The C1 and C2 Domains of Protein Kinase C Are Independent Membrane Targeting Modules, with Specificity for Phosphatidylserine Conferred by the C1 Domain†

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ABSTRACT: Protein kinase C is specifically activated by binding two membrane lipids: the second messenger, diacylglycerol, and the amino phospholipid, phosphatidylserine. This binding provides the energy to release an autoinhibitory pseudosubstrate from the active site. Interaction with these lipids recruits the enzyme to the membrane by engaging two membrane-targeting modules: the C1 domain (present as a tandem repeat in most protein kinase Cs) and the C2 domain. Here we dissect the contribution of each domain in recruiting protein kinase C βII to membranes. Binding analyses of recombinant domains reveal that the C2 domain binds anionic lipids in a Ca^{2+}-dependent, but diacylglycerol-independent, manner, with little selectivity for phospholipid headgroup beyond the requirement for negative charge. The C1B domain binds membranes in a diacylglycerol/phorbol ester-dependent, but Ca^{2+}-independent manner. Like the C2 domain, the C1B domain preferentially binds anionic lipids. However, in striking contrast to the C2 domain, the C1B domain binds phosphatidylserine with an order of magnitude higher affinity than other anionic lipids. This preference for phosphatidylserine is, like that of the full-length protein, stereoselective for *sn*-1,2-phosphatidyl-L-serine. Quantitative analysis of binding constants of individual domains and that of full-length protein reveals that the full-length protein binds membranes with lower affinity than expected based on the binding affinity of isolated domains. In addition to entropic and steric considerations, the difference in binding energy may reflect the energy required to expel the pseudosubstrate from the substrate binding cavity. This study establishes that each module is an independent membrane-targeting module with each, independently of the other, containing determinants for membrane recognition. The presence of each of these modules, separately, in a number of other signaling proteins epitomizes the use of these modules as discreet membrane targets.

Reversible membrane recruitment is one of the most common mechanisms for regulating the function of signal-transducing enzymes. To this end, an abundance of signals use lipid second messengers to communicate extracellular information within the cell by modulating the subcellular location of signaling proteins. Generation of lipids such as diacylglycerol and 3′-phosphoinositides activates a multitude of intracellular signaling pathways by recruiting effector enzymes to specific membrane locations. These enzymes are recruited to membranes via specific membrane-targeting modules that specifically recognize the relevant lipid second messenger. PH and FYVE domains, which bind 3′-phosphoinositides, C1 domains, which bind diacylglycerol, and C2 domains, which bind phospholipids, are the best characterized modules (*1, 2*). These modules are present in both the enzymes that catalyze the production of lipid second messengers and the enzymes that are activated by lipid second messengers. Reversibility and specificity in membrane recruitment is often achieved by the coordinated use of two membrane targeting modules, each binding with relatively low affinity so that anchorage of both domains is required for a high-affinity membrane interaction (*3, 4*). Thus, disruption of one anchor, for example, by metabolism of diacylglycerol in the case of a C1 domain, effectively releases the enzyme from the membrane.

Protein kinase C serves as a paradigm for the use of two membrane-targeting modules to achieve specificity and reversibility in its membrane interaction (*4*). Members of this family of serine/threonine kinases transduce the myriad of signals that result in generation of diacylglycerol (*5*). In unstimulated cells, the enzyme localizes primarily to the cytosol, where interactions with binding proteins may fine-tune its exact location (*6*). Generation of diacylglycerol recruits the enzyme to the membrane by engaging, for conventional and novel isozymes, the C1 and C2 domains which are present in the amino-terminal regulatory moiety of the enzyme (*7*). The C1 domain is a small globular module with two pulled-apart β-sheets that encompass the binding pocket for C1 domain ligands such as diacylglycerol and phorbol esters (*2*). The C2 domain binds anionic lipids and, for conventional protein kinase Cs, Ca^{2+} (*8*). In this β-strand-rich globular domain, loops at opposite ends of the sequence come together to form an aspartate-rich Ca^{2+} binding site. In novel protein kinase Cs, critical aspartates are absent and this module does not bind Ca^{2+}. Although the elucidated

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structures show no defined lipid binding pocket in the C2 domain, numerous biochemical studies have localized the binding site for lipids to the area surrounding the Ca^{2+} binding site (*9*−*12*).

The activity of all isozymes of protein kinase C is stimulated by phosphatidylserine. Conventional isozymes (α, βI, βII, γ) are additionally regulated by two second messengers, diacylglycerol and Ca^{2+}; novel isozymes (δ, ϵ, η) are regulated by diacylglycerol; and no additional second messengers have been identified for atypical isozymes (ζ, λ) (*7, 13*). In addition, all isozymes are rendered catalytically competent by a priming phosphorylation by the phosphoinositide-dependent kinase, PDK-1 (*14*−*16*). The stimulation of protein kinase C activity by phosphatidylserine has served as a hallmark for describing this enzyme since its initial discovery 2 decades ago (*17*−*19*). Recently, experiments with enantiomeric lipids established that the requirement for phosphatidylserine reflects a stereospecific interaction of determinants on the protein with this lipid rather than the ability of this lipid to structure membranes in such a way as to provide an optimal binding surface (*20*). The stereospecific binding to this lipid and to *sn*-1,2-diacylglycerol (or phorbol esters, functional analogues) activates the enzyme by inducing a conformational change that expels an autoinhibitory pseudosubstrate sequence from the active site (*21*). Although structural studies have established that the C1 and C2 domains contain the ligand binding pockets for phorbol esters/diacylglycerol and Ca^{2+}, respectively, the molecular basis for the phosphatidylserine selectivity has evaded detection (*20*).

In this study, we examine the mechanism of membrane interaction of the isolated C1B and C2 domains of protein kinase C β in order to dissect the contribution of each in the lipid regulation of protein kinase C. We find that the C1 domain interaction is regulated by phorbol esters/diacylglycerol and not Ca^{2+}, as expected, but that, surprisingly, it is also regulated by anionic lipids with a 10-fold preference for phosphatidylserine. The C2 domain is regulated by Ca^{2+} and anionic lipids, with a slight preference for phosphatidylserine that is not stereospecific. Thus, the determinants for specific recognition of phosphatidylserine reside in the C1 domain. Comparison of binding constants of the domains with those of the full-length protein reveals that the C1 domain binds membranes with comparable affinity either as an isolated domain or in the context of the full-length protein. In contrast, the C2 domain binds membranes with slightly higher affinity as an isolated domain compared with its affinity in the full-length protein. Importantly, this study shows that the isolated C1 and C2 domains of protein kinase C can, separately, recreate the specificity in the cofactor regulation of the native enzyme, revealing that each module operates independently of the other.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoylphosphatidyl-L-serine, 1-palmitoyl-2-oleoylphosphatidylcholine, 1-palmitoyl-2-oleoylphosphatidylglycerol, 1-palmitoyl-2-oleoylglycerol, dioleoylphosphatidic acid, phosphatidylinositol (liver), and phosphatidylethanolamine (egg) were purchased from Avanti Polar Lipids. 2-Oleoyl-3-palmitoyl-*sn*-phosphatidylcholine and 2-oleoyl-3-palmitoyl-*sn*-phosphatidyl-D-serine were kindly synthesized by M. Zimmerman and Dr. D. Daleke (Indiana University) as described previously (*20*). 2-Oleoyl-3-palmitoyl-*sn*-glycerol was synthesized as described previously (*20*). Lipid purity was analyzed by thin-layer chromatography, and concentrations were determined by phosphate analysis (*22*). Protein kinase C βII-pTB653 was a gift from Dr. Y. Ono, Kobe University (*23*). pGEX-KG vector was from American Type Culture Collection. Glutathione, phorbol myristate acetate, ATP, and protamine sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-^{32}P]ATP (3 mCi mmol^{-1}) and [^{3}H]dipalmitoylphosphatidylcholine (DPPC) (30 Ci mmol^{-1}) were from NEN Life Sciences. Restriction enzymes and other DNA-modifying enzymes were from New England Biolabs or Gibco/BRL. PCR primers were prepared by Gibco/BRL. Thrombin and glutathione−Sepharose 4B were from Pharmacia Biotech. Electrophoresis reagents were from Calbiochem or Biorad. Isopropyl-β-D-thiogalactopyranoside (IPTG),[1] Triton X-100, and leupeptin were from Calbiochem. Protein kinase C βII was purified to homogeneity from the baculovirus expression system and was stored at −20 °C in 10 mM Tris (pH 7.5 at 4 °C), 0.5 mM EDTA, 0.5 mM EGTA, 150 mM KCl, 0.5 mM DTT, and 50% (v/v) glycerol, as described (*21*). All other chemicals were reagent grade.

Preparation of Constructs. Expression vectors for GST-C1B (residues 91−161 of protein kinase C βII) and GST-C2 domains (residues 157−289 of protein kinase C βII) were constructed by PCR amplification using protein kinase C βII-pTB653 as a template. For the C1B, the 5′ primer was 5′CGGGATCCGGCCCGGCCTCTGATGAC, which contains a *Bam*HI restriction site, and the 3′ primer was 5′CCCAAGCTTAGCGGCCACGGCGTTCTGTGTGGTCGG, which contains a stop codon and a *Hin*dIII restriction site. The PCR-amplified product was cloned into the *Bam*HI and *Hin*dIII sites of the pGEX expression vector. For C2, the 5′ primer was GCCCATGGAACGCCGTGGCCGC, which contains an *Nco*I restriction site, and the 3′ primer was 5′GGCAAGCTTACGGCACATTAAAGCTTACG, which contains a stop codon and a *Hin*dIII restriction site. The PCR-amplified product was cloned into the *Nco*I and *Hin*dIII sites of the pGEX expression vector. Constructs were confirmed by sequencing.

Expression and Purification of Free C1B and C2 Domains. *E. coli* strain BL21pLysS (containing the lysozyme gene) was used as a host for protein expression. A culture in 50 mL of LB medium was grown overnight at 37 °C to saturation, and then diluted 10-fold and grown at 37 °C to an optical density of 0.8 at 600 nm. After 15 min on ice, cells were induced with IPTG (0.5 mM). After 5 h at 24 °C, the cells were harvested by centrifugation (4000*g*) and resuspended in cold buffer A [PBS, pH 7.5, 1% (w/v) Triton X-100, 85 μM leupeptin, 2 mM benzamidine, 0.4 mM PMSF]. Cells were frozen at −80 °C and then thawed to induce lysis. All remaining procedures were performed on ice unless indicated. DNase (10 μg/mL) was added, and the

[1] Abbreviations: PC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; PS, 1-palmitoyl-2-oleoyl-*sn*-phosphatidyl-L-serine; PG, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylglycerol; PA, dioleoylphosphatidic acid; DG, 1-palmitoyl-2-oleoyl-*sn*-glycerol; DTT, dithiothreitol; GST, glutathione-*S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PMA, phorbol myristate acetate.

viscous solution was passed through a 25 1/2 G syringe needle to reduce viscosity. Insoluble material was removed by centrifugation (10000*g*, 30 min, 4 °C). GST-C1B and GST-C2 in the soluble fraction were bound to glutathione−Sepharose (2 mL of a 50% slurry equilibrated in buffer A) during an overnight incubation at 4 °C. Glutathione−Sepharose beads were sedimented, washed with buffer A, PBS, and then buffer B (50 mM Tris, pH 7.5, 150 mM NaCl), and resuspended in an equal volume buffer B. C1B and C2 were released from GST and glutathione−Sepharose by incubation with thrombin (10 units) at 25 °C for 1 h. Purity and concentration of the isolated domains were analyzed by SDS−PAGE using a Tricine gel system, followed by densitometry of the Coomassie stain relative to a lysozyme standard. Circular dichroism spectra were recorded (AVIV Instruments, Inc., model 202) to analyze secondary structure. Protein stocks (∼1 mg mL^{-1}) were stored at −20 °C for up to 6 months without detectable change in binding activity.

Lipid Vesicle Preparation. Sucrose-loaded large unilamellar vesicles containing trace [^{3}H]DPPC were prepared by drying mixtures of lipids in chloroform/methanol (2:1) under a stream of nitrogen, followed by evacuation under vacuum for 1 h, suspension in 20 mM HEPES, pH 7.5, 170 mM sucrose, and then 5 freeze−thaw cycles followed by extrusion using a Liposofast microextruder (Avestin, Inc.) as described (*24*). Extruded vesicles were suspended in 100 mM KCl, 20 mM HEPES and centrifuged at 100000*g* for 30 min at 25 °C in a Beckman centrifuge to dilute the free sucrose. PMA, prepared in a DMSO stock, was subsequently added with vigorous vortexing such that the end concentration of DMSO was <5%.

Membrane Binding Assay for Full-Length Protein Kinase C. The binding of protein kinase C to sucrose-loaded vesicles was measured as described (*24*, *25*). Briefly, protein kinase C (∼100 ng in 120 μL) was incubated with sucrose-loaded vesicles (0.025−3.2 mM lipid) for 10 min in the presence of 0.2 mM Ca^{2+}, 0.3 mg mL^{-1} BSA, 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.4. Membrane-bound protein kinase C was separated from free enzyme by centrifugation at 100000*g* for 30 min at 25 °C. The fraction of sedimented vesicles was determined from radioactivity. The fraction of protein kinase C that sedimented with the vesicles was determined by assaying the activity of both the supernatant and pellet fractions under identical conditions using the cofactor-independent substrate protamine sulfate (0.2 mg mL^{-1}). A control experiment in which the enzyme was quantified by activity assay and by SDS−PAGE analysis (see below) yielded the same results regardless of the assay method used. Thus, results obtained with full-length protein kinase C using the activity assay can be compared to those obtained with domains (see below). The vesicle-associated protein kinase C activity, A_v, was calculated according to eq 1:

$$A_v = \frac{\beta A_b + (\beta - 1)A_t}{\alpha + \beta - 1} \quad (1)$$

where A_b and A_t are the measured activities of the bottom and top fractions, respectively, and β is the fraction of protein kinase C in the supernatant in the absence of vesicles and α is the fraction of vesicles in the pellet fraction. The fraction of bound protein kinase C, *B*, was calculated according to eq 2:

$$B = \frac{A_v}{A_b + A_t} \quad (2)$$

Membrane Binding Assay for C1B and C2 Domains. Purified domains (1−3 μg in 120 μL) were incubated with sucrose-loaded vesicles for 10 min in the presence of 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.4, 0.3 mg mL^{-1} BSA, unless otherwise indicated. Experiments with the C1B domain included 1 mM lipid, and those with the C2 domain included 0.5 mM lipid and 0.3 mM $CaCl_2$ unless otherwise indicated. Membrane-bound protein was separated from free protein by centrifugation at 100000*g* for 30 min at 25 °C. The fraction of sedimented vesicles was determined from radioactivity. Protein in each fraction was determined by analysis of the supernatant and pellet by SDS−PAGE using a Tricine gel system. Gels were Coomassie- or silver-stained, and analyzed by densitometry relative to a lysozyme standard. The resulting values were used in place of "activity" in the above calculations to determine fraction bound and membrane affinity.

Calculation of Binding Constants. The values for apparent K_a were calculated according to eq 3:

$$K_a = \frac{B}{FL} \quad (3)$$

where *F* is the fraction of free enzyme and *L* is the total lipid concentration (note that the concentration of total lipid greatly exceeded that of the protein kinase C and was consequently essentially equal to the concentration of the free lipid). The apparent K_a is a relative number describing the partition coefficient of protein kinase C or its domains with various membranes and is of value in comparative, rather than absolute, analyses.

RESULTS

Expression of C1A and C2 Domain Constructs. Constructs of the C1B and C2 domain were designed based on the crystal structures of these domains (*26*, *27*). The C1B domain construct comprised residues 92−161 of protein kinase C βII, where residues 102 and 152 represent the His and Cys, respectively, bracketing the C1B domain (*28*). The C2 domain construct spanned residues 157−289 and contained the core C2 domain (159−280) plus a short carboxyl-terminal helix that begins the connection to the hinge preceding the kinase core; this construct corresponds to the one whose structure was determined by Sutton and Sprang (*27*). Each construct contained GST fused to the amino terminus; the C2 domain construct contained an additional 13 amino acid Gly-rich linker. Both constructs were expressed in bacteria, the soluble protein was purified by glutathione−Sepharose chromatography, and the domains were released from GST by thrombin treatment. Figure 1B shows a Coomassie blue-stained gel of the purified and cleaved C1B (lanes 2 and 3) and C2 domains (lanes 4 and 5). Both domains were primarily β-sheet structure as assessed by the single, rather than double, minimum at approximately 217 nm that was observed using circular dichroism spectroscopy (Figure 1C), consistent with correctly folded domains (*26*, *27*).

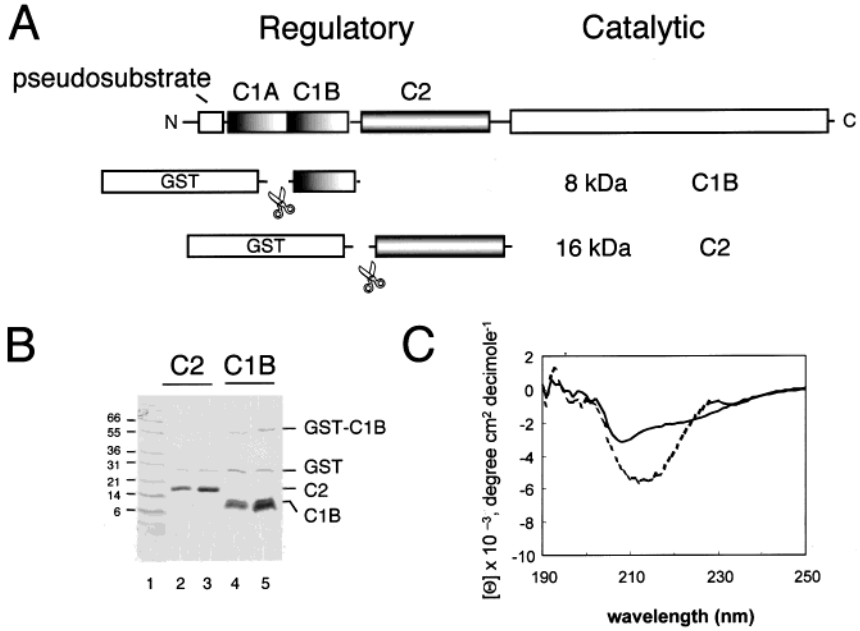

FIGURE 1: C1B and C2 domain constructs. (A) Schematic representation of C1B domain (residues 92−161 of protein kinase C βII) and C2 domain (residues 157−289) constructs used in this study. Proteins were expressed as fusion proteins with GST, which was subsequently removed as described under Materials and Methods. (B) Coomassie-stained gel of the purified C2 (lanes 2 and 3, 1.3 and 3.3 μg) and C1B domains (lanes 4 and 5, 3 and 7.5 μg). (C) Circular dichroism spectra of the purified C2 (dashed line) and C1B (solid line) domains, measured as described under Materials and Methods. Protein concentrations were 0.65 and 1.5 mg mL $^{-1}$ for the C2 and C1B domains, respectively.

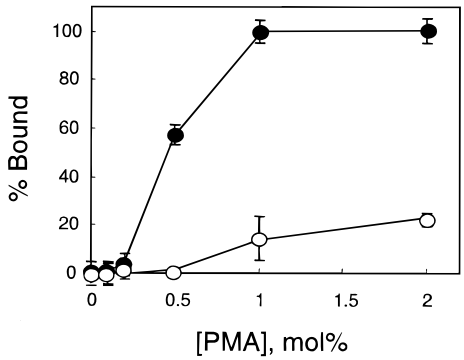

FIGURE 2: PMA recruits the C1B domain to neutral and anionic membranes. Binding of the C1B domain to phosphatidylcholine (open symbols) or phosphatidylserine/phosphatidylcholine vesicles (40:60 mole fraction; solid symbols) containing 0−2 mol % PMA was measured as described under Materials and Methods. Total lipid concentration was 1 mM. Membrane binding was measured in the presence of 100 mM KCl and 2.5 mM $MgCl_2$. Data represent the average ± range of duplicate determinations from a representative experiment.

Membrane Binding Properties of the C1B Domain. Figure 2 shows that the isolated C1B domain bound neutral membranes composed of phosphatidylcholine in a phorbol ester-dependent manner (open symbols). This binding was relatively weak, but readily detectable in the presence of 1 mM lipid[2] (22% of the protein was bound to membranes containing 2 mol % PMA). Compilation of data from three independent experiments revealed an apparent membrane association constant of (8.0 ± 0.6) × 10^2 M^{-1} for the interaction of the C1B domain with neutral membranes containing 2 mol % PMA (Table 1). No binding to neutral membranes was detected in the absence of PMA, indicating an affinity <20 M^{-1} based on the detection limit of the assay. Inclusion of phosphatidylserine markedly enhanced the binding of the C1B domain to vesicles. The apparent membrane association constant for binding to membranes containing 40 mol % phosphatidylserine and 2 mol % PMA was (1.27 ± 0.01) × 10^5 M^{-1} (Table 1). Thus, the presence of phosphatidylserine increased the affinity of the C1B domain for PMA-containing membranes by over 2 orders of magnitude.

Figure 3 shows that diacylglycerol also promoted the binding of the C1B domain to membranes. Inclusion of 5 mol % diacylglycerol in membranes composed of phosphatidylserine and phosphatidylcholine resulted in at least a 15-fold increase in the apparent membrane affinity, from <30 M^{-1} to (4.5 ± 0.5) × 10^2 M^{-1}. This affinity is approximately 280-fold weaker than that measured in the presence of 2 mol % PMA (see above), revealing that diacylglycerol is over 300-fold weaker, on a molar basis, than PMA in recruiting the C1B domain to membranes. This difference in potency of the two ligands on the isolated C1B domain is the same as the difference in potency observed for the full-length protein (*29*). Figure 3 also shows that the interaction of the C1B domain with anionic membranes was not affected by Ca^{2+}: similar binding was observed in the presence of 0.3 mM Ca^{2+} (filled bars) or 0.5 mM EGTA (open bars).

To determine whether the interaction with phosphatidylserine reflected a nonspecific interaction with anionic membranes, or selective recognition of the serine headgroup, we examined the binding to membranes containing another monovalent anionic lipid, phosphatidylglycerol. Figure 4 compares the binding of the C1B domain to membranes containing 40 mol % phosphatidylglycerol or phosphati-

[2] The binding of protein kinase C, and its isolated domains, to membranes obeys the law of mass action. Thus, under the conditions of our assay where the concentration of lipid far exceeds that of protein kinase C, the ratio of bound to free protein kinase C is linearly related to the total lipid concentration (*29*).

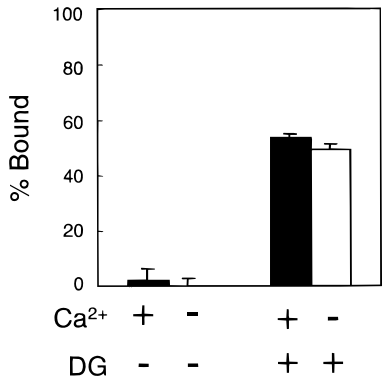

FIGURE 3: Diacylglycerol, but not Ca^{2+}, modulates the affinity of the C1B domain for anionic membranes. Binding of the C1B domain to phosphatidylcholine vesicles containing phosphatidylserine (40 mol %), with or without 5 mol % diacylglycerol (DG), was measured in the presence of 0.3 mM Ca^{2+} (solid bars) or 0.5 mM EGTA (open bars) as described under Materials and Methods. Total lipid concentration was 1 mM. Membrane binding was measured in the presence of 100 mM KCl and 2.5 mM $MgCl_2$. Data represent the average ± range of duplicate determinations from a representative experiment.

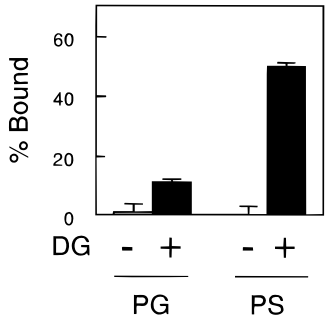

FIGURE 4: C1B domain selectively binds phosphatidylserine-containing membranes. (A) The binding of the C1B domain to phosphatidylcholine vesicles containing 40 mol % phosphatidylglycerol (PG) (left) or 40 mol % phosphatidylserine (PS) (right) was measured in the presence or absence of 5 mol % diacylglycerol (DG) as described under Materials and Methods. Total lipid concentration was 1 mM. Binding measurements were conducted in the presence of 100 mM KCl and 2.5 mM $MgCl_2$. Data represent the average ± SEM of 4−6 determinations.

dylserine in the presence (filled bars) or absence (open bars) of 5 mol % diacylglycerol. Binding to membranes lacking the diacylglycerol was barely detectable under these assay conditions, with an apparent membrane association constant of approximately 30 M^{-1} (see Table 1). Inclusion of 5 mol % diacylglycerol caused a 15-fold increase in the affinity of the C1 B domain for vesicles containing phosphatidylserine [apparent membrane affinity increased to $(4.5 \pm 0.5) \times 10^2$ M^{-1}], but only a modest increase in the affinity for vesicles containing phosphatidyglycerol [apparent membrane affinity increased to $(0.8 \pm 0.1) \times 10^2$ M^{-1}]. Similarly, the C1B domain bound anionic membranes containing PMA with 15-fold higher affinity if the anionic lipid was phosphatidylserine [apparent $K_a = (1.27 \pm 0.01) \times 10^5$ M^{-1}] compared with phosphatidylglycerol [apparent $K_a = (8.4 \pm 0.2) \times 10^3$ M^{-1}] (Table 1). Thus, the presence of ligand (diacylglycerol or PMA) caused the C1B domain to bind phosphatidylserine-containing membranes with an order of magnitude higher affinity than phosphatidylglycerol-containing membranes.

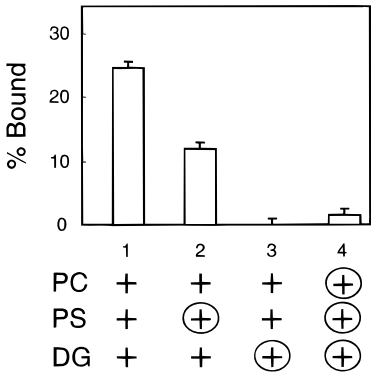

FIGURE 5: Binding of the C1B domain to phosphatidylserine is stereospecific. The binding of the C1B domain to diacylglycerol/phosphatidylserine/phosphatidylcholine (5:40:55 mole fraction) vesicles containing various stereoisomers was measured as described under Materials and Methods. (1) Vesicles contained natural isomers (*sn*-1,2-phosphatidyl-L-serine, *sn*-1,2-diacylglycerol, *sn*-1,2-phosphatidylcholine); (2) the phosphatidylserine enantiomer (*sn*-2,3-phosphatidyl-D-serine, *sn*-1,2-diacylglycerol, *sn*-1,2-phosphatidylcholine); (3) the diacylglycerol enantiomer (*sn*-1,2-phosphatidyl-L-serine, *sn*-2,3-diacylglycerol, *sn*-1,2-phosphatidylcholine); or (4) enantiomers of all three components (*sn*-2,3-phosphatidyl-D-serine, *sn*-2,3-diacylglycerol, *sn*-2,3-phosphatidylcholine). The total lipid concentration was 1 mM. Membrane binding was measured in the presence of 100 mM KCl and 2.5 mM $MgCl_2$. Data represent the average ± SEM of 6 determinations. Circled lipids indicate the *sn*-2,3 stereochemistry.

We next addressed whether the selective recognition of phosphatidylserine by the C1B domain was stereospecific for *sn*-1,2-phosphatidyl-L-serine. Figure 5 shows the binding of the C1B domain to diacylglycerol/phosphatidylserine/phosphatidylcholine (5:40:55, mole fraction) membranes which differ only in the stereochemistry of the lipids. Column 1 shows the binding to membranes containing the naturally occurring *sn*-1,2- phospholipids; the apparent membrane affinity was $(3.3 \pm 0.2) \times 10^2$ M^{-1}. Replacement of the phosphatidylserine with its enantiomer, *sn*-2,3-phosphatidyl-D-serine, reduced binding approximately 2-fold (column 2) to yield an apparent membrane affinity of $(1.4 \pm 0.2) \times 10^2$ M^{-1}. Replacement of the physiological *sn*-1,2-diacylglycerol with its enantiomer, *sn*-2,3-diacylglycerol, abolished binding of the C1B to membranes (column 3) (apparent membrane affinity <20 M^{-1}), demonstrating strict stereospecificity in the domain's interaction with diacylglycerol.

Membrane Binding Properties of the C2 Domain. Figure 6 shows that the isolated C2 domain bound anionic lipids in a calcium-dependent manner. Binding to vesicles containing either 40 mol % phosphatidylserine or 40 mol % phosphatidylglycerol depended on Ca^{2+}, with no detectable binding observed in the absence of this cation (apparent membrane affinity <50 M^{-1} based on detection limit of assay). In contrast, binding was unaffected by the presence of diacylglycerol: similar binding to vesicles containing either 40 mol % phosphatidylserine or 40 mol % phosphatidylglycerol was observed in the presence (open bars) or absence (filled bars) of 5 mol % diacylglycerol. Apparent membrane affinities for phosphatidylserine and phosphatidylglycerol were $(4.4 \pm 0.5) \times 10^3$ M^{-1} and $(1.4 \pm 0.2) \times 10^3$ M^{-1}, respectively, illustrating some preference for the serine headgroup.

Figure 6B shows that the binding of the C2 domain to membranes depended on the presence of anionic lipid. Under

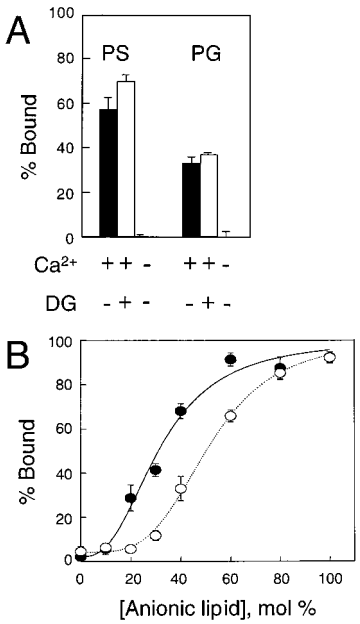

FIGURE 6: Binding of the C2 domain to membranes is dependent on anionic lipid and Ca^{2+}. (A) The binding of the C2 domain to phosphatidylcholine vesicles containing 40 mol % phosphatidylserine (PS) or phosphatidylglycerol (PG) was measured in the absence (solid bars) or presence (open bars) of 5 mol % diacylglycerol (DG) and in the presence of 0.3 mM Ca^{2+} (open and solid bars) or 0.5 mM EGTA, as described under Materials and Methods. The total lipid concentration was 0.5 mM. Membrane binding was measured in the presence of 100 mM KCl and 5 mM $MgCl_2$. Data are average ± range of duplicate determinations from a representative experiment. (B) The binding of the C2 domain to phosphatidylcholine vesicles containing the indicated amount of phosphatidylglycerol (open symbols) or phosphatidylserine (solid symbols) was measured as described under Materials and Methods. The total lipid concentration was 0.5 mM. Assays contained 0.3 mM Ca^{2+}, 100 mM KCl, and 5 mM $MgCl_2$. Data represent the average ± SEM of 4−6 determinations. The curves shown are predicted for the data from a modified Hill equation using Sigma plot, as described previously (*43*).

the conditions of the assay in Figure 6B, half-maximal binding of the isolated C2 domain required approximately 30 mol % phosphatidylserine and approximately 45 mol % phosphatidylglycerol, further demonstrating the modest preference for the serine headgroup.

Lipid specificity was further analyzed in Figure 7. The C2 domain bound anionic membranes with little preference for headgroup beyond the requirement for negative charge: similar binding to membranes containing 40 mol % phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid was observed. As in Figure 6, there was an approximately 2-fold preference for the serine headgroup. The C2 domain did not bind the neutral lipids phosphatidylcholine or phosphatidylethanolamine. Binding to anionic membranes was abolished in the presence of EGTA.

To determine whether the slight preference for phosphatidylserine reflected a nonspecific interaction or stereospecific recognition of the serine headgroup, we compared the binding of the C2 domain to membranes containing the naturally occurring lipids or their stereoisomers. Figure 8 shows that the C2 domain bound equally well to membranes containing the naturally occurring lipids *sn*-1,2-phosphatidyl-L-serine/*sn*-1,2-diacylglycerol/*sn*-1,2-phosphatidylcholine (40:5:55; column 1) or membranes in which the phosphatidylserine (column 2), diacylglycerol (column 3), or all three lipids

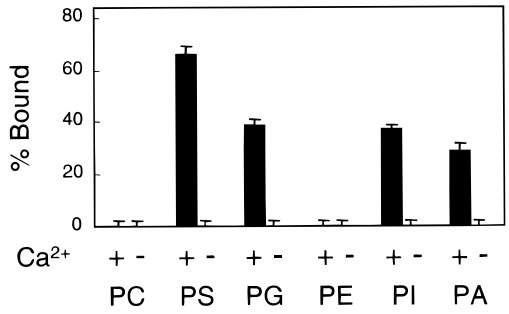

FIGURE 7: Lipid selectivity in the interaction of the C2 domain with membranes. The binding of the C2 domain to phosphatidylcholine vesicles (PC) or phosphatidylcholine vesicles containing 40 mol % phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), or phosphatidic acid (PA) was measured in the presence of 0.3 mM calcium (solid bars) or 0.5 mM EGTA (open bars) as described under Materials and Methods. The total lipid concentration was 0.5 mM. Membrane binding was measured in the presence of 100 mM KCl and 5 mM $MgCl_2$. Data represent the average ± SEM of 6 determinations.

(phosphatidylserine, diacylglycerol, phosphatidylcholine) were replaced with their enantiomeric counterparts (column 4). Thus, the modest preference of the C2 domain for phosphatidylserine over other anionic lipids does not result from specific determinants on the protein recognizing the molecular architecture of this lipid, but must arise because of unique properties of phosphatidylserine in structuring membranes.

To determine which other factors may account for the C2 domain's slight preference for phosphatidylserine, we tested how its binding to anionic vesicles responded to changes in various ions present in the assay. Figure 9A shows that the Ca^{2+} dependence for binding to either phosphatidylserine (closed circles) or phosphatidylglycerol (open circles) differed by a factor of 4, similar to the difference in affinity for the two lipids: half-maximal binding to phosphatidylserine was observed in the presence of 40 μM Ca^{2+}, and half-maximal binding to phosphatidylglycerol required 170 μM Ca^{2+}. The ionic strength dependence was similar: KCl was equally effective at reducing the binding of the C2 domain to membranes containing phosphatidylserine (closed symbols) or phosphatidylglycerol (open symbols; Figure 9B). However, Mg^{2+} appeared more effective at reducing the affinity of the C2 domain for membranes composed of phosphatidic acid (open triangles) or phosphatidylglycerol (open circles) than phosphatidylserine (closed circles; Figure 9C). Thus, the modest selectivity of the C2 domain for phosphatidylserine was unaffected by Ca^{2+} or KCl, but was enhanced by Mg^{2+}.

Comparison of Free Domains with Full-Length Protein Kinase C. To determine how the C1B and C2 domains function in the context of the whole protein, we compared the binding of the isolated domains and of full-length protein kinase C to membranes of different composition. Table 1 shows that the apparent membrane affinity for the isolated C1B domain or full-length protein kinase C βII for PMA-containing neutral membranes was similar (approximately 10^3 M^{-1}). This suggests that the ligand binding pocket of the C1B domain is equally accessible to phosphatidylcholine membranes as an isolated domain or in the full-length

Table 1: Apparent Membrane Binding Constants (K_a) for the Interaction of Individual C1B and C2 Domains and Full-Length Protein Kinase C βII with Phosphatidylcholine Vesicles Containing 0 or 2 mol % PMA and 0 or 40 mol % Anionic Lipid[a]

	vesicle composition			
construct	PMA/PC	PMA/PG/PC	PMA/PS/PC	PG/PC
C1B domain	$(8.0 \pm 0.6) \times 10^2$	$(8.4 \pm 0.2) \times 10^3$	$(1.27 \pm 0.01) \times 10^5$	$(3.0 \pm 0.7) \times 10^1$
C2 domain	nd	$(3.5 \pm 0.6) \times 10^2$	$(1.0 \pm 0.1) \times 10^3$	$(2.1 \pm 0.2) \times 10^2$
full-length enzyme	$(1.26 \pm 0.02) \times 10^3$	$(5.7 \pm 0.1) \times 10^4$	$(9.5 \pm 0.1) \times 10^5$	$(4.1 \pm 0.3) \times 10^2$

[a] Membrane binding was measured in the presence of 0.2 mM Ca^{2+}, 5 mM $MgCl_2$, 100 mM KCl, 0.3 mg mL^{-1} BSA, and 20 mM HEPES, pH 7.4. The total lipid concentration ranged from 0.025 to 3.2 mM. The values for K_a were calculated using the formula under Materials and Methods and are expressed in units of M^{-1}. Data are presented as a weighted average ± SD of the K_a values obtained from at least two independent experiments, each performed in triplicate. nd, not detectable.

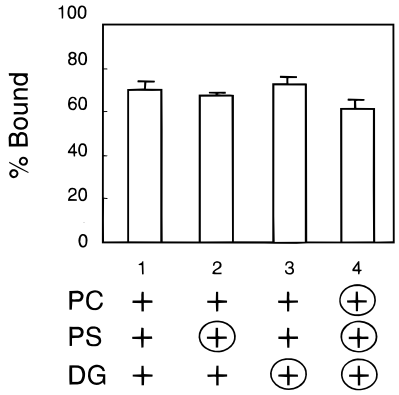

FIGURE 8: Interaction of the C2 domain with phosphatidylserine is not stereospecific. The binding of the C2 domain to diacylglycerol/phosphatidylserine/phosphatidylcholine (5:40:55, mole fraction) vesicles containing various stereoisomers was measured as described under Materials and Methods. (1) Vesicles contained natural isomers (*sn*-1,2-phosphatidyl-L-serine, *sn*-1,2-diacylglycerol, *sn*-1,2-phosphatidylcholine); (2) the phosphatidylserine enantiomer (*sn*-2,3-phosphatidyl-D-serine, *sn*-1,2-diacylglycerol, *sn*-1,2-phosphatidylcholine); (3) the diacylglycerol enantiomer (*sn*-1,2-phosphatidyl-L-serine, *sn*-2,3-diacylglycerol, *sn*-1,2-phosphatidylcholine); or (4) enantiomers of all three components (*sn*-2,3-phosphatidyl-D-serine, *sn*-2,3-diacylglycerol, *sn*-2,3-phosphatidylcholine). The total lipid concentration was 0.5 mM. Membrane binding was measured in the presence of 0.3 mM Ca^{2+}, 100 mM KCl, and 5 mM $MgCl_2$. Data represent the average ± SEM of 6 determinations. Circled lipids indicate the *sn*-2,3 stereochemistry.

protein. Addition of phosphatidylglycerol increased the binding affinity of the C1B domain by an order of magnitude, and addition of phosphatidylserine increased the binding by an additional order of magnitude (to 10^5 M^{-1}). Thus, the isolated C1B domain retained the specificity for phosphatidylserine observed for full-length protein.

In the presence of 0.2 mM Ca^{2+}, the affinity of the C2 domain for membranes containing phosphatidylglycerol (approximately 10^2 M^{-1}) was an order of magnitude greater than that of the C1B domain (approximately 10^1 M^{-1}) for such anionic membranes. In contrast to the C1B domain, the C2 domain bound these membranes by an interaction that was insensitive to PMA and only modestly sensitive to phosphatidylserine. Whereas the C1B domain bound PMA-containing membranes with 15-fold higher affinity if the anionic lipid was phosphatidylserine instead of phosphatidylglycerol, the C2 domain bound such membranes with only 3-fold higher affinity if the acidic lipid was phosphatiylserine instead of phosphatidylglycerol. [This 3-fold selectivity for the C2 domain was also observed in the absence of PMA, which did not significantly alter the affinity of the C2 domain for anionic membranes (data not shown).]

In cases where both the C1B and the C2 domains were engaged on the membrane (anionic lipids and PMA present), the membrane affinity of the full-length protein was at least an order of magnitude less than the product of the affinities of the isolated C1B and C2 domains. The product of the apparent membrane binding constants for the interaction of the C1B and C2 domains with phosphatidylglycerol, phosphatidylglycerol/PMA membranes, or phosphatidylserine/PMA membranes was 15, 50, or 130 times greater that the apparent membrane binding constant for the full-length protein.

DISCUSSION

Analysis of the membrane interaction of the isolated C1B and C2 domains of protein kinase C has revealed that both domains function as independent membrane-targeting modules. The interaction of the C1B domain with membranes is driven by at least three mechanisms: by binding its ligand, diacylglycerol or phorbol esters; by a nonspecific electrostatic interaction with anionic lipids; and by a stereoselective interaction with phosphatidylserine. This interaction is insensitive to physiological Ca^{2+}. The interaction of the C2 domain is driven by binding its ligand, Ca^{2+}, and by a nonspecific electrostatic interaction with anionic lipids. The C2 domain interaction is insensitive to diacylglycerol and displays no stereospecificity for phosphatidylserine.

Specificity for Phosphatidylserine Resides in the C1B and Not the C2 Domain. A unifying feature of all protein kinase C isozymes is their specific activation by phosphatidylserine (*7*, *18*). Binding studies with full-length protein kinase C have revealed that the enzyme displays a 10-fold selectivity for phosphatidylserine over other anionic lipids in the presence, but not absence, of diacylglycerol (*30*). This specificity primarily reflects interaction of specific determinants on protein kinase C with specific determinants on the lipid, rather than reflecting recognition of unique membrane-structuring properties of phosphatidylserine. The enzyme specifically binds membranes containing *sn*-1,2-phosphatidyl-L-serine and is unable to bind enantiomeric membranes that maintain identical physical properties but differ only in the stereochemistry of the lipids (*20*). Here we show that the determinants that dictate this specificity reside in the C1B domain: the affinity of both the isolated domain and the full-length protein is 1 order of magnitude greater for phosphatidylserine-containing membranes compared with membranes containing phosphatidylglycerol. Studies with enantiomeric lipids reveal that a significant part of this increase arises from stereospecific interactions with specific determinants on the phospholipid. Curiously, a separate study

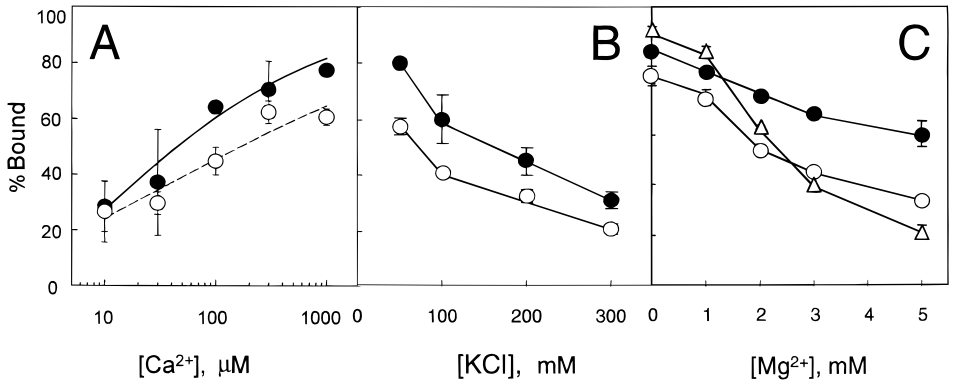

FIGURE 9: Dependence of the C2 domain/membrane interaction on calcium, ionic strength, and magnesium. The binding of the C2 domain to phosphatidylcholine vesicles containing 40 mol % phosphatidylglycerol (open circles), phosphatidylserine (closed circles), or phosphatidic acid (open triangles) was measured in the presence of various ion concentrations as described under Materials and Methods. The total lipid concentration was 0.5 mM. Unless otherwise indicated, ion concentrations were 100 mM KCl, 5 mM $MgCl_2$, and 0.3 mM calcium. Data represent the average ± range of duplicate determinations from representative experiments. (A) The binding of the C2 domain to membranes was measured in the presence of 10−1000 μM free Ca^{2+} using 0.5 mM EGTA as a buffer, as described (*44*). (B) The binding of the C2 domain to membranes was measured in the presence of 50−300 mM KCl. (C) The binding of the C2 domain to membranes was measured in the presence of 0−5 mM $MgCl_2$.

of the interaction of a GST-fusion protein encompassing the C1A and C1B domains of protein kinase C α concluded that a tandem C1A/B construct did not disciminate between phosphatidylserine and phosphatidylglycerol (*31*). This study differs from ours in that the construct was refolded from inclusion bodies and contained a His tag. In addition to isozyme or folding differences, one possibility is that electrostatic interactions of the basic tag dominated in the membrane interaction. In our study, the entire population of C1B domain was functional as assessed by phorbol esters causing complete membrane binding.

In contrast to the C1B domain, the C2 domain displays no stereospecificity in its interaction with phosphatidylserine. Although this domain binds phosphatidylserine-containing membranes with 2−3-fold greater affinity than membranes composed of other anionic lipids, the domain does not discriminate between enantiomers of phosphatidylserine. Thus, the major driving force in the interaction of the C2 domain with membranes is a Ca^{2+}-dependent electrostatic interaction.

The C2 domain, does, nonetheless display a slight preference for phosphatidylserine over other anionic lipids. This selectivity over other anionic lipids was also observed for the C2 domain of protein kinase C α (*31*). In this study, we show that the interaction with phosphatidylserine is not stereospecific. Thus, the selectivity may arise from unique properties of phosphatidylserine in structuring membranes to provide an optimal surface for C2 domain binding. A second possibility is that the concentration of Ca^{2+} at the membrane interface is different for phosphatidylserine-containing membranes compared with membranes composed of other anionic lipids. In support of this, Mg^{2+}, which competes with Ca^{2+} for binding to phosphatidylserine (*32*), increases the observed selectivity for phosphatidylserine. Thus, the slight selectivity for phosphatidylserine could reflect an increased affinity of this lipid for Ca^{2+} compared with other anionic phospholipids.

The Affinity of the Isolated C1B Domain for PMA-Containing Neutral Membranes Is the Same as That of Full-Length Protein Kinase C. In this study, we find that the apparent membrane binding affinity of the isolated C1B domain to neutral membranes containing PMA is similar to that of the full-length protein. If the interaction of the full-length protein is driven only by the C1B domain, with little participation of the C1A domain, the similarity in binding constants reveals that the domain is equally accessible to neutral membranes alone or in the context of the full-length protein. Several lines of evidence suggest that this domain is primarily, if not exclusively, responsible for recruiting protein kinase C to membranes. First, the stoichiometry of phorbol ester binding to protein kinase C has been reported to be one ligand per protein kinase C for diverse isoforms (*29, 33*−*35*), although one group has suggested that, under some conditions, two ligands may bind to protein kinase C α (*36*). Second, mutagenesis of the C1A or C1B domain to impair phorbol ester binding has revealed that the C1B domain, and not the C1A domain, is primarily responsible for the PMA-dependent translocation of protein kinase C δ in vivo (*37*). Binding studies with GST-fusion constructs of the C1A and C1B domains of protein kinase C δ suggested that only the C1B domain binds PMA (*38*). In contrast, a similar study with the isolated domains of protein kinase C γ reported that both the C1A and C1B domains bound phorbol esters with similar affinities (*39*). Thus, it is not clear if the dominating interaction of the C1B domain in the full-length protein arises because the C1A domain is masked, or whether the C1A has a reduced affinity for ligand. Because the sum of the binding energies of individual domains is greater than the binding energy for full-length protein, this suggests that the C1A is unlikely to contribute significantly to the membrane interaction.

The Affinity of the C1B and C2 Domains for Anionic Membranes Is Reduced in the Context of the Full-Length Protein. The sum of the observed binding energies of two isolated domains (e.g., C1B and C2) to a surface typically differs from the binding energy measured when the two domains are tethered (e.g., as in full-length protein kinase C) as a result of entropic and steric considerations (*40*). In the case of protein kinase C, this study shows that the binding energy of full-length protein kinase C to anionic membranes is less than that expected from the sum of the binding energies of the isolated C1B and C2 domains. This difference

is most pronounced under conditions resulting in protein kinase C activation. In addition to entropic considerations, one contibution to this difference may be the energy required to expel the pseudosubstrate from the substrate binding cavity. For example, the sum of the binding energies for the isolated C1B (7 kcal mol^{-1}; apparent K_a of 10^5 M^{-1}) and C2 (4 kcal mol^{-1}; apparent K_a of 10^3 M^{-1}) domains to 'activating membranes' (PMA/phosphatidylserine) is approximately 3 kcal mol^{-1} greater than the binding energy of full-length protein to the same membranes (8 kcal mol^{-1}; apparent K_a of 10^6 M^{-1}). If one also takes into account the binding affinity of the isolated pseudosubstrate sequence for anionic lipids (10^4 M^{-1}) (*41*), then closer to 8 kcal mol^{-1} may be required to release the pseudosubstrate from the kinase core. Thus, a major role of the membrane-targeting modules of protein kinase C may be to provide the energy to displace the pseudosubstrate form the substrate binding cavity.

Other contributions to the decreased membrane affinity of the full-length protein compared with that of the isolated domains is that steric constraints introduced by tethering both domains to the membranes reduce the affinity of one, or both, domain to the membrane. For example, interaction of the C2 domain with membranes could reduce accessibility of the C1B domain to membranes, or the reverse. An elegant study by Oancea and Meyer suggested that the C2 domain binds membranes before the C1 domain. This was based on the finding that a GFP−C1 domain fusion protein was recruited to the plasma membrane of PMA-treated cells with faster kinetics than GFP−full-length protein kinase C γ (*42*). In their study, a GFP−C2 domain fusion protein bound the plasma membrane of Ca^{2+}/ionophore-treated cells with similar kinetics as GFP−full-length protein. This contrasts with our study which suggests that the C1 domain binds membranes equally well as an isolated domain or as part of protein kinase C. This difference could arise from isozyme differences, from the presence of the GFP tag, or because of factors in the cell (e.g., binding proteins) not present in our model membrane study. In addition, because off rates were not measured in the kinetic study, it is possible that the affinities of the isolated domain could be the same as that of the domain in the full-length protein.

CONCLUSIONS

The membrane interaction of protein kinase C is regulated by two membrane-targeting modules: the C1 and C2 domains. Each domain alone is capable of recruiting protein kinase C to membranes by a relatively weak interaction (binding constants on the order of $10^2 - 10^5$ M^{-1}); however, the tethering of both is required to produce the energy to remove the autoinhibitory pseudosubstrate sequence from the active site. The C1 domain interaction is driven by a specific interaction with diacylglycerol or PMA, which is approximately 300-fold more potent than diacylglycerol on a molar basis, by a nonspecific electrostatic interaction with anionic lipids, and by a specific recognition of phosphatidylserine. The C2 domain interaction is driven by a Ca^{2+}-dependent interaction with anionic phospholipids which displays no significant headgroup selectivity beyond the requirement for negative charge. When both the C1 and C2 domains are engaged on the membrane, the membrane affinity of the full-length protein is less than that expected if each domain bound independently of the other. Part of this reduction could be explained by the energy required to release the pseudosubstrate from the substrate binding site.

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REFERENCES

1. Fruman, D. A., Rameh, L. E., and Cantley, L. C. (1999) *Cell 97*, 817−820.
2. Hurley, J. H., and Misra, S. (2000) *Annu. Rev. Biophys. Biomol. Struct. 29*, 49−79.
3. McLaughlin, S., and Aderem, A. (1995) *Trends Biochem. Sci. 20*, 272−276.
4. Newton, A. C., and Johnson, J. E. (1998) *Biochim. Biophys. Acta 1376*, 155−172.
5. Nishizuka, Y. (1995) *FASEB J. 9*, 484−496.
6. Mochly-Rosen, D. (1995) *Science 268*, 247−251.
7. Newton, A. C. (1997) *Curr. Opin. Cell Biol. 9*, 161−167.
8. Nalefski, E. A., and Falke, J. J. (1996) *Protein Sci. 5*, 2375−2390.
9. Edwards, A. S., and Newton, A. C. (1997) *Biochemistry 36*, 15615−15623.
10. Nalefski, E. A., and Falke, J. J. (1998) *Biochemistry 37*, 17642−17650.
11. Perisic, O., Fong, S., Lynch, D. E., Bycroft, M., and Williams, R. L. (1998) *J. Biol. Chem. 273*, 1596−1604.
12. Medkova, M., and Cho, W. (1998) *J. Biol. Chem. 273*, 17544−17552.
13. Toker, A. (1998) *Front. Biosci. 3*, D1134−D1147.
14. Chou, M. M., et al. (1998) *Curr. Biol. 8*, 1069−1077.
15. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) *Science 281*, 2042−2045.
16. Dutil, E. M., Toker, A., and Newton, A. C. (1998) *Curr. Biol. 8*, 1366−1375.
17. Kaibuchi, K., Takai, Y., and Nishizuka, Y. (1981) *J. Biol. Chem. 256*, 7146−7149.
18. Bell, R. M., and Burns, D. J. (1991) *J. Biol. Chem. 266*, 4661−4664.
19. Newton, A. C. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 1−25.
20. Johnson, J. E., Zimmerman, M. L., Daleke, D. L., and Newton, A. C. (1998) *Biochemistry 37*, 12020−12025.
21. Orr, J. W., Keranen, L. M., and Newton, A. C. (1992) *J. Biol. Chem. 267*, 15263−15266.
22. Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.
23. Ono, Y., et al. (1987) *Science 236*, 1116−1120.
24. Mosior, M., and Newton, A. C. (1995) *J. Biol. Chem. 270*, 25526−25533.
25. Rebecchi, M., Peterson, A., and McLaughlin, S. (1992) *Biochemistry 31*, 12742−12747.
26. Zhang, G., Kazanietz, M. G., Blumberg, P. M., and Hurley, J. H. (1995) *Cell 81*, 917−924.
27. Sutton, R. B., and Sprang, S. R. (1998) *Structure 6*, 1395−1405.
28. Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1997) *Protein Sci. 6*, 477−480.
29. Mosior, M., and Newton, A. C. (1996) *Biochemistry 35*, 1612−1623.
30. Newton, A. C., and Keranen, L. M. (1994) *Biochemistry 33*, 6651−6658.
31. Medkova, M., and Cho, W. (1999) *J. Biol. Chem. 274*, 19852−19861.
32. McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G., and McLaughlin, A. (1981) *J. Gen. Physiol. 77*, 445−473.
33. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., and Nishizuka, Y. (1983) *J. Biol. Chem. 258*, 11442−11445.

34. König, B., DiNitto, P. A., and Blumberg, P. M. (1985) *J. Cell. Biochem. 29*, 37−44.
35. Hannun, Y. A., and Bell, R. M. (1986) *J. Biol. Chem. 261*, 9341−9347.
36. Slater, S. J., Kelly, M. B., Taddeo, F. J., Rubin, E., and Stubbs, C. D. (1994) *J. Biol. Chem. 269*, 17160−17165.
37. Szallasi, Z., Bogi, K., Gohari, S., Biro, T., Acs, P., and Blumberg, P. M. (1996) *J. Biol. Chem. 271*, 18299−18301.
38. Hunn, M., and Quest, A. F. G. (1997) *FEBS Lett. 400*, 226−232.
39. Quest, A. F. G., and Bell, R. M. (1994) *J. Biol. Chem. 269*, 20000−20012.
40. Jencks, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4046−4050.
41. Mosior, M., and McLaughlin, S. (1991) *Biophys. J. 60*, 149−159.
42. Oancea, E., and Meyer, T. (1998) *Cell 95*, 307−318.
43. Orr, J. W., and Newton, A. C. (1992) *Biochemistry 31*, 4661−4667.
44. Keranen, L. M., and Newton, A. C. (1997) *J. Biol. Chem. 272*, 25959−25967.

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