.” Data represent the average of two independent experiments performed in triplicate. Midpoints and Hill coefficients were 22.2 ± 0.3 mol % PS and 6.0 ± 0.4 for PKCβII in Ca2+, 45 ± 4 mol % PS and 4.6 ± 1.7 for PKCδ in Ca2+, and 48 ± 3 mol % PS and 4.2 ± 1.0 for PKCδ in EGTA.
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FIGURE 6. Binding of the C1A + C1B domains or the C1B domain of PKC/JII or PKCδ to membranes containing 1% PMA and increasing amounts of PS, with the remainder of lipid as PC. Binding was measured as described under “Experimental Procedures.” Curves were fit to Equation 3 under “Experimental Procedures.” Data represent the average of two independent experiments performed in duplicate. Hill coefficients were 2.6 ± 0.8, 3.1 ± 0.5, 3 ± 1, and 3.0 ± 0.5 for the C1B domain of PKC/JII and the C1B domain and C1A + C1B domains of PKC/JII and PKCδ.

FIGURE 7. A, binding of full-length PKCe and C2-deleted PKCe to membranes containing 5 mol % DAG with increasing amounts of PS. Binding was measured as described under “Experimental Procedures.” Curves were fit to Equation 3 under “Experimental Procedures.” Data represent the average of two independent experiments performed in duplicate. B, replot of C2-deleted PKCe, C2-deleted PKCδ, and full-length PKC/JII from A and Fig. 1 (A and C), respectively.

domain of PKCβII. These data reveal the C1B domain of PKCδ has an ~3-fold higher intrinsic affinity for membranes compared with the C1B domain of PKCβII at this lipid composition. Assays with isolated domains and mutant proteins (Fig. 6) were performed with 1% PMA in the membrane versus 0.03% PMA for full-length proteins (Fig. 5). The Hill coefficients were 2.6 ± 0.8, 3.1 ± 0.5, 3 ± 1, and 3.0 ± 0.5 for constructs that use the C1B domain or C1A + C1B domains of PKCβII and the C1B domain or C1A + C1B domains of PKCδ, respectively.

PKCe Uses Its C1 Domain to an Intermediate Extent Compared with PKCβII and PKCδ—Finally, we examined the effect of deleting the C2 domain on the membrane binding of another novel PKC isozyme, PKCe. Fig. 7A shows that deletion of the C2 domain of PKCe caused a slight decrease in binding to PS/DAG membranes in contrast to deletion of the C2 domain of PKCδ, which modestly enhanced binding. Fig. 7B presents a comparison of the binding data from Figs. 1 (A and C) and 7A for PKCe, PKCδ, and PKCβII under conditions in which only the C1 domain drives the membrane interaction. The membrane binding affinity of the C1 domain of PKCe was slightly weaker than that of PKCδ, reaching half-maximal binding at 34 versus 19% PS, but much stronger than that of PKCβII, which barely bound under the conditions used (5 mol % DAG with increasing mol % PS). These data reveal that the membrane binding affinity of the C1 domain of novel PKCs is higher than that of conventional isoforms to compensate for the lack of contribution of the C2 domain and that, within subclasses, the membrane affinity is further fine-tuned.

DISCUSSION

In this study, we have shown that the C2 domain of PKCδ has no intrinsic affinity for membranes and does not contribute to either the membrane translocation or cellular activity of this isozyme. Furthermore, we have shown that this isoform compensates for the nonfunctional C2 domain by having a C1 domain that binds membranes 2 orders of magnitude more tightly than the C1 domain of conventional PKC isoforms, which have two membrane tethers. Thus, our results reveal a model in which determinants on the C1 domain tune the affinity with which PKC isoforms bind membranes. Most important, isoforms whose membrane translocation is driven by a single membrane-targeting module and whose membrane translocation is driven by two membrane-targeting modules adjust the affinities of individual modules so that the net binding is comparable.

The C2 Domain of PKCδ Is Not a Membrane-targeting Module—The C2 domain is present in a variety of proteins and serves the function of membrane-targeting module as well as protein-interacting module. Most C2 domains bind Ca$^{2+}$ in an aspartic acid-lined cavity formed between loops in the β-strand-rich domain. Binding of Ca$^{2+}$ neutralizes the negative electrostatic potential surrounding the Ca$^{2+}$-binding site and promotes binding to either neutral (as occurs for the C2 domain of phospholipase A$_2$) or anionic (as occurs for conventional PKCs) phos-
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Phospholipids. The C2 domain of novel PKCs lacks key aspartic acid residues required for Ca\(^{2+}\) coordination and is thus unable to bind Ca\(^{2+}\). Whether this module constitutively binds anionic membranes has not been established. Elucidation of the crystal structure of the C2 domain of PKCθ suggested several possible membrane-interacting sites, either clusters of positively charged residues or regions of neutral/hydrophobic residues (15). In addition, electrostatic calculations predicted a very weak affinity of the C2 domain of PKCθ for PS that also involved hydrophobic interactions (20).

Here, we have shown that, under all conditions tested, the C2 domain of PKCθ does not interact with membranes. In fact, deletion of the C2 domain modestly facilitates the membrane binding of PKCθ. Such autoinhibition by the C2 domain has also been reported for the Aplysia novel PKC (21). One possibility is that the non-membrane-binding C2 domain sterically hinders access of the C1 domain to membranes. A second possibility is that electrostatic repulsion by the novel C2 domain hinders the access of PKCθ to membranes. This latter possibility is supported by the thermodynamic calculations showing electrostatic repulsion of the C2 domain of PKCθ from anionic membranes (Fig. 3). In this case, repulsion from membranes can be overcome by engagement of the C1 domain on membranes.

Comparison of the structure of the C2 domain of PKCθ with C2 domains that do not bind Ca\(^{2+}\) but do bind lipids reveals that several important lipid-binding residues/regions are not present in PKCθ. The C2 domains of PKCθ and PKCe share only 19% sequence identity and are proposed to form two distinct subclasses of novel C2 domains (22). The C2 domain of PKCe has recently been shown to bind weakly to PS, phosphatidic acid, and phosphatidylglycerol (23). The lipid-binding regions were localized to residues in loops 1 and 3 of the C2 domain of PKCe that are not conserved in the C2 domain of PKCθ. There is also an extra α-helix in PKCe that is not found in PKCθ (8). The C2 domain of PKCθ is also similar in structure to that of PTEN, which also does not bind Ca\(^{2+}\), but does bind PS/PC. However, the C2 domain of PTEN has a lysine-rich sequence in the CBR3-like region, which has been implicated in membrane binding (8). This sequence is absent in the C2 domain of PKCθ, which contains acidic residues instead (8).

Our results are consistent with a recent study that compared the binding abilities of the PKCθ C1A and C1B domains and also looked briefly at a C2-deleted construct of PKCθ that bound similarly to full-length PKCθ (24). However, our studies go further than these conclusions. Our results reveal that the C2 domain of PKCθ also does not bind phosphatidylglycerol, ceramide, and cardiolipin in vitro, lipids that have been proposed to modulate the function of this isozyme. Specifically, PKCθ has been shown to bind ceramide in vitro (25) and to translocate to the Golgi after ceramide treatment, where it is tyrosine-phosphorylated and activated (26). PKCθ has also been shown to translocate to mitochondria in response to oxidative stress (27). Cardiolipin is found in the inner mitochondrial membrane and could possibly interact with the C2 domain. However, our data indicate that binding to cardiolipin is through the C1 domain as well (data not shown).

The C2 domain of PKC type II from Aplysia, which has the highest degree of homology to mammalian PKCe, has been reported to have no significant affinity for membranes unless it is phosphorylated at Ser\(^{37}\). It was concluded that the phosphate at position 37 causes two loops between strands to pull apart, thus exposing a new lipid-binding site (21). Mutation of a similarly positioned Ser to Glu (S16E and S24E) in the C2 domain of PKCθ did not confer lipid binding (data not shown). Phosphorylation by protein kinase A, which is predicted to phosphorylate Thr\(^{20}\) (28), did not promote membrane binding. The above results indicate that the PKCθ C2 domain does not bind to lipids in its native state or following phosphorylation at Ser/Thr residues. Rather, this module likely serves as a protein-interacting domain, and some studies have found binding partners for it such as actin and GAP-43 (29, 30). A recent study reported that the C2 domain of PKCθ is in fact a phosphotyrosine-binding domain and that the C2 domain binds directly to a glycoprotein, CDCP1, following phosphorylation by Src or H\(_{2}\)O\(_{2}\), treatment, the latter of which broadly promotes tyrosine phosphorylation (31). We wondered if binding of the C2 domain to phosphoproteins could cause translocation or activation of PKCθ in cells. In our studies, activation in response to peroxide (as measured by CKAR) was observed for both full-length PKCθ and C2-deleted PKCθ, suggesting that the C2 domain is not crucial for this. Very little, if any, translocation was observed in response to peroxide with either construct. This is in agreement with a previous study that showed a 3-fold increase in activation of PKCθ in response to H\(_{2}\)O\(_{2}\), but no translocation (32).

Phorbol ester-induced translocation of full-length PKCθ and C2-deleted PKCθ in COS-7 cells is not significantly different. Both constructs translocate to the Golgi in ~2–3 min. Very subtle differences can be seen in the translocation patterns of full-length versus C2-deleted PKCθ (Fig. 4A). The overlay shows more nuclear staining with C2-deleted PKCθ than with full-length PKCθ. It is possible that the C2 domain recognizes protein binding partners that are localized to the nucleus. Moreover, the destination of PKCθ in the cell depends on the hydrophobicity of the phorbol ester used (33, 34). In fact, PKCθ has been shown to translocate to the plasma membrane, nuclear membrane, Golgi, endoplasmic reticulum, and mitochondria in various cell types and in response to different stimuli (27, 35, 36). However, the C2 domain is not needed for translocation of PKCθ in COS-7 cells. It has been shown that the V5 region (specifically the last few residues of the catalytic domain) of PKCθ is responsible for some of its localization, translocation, and downstream effects (37). The above literature agrees with our observations that the C2 domain of PKCθ does not contain determinants for localization of PKCθ, and in fact, PKCθ is also activated by tyrosine phosphorylation and in this way does not even require membrane binding (17).

**Mechanism of Apparent Cooperativity in Lipid Binding**—The binding of conventional PKCs to membranes has long been known to show steep sigmoidal dependence on the mol fraction of PS (38). This steep sigmoidal dependence is observed only in membranes containing the C1 domain ligands (DAG or phorbol esters) and only when the anionic lipid is sn-1,2-phosphatidyl-i-serine. Kinetic analyses have revealed that conventional PKCs bind eight PS molecules in large unilamellar vesicles with Hill coefficients of 4–8 describing this binding (39, 40). Based on these observations, we have hypothesized that PKCs bind multiple anionic phospholipids, with stereospecific binding to at least one PS molecule (41). The isolated C1 (but not C2) maintains domain stereospecific binding to PS, revealing that the C1 domain contains determinants that specifically recognize sn-1,2-phosphatidyl-i-serine (5). Some of this high cooperativity results from the reduction in dimensionality that occurs when PKC translocates from the cytosol to the membrane upon engaging the first lipid. Beyond this contribution to cooperativity, the remaining cooperativity has yet to be accounted for.

Here, we have shown that a novel PKCθ isozyme that uses only the C1 domain for membrane binding displays markedly reduced cooperativity compared with isozymes that can also use a C2 domain. Specifically, a Hill coefficient of 12 describes the apparent cooperativity in the binding of PKCθ to PS-containing membranes compared with 4 for the binding of PKCθ to equivalent PS-containing membranes. These data suggest that the coordinated recruitment of the C1 and C2 domains to membranes accounts for some of the high apparent cooperativity in PS
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binding for PKCβII. This high apparent cooperativity is reduced when only one domain engages on the membranes. Our current data support a model in which the C1 domain specifically binds at least one PS molecule, in which the binding of this first lipid increases the probability of binding additional lipids through reduction in dimensionality, and in which the involvement of the C2 domain at least doubles the number of anionic lipids that retain PKC at membranes.

The C1 Domain of PKC6 Has Higher Membrane Affinity than the C1 Domain of Conventional PKC6s—Analysis of membrane binding of PKCβII in the presence or absence of Ca2+ revealed that the C2 domain is the major determinant that recruits this isoform to membranes even in the presence of high concentrations of DAG. For example, the C2 domain contributes >99% of the binding energy to membranes containing 5 mol % DAG and 20 mol % PS (Kd = 104 M−1 when the C2 domain is engaged with 200 μM Ca2+ and the C1 domain is engaged with 5 mol % DAG, and Kd = 105 M−1 when only the C1 domain is engaged) (Fig. 1C). In marked contrast, the C1 domain alone drives the membrane interaction of PKCβ (Kd = 104 M−1 at 20 mol % PS and 5 mol % DAG) (Fig. 1A). Therefore, the C1 domain of PKC6 binds ~2 orders of magnitude more tightly to membranes than that of PKCβII, thus compensating for the lack of a membrane-binding C2 domain. We have previously shown that the C1 and C2 domains of PKCβII function as independent membrane-targeting modules; the energy released by binding both modules is just slightly less than the sum of binding each individual module (5). Consistent with this, it was shown that both the C1 and C2 domains of PKCβII are required for efficient translocation of the enzyme to the membrane in cells following agonist stimulation; deletion of either domain of PKCβII impairs translocation in response to physiological stimuli, an impairment that can be rescued by elevating Ca2+ or adding phorbol esters to tighten the membrane affinity of the functional domain (42).

The C2 Domain of PKC6 Has Modest Membrane Affinity—Deletion of the C2 domain of PKC6 caused a modest decrease in the PS dependence of membrane binding. This is consistent with a report that the isolated domain has a weak affinity for anionic membranes (23). Similarly, a recent report showed that the isolated C2 domain of PKC6 has a weak affinity for membranes, although it was also reported that the affinity of full-length PKC6 for membranes increases when the C2 domain is deleted (34).

Comparison of the membrane affinity of C2-deleted constructs of PKCβ and PKCε reveals that C2-deleted PKC6β binds membranes with ~5-fold higher affinity (for example, Kd = 104 M−1 (Fig. 1A) and 2 × 103 M−1 (Fig. 7A) at 20 mol % PS for PKCβ6 and PKCε, respectively). For the three PKC6 isoforms tested, the affinity of the C1 domain for membranes is tuned according to the affinity of the C2 domain. Thus, the affinity of the C1 domain of PKC6 (C2 domain with relatively no affinity for membranes) is the strongest, followed by that of PKC6 (C2 domain with relatively low affinity) and then PKCβ (C2 domain with relatively high affinity). However, the Kd for the binding of the C2 domain of PKCβII is 7.3 × 10−10 M with vesicles containing 40 mol % PS (10), whereas the Kd for binding of the C2 domain of PKCε is 5.4 × 10−5 M with the same vesicle composition (23), and the C2 domain of PKCβ does not bind at all. Although the Kd values were obtained from different assays, they are still an indication of the relative binding abilities of each domain. The Hill coefficient for the C2 domain of PKC6ε is 2.52 (23), which fits in well with the estimated two binding sites for PS on the C2 domain of PKCβII. Therefore, it is possible that the C2 domains of PKCβII and PKCβ bind to the same number of PS molecules, even though their binding strengths vary greatly.

Conclusion—Here, we have shown that the C2 domain of PKC6 does not function as a membrane-targeting module. For this isoform, high affinity membrane binding is achieved exclusively by the use of the C1 domain, which binds membranes 2 orders of magnitude more tightly than the C1 domain of conventional PKC isoforms. Our data reveal that the affinity of the C1 domain is tuned to allow comparable membrane affinity of both Ca2+-regulated and Ca2+-independent isoforms. The kinetics of membrane translocation are faster for conventional PKC isoforms that can take advantage of pre-targeting to membranes by Ca2+, but the affinities with which these isoforms bind membranes are comparable at saturating ligand concentrations.

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