A Comparison of the Membrane Binding Properties of C1B Domains of PKCγ, PKCδ, and PKCε

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ABSTRACT The C1 domains of classical and novel PKCs mediate their diacylglycerol-dependent translocation. Using fluorescence resonance energy transfer, we studied the contribution of different negatively charged phospholipids and diacylglycerols to membrane binding. Three different C1B domains of PKCs were studied (the classical γ, and the novel δ and ε), together with different lipid mixtures containing three types of acidic phospholipids and three types of activating diacylglycerols. The results show that C1Bγ and C1Bε exhibit a higher affinity to bind to vesicles containing 1-palmitoyl-2-oleoyl-*sn*-phosphatidic acid, 1-palmitoyl-2-oleoyl-*sn*-phoshatidylserine, or 1-palmitoyl-2-oleoyl-*sn*-phosphatidylglycerol, with C1Bε being the most relevant case because its affinity for POPA-containing vesicles increased by almost two orders of magnitude. When the effect of the diacylglycerol fatty acid composition on membrane binding was studied, the C1Bε domain showed the highest binding affinity to membranes containing 1-stearoyl-oleoyl-*sn*-glycerol or 1,2-*sn*-dioleoylglycerol with POPA as the acidic phospholipid. Of the three diacylglycerols used in this study, 1,2-*sn*-dioleoylglycerol and 1-stearoyl-oleoyl-*sn*-glycerol showed the highest affinities for each isoenzyme, whereas 1,2-*sn*-dipalmitoylglycerol; showed the lowest affinity. DSC experiments showed this to be a consequence of the nonfluid conditions of 1,2-*sn*-dipalmitoylglycerol;-containing systems.

INTRODUCTION

PKC is a large family of phospholipid-dependent serine/threonine kinases, which are activated by many extracellular signals, and which play a critical role in several signaling pathways in the cell. Mammalian isoenzymes have been grouped into three subfamilies according to their enzymatic properties. The first group, called classical or conventional isoenzymes [cPKCs (classical/conventional PKCs)], includes PKCα, βI, βII, and γ, all of which contain the conserved C1 and C2 domains in the regulatory region. These isoenzymes are regulated by Ca^{2+} and acidic phospholipids, which interact with the C2 domain, and by DAG, which interacts with the C1 domain (1,2). Translocation of classical PKC molecules to their target, the plasma membrane, is mediated by the C1 and C2 domains, with the initial membrane affinity mainly determined by C2 domain-membrane interactions through Ca^{2+} bridging (3), and PIP_2 binding (4–6), followed by C1 domain-diacylglycerol interactions (1). On the other hand, the enzymes of the novel PKC subfamily (δ, ε, θ, and η) do not bind Ca^{2+} and are activated through diacylglycerol binding to the C1 domain (2), although the C2 domain may also play a role through interaction with ligands like phosphatidic acid (7).

A common feature of classical and novel PKCs is the presence of two C1 subdomains located in tandem within the regulatory region. Various studies have shown that, besides other differences between conventional and novel isoenzymes, the C1 subdomains are not functionally equivalent. Thus, the two C1 subdomains of some isoenzymes exhibit different phorbol ester binding affinities, which are inverted when diacylglycerol is used as a ligand (1,8–11).

Extensive studies performed by authors like Burns and Bell (8) or Irie et al. (9) and Shindo et al. (11) have indicated that despite high sequence homology, isolated C1 domains of conventional and novel PKCs have different intrinsic phorbol ester and diacylglycerol affinities. Differential roles in the membrane targeting and activation of PKCs have been attributed ascribed to the different DAG affinities of C1A and C1B domains (12,13). For PKCα (12,14), the C1A domain, which shows a much higher DAG affinity than the C1B domain, plays a predominant role in these processes. For PKCδ, conflicting results have been published; one group (9) claims that C1B has a much higher binding affinity for diacylglycerol than C1A, whereas another group (13) maintains that the opposite is true (13). In regard to PKCγ (12), whereby the C1A and C1B domains have comparable DAG affinities, both C1 domains participate in the processes. PKCε is similar to PKCγ in that C1A and C1B domains are involved in membrane binding and activation, although the C1A domain has approximately a threefold higher DAG affinity than the C1B domain (15).

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Abbreviations: DAG, diacylglycerol; DOG, 1,2-*sn*-dioleoylglycerol; DPG, 1,2-*sn*-dipalmitoylglycerol; ECFP, enhanced cyan fluorescent protein; FRET, fluorescence resonance energy transfer; OG-PE, *N*-(5-dimethylamino-naphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; PA, phosphatidic acid; PIP_2, phosphatidylinositol-4,5-bisphosphate; PG, phosphatidylglycerol; PKC, protein kinase C; POPA, 1-palmitoyl-2-oleoyl-*sn*-phosphatidic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylglycerol; POPS, 1-palmitoyl-2-oleoyl-*sn*-phoshatidylserine; PS, phosphatidylserine; SAG, 1-stearoyl-oleoyl-*sn*-glycerol.

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The DAG-preferences of PKCs have previously been studied by several authors, for the whole enzymes and for isolated C1 domains (16,17). PKCα (18) was used in a study in which it was shown that if the membrane is in a fluid state, 1,2-*sn*-isomers have a similar capacity to activate regardless of the chain length or the saturation/unsaturation level of their fatty acyl chains.

It has long been known that PS enhances the membrane affinity and activity of PKCs (19); although it has also been claimed that the PS dependence may vary significantly among PKC isoforms (12,20). Among conventional PKCs, PKCα (20) and PKCβ_{II} (21) prefer PS to PG, whereas PKCγ shows little preference between PS and PG (12). Among novel PKCs, PKCδ shows a certain degree of PS selectivity (13), whereas PKCε shows no significant PS selectivity (15,20).

Nevertheless, it should be taken into account that the C1 and the C2 domains bind anionic phospholipids, and so it is difficult to use whole-enzyme activity to discern from which domain the observed specificity arises. For this reason, it is important to study the isolated domains. In the case of the C1 domain, only a limited number of studies have been carried out for this purpose; and among the findings it may be mentioned that the C1B domain of PKCβII has a dissociation constant of 2.22 mM in the presence of PS and 12.5 mM in the presence of PG (22). However, when a Tyr residue was mutated to Trp, the C1B domain showed a dissociation constant of 7.7 μM for a membrane containing PS and 15 μM for a membrane containing PG, i.e., although the constant was considerably reduced, the preference was not pronounced for PS (23). In the case of C1Bδ, it was shown that the preference was for PS (K_d of 35 μM) rather than for PG (K_d of 700 μM) (22).

In this work, we characterize the binding of isolated C1B domains of three different PKCs (the classical γ, and the novel δ and ε) to liposomes of different compositions, using three different DAGs (DOG, SAG, and DPG) and three different anionic phospholipids (PS, PG, and PA) to prepare the model vesicles. C1Bε was found to have the highest binding affinity to vesicles containing PA as acidic phospholipid and DOG or SAG as diacylglycerol. In general, DOG and SAG lead to a higher membrane binding affinity than DPG in all C1B domains.

MATERIALS AND METHODS

Materials

All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Oregon Green 488-dihexadecanoylphosphatidylethanolamine (OG-PE) was obtained from Invitrogen (Barcelona, Spain).

Construction of the expression plasmids

C-terminal fusions of isolated C1 domains were generated by inserting cDNAs into the multiple cloning site of the pECFP vector modified and described by Marin-Vicente et al. (2005).

Briefly, cDNAs encoding C1B domains of PKCγ, δ, and ε were amplified by PCR using the following primers:

$C1B\gamma$: 5′CCGAAGCTTCGCAACAAGCACAAG

$C1B\gamma$: 3′CGCCGCACCTGACTGGGCCCTAGG

$C1B\delta$: 5′TATAAGCTTGACATGCCTCACCGA

$C1B\delta$: 3′GACACACCATAGTTGACTCCTAGGAA

$C1B\varepsilon$: 5′CCGAAGCTTAACATGCCCCACAAG

$C1B\varepsilon$: 3′GTTAACACCCCACCTGACTGGGCCCTAAA

C1Bγ and ε were digested with *Hin*dIII/*Xma*I, and C1Bδ was digested with *Hin*dIII/*Bam*HI. The resulting fragments were ligated with their corresponding digested vectors to generate the different fusion constructs.

All constructs were confirmed by DNA sequencing in the Research and Development Support Center, Universidad de Murcia (Spain).

Cell culture and transfections

HEK 293 cells (European Collection of Cell Cultures) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected with 2 μg DNA/6 cm plate using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. The cells were lysed 24 h after transfection in ice-cold hypotonic buffer (10 mM Tris pH 7.4, 10 mM NaF, 1 mM orthovanadate, 1 mM PMSF, and 10 μg/mL each aprotinin and leupeptin) and incubated on ice (20 min). Cells were lysed by 15 passages through a 30-gauge needle; lysates were centrifuged (15,000 $\times$ g, 15 min) to remove nuclei and cell debris. Supernatants were collected and used in the fluorescence experiments.

Binding of C1B-ECFP domains to lipid vesicles

Binding experiments were carried out using small unilamellar vesicles obtained by sonication of multilamellar vesicles. These multilamellar vesicles were prepared by desiccating mixtures of chloroform solutions of lipids in the suitable proportions and extensive vortexing. Lipid mixtures contained phospholipids POPC/POPX/OG-PE in the desired proportions, whereby POPX stands for POPS, POPA, or POPG and diacylglycerols (DOG, SAG, or DPG). Binding experiments were performed at 25°C, using a FluroMax-3 (Jobin Yvon, Horiba, Edison, NJ), with a 20 mM Hepes (pH 7.4) and 100 mM KCl buffer. The excitation and emission windows were set to 3 nm and 4 nm, respectively. To normalize the amount of protein between different experiments, cell lysates were added to the fluorescence

cuvette to reach the same starting ECFP fluorescence intensity. FRET was measured as the fluorescence variation of ECFP as a function of lipid concentration. The intensity of ECFP fluorescence was measured using an excitation wavelength of 433 nm and collected at 473 nm. To correct for the fluorescence attenuation produced by the lipidic vesicles, a control was measured in which vesicles containing only POPC and OG-PE were added to a cuvette with C1B-ECFP, whereas the values obtained were subtracted from these of the above experiments. Additionally, the dilution effect produced by the addition of vesicles was corrected by subtracting the values obtained after adding the same volumes of buffer without lipid vesicles to a cuvette containing the C1B domain. The mean values of three different experiments are shown.

Equilibrium binding data were best-fitted using the Hill equation (1):

$$\Delta F = \Delta F_{\max}\left(X^{H}/K_{D}^{H} + X^{H}\right) \quad (1)$$

whereby ΔF_{max} represents the calculated maximal fluorescence change (normalized to unity to simplify graphical representation), H is the Hill coefficient, X represents the free diacylglycerol concentration corrected for the leaflet effect (for phospholipid titrations), and K_D represents the apparent equilibrium dissociation constant for lipid binding and corresponds to the inverse of the affinity constant. Note that the amount of protein added is low because it comes from the cell lysates of C1B-overexpressing HEK293 cells, and from the first lipid additions the [protein]<<[lipid]. Therefore, free concentrations of DAG can be assumed to be approximately the same as total lipid concentrations.

Differential scanning calorimetry

A high-sensitivity MicroCal VP scanning calorimeter (Northampton, MA) was used in these experiments. Scan rates were 60°/h. The low temperature mode was used. Samples (160 μL) contained 10 mg of phospholipids and the necessary amount of DAG to give a 5 mol % or a 15 mol % composition. The lipid mixtures were resuspended in 25 mM Hepes pH 7.4, and 0.5 mM EGTA. Multilayered vesicles were generated by vigorous mixing. A buffer profile was subtracted from the sample scans. Baselines were created by a cubic spline and subtracted.

RESULTS

The aim of this work was to study the binding affinity of C1 domains of PKCs for different DAGs and for different anionic phospholipids. C1B subdomains known to have an affinity for diacylglycerols were used, including those from PKCα, PKCγ, PKCδ, and PKCε.

Because these C1B domains lack suitable residues to induce fluorescence energy transfer between a membrane probe and Trp residues, a new FRET method was developed, in which the acceptor was phosphatidylethanolamine labeled with Oregon Green-488 (OG-PE) located in the membrane, and the donor was the C1 domain fused to ECFP (Fig. 1).

The influence of acidic phospholipids on the binding of C1B domain to membranes

Using this method, the binding of C1B domains to small unilamellar vesicles containing different lipid compositions was studied. Fig. 2 shows the binding of the C1Bγ domain to increasing concentrations of POPC/POPX/DOG vesicles, whereby POPX stands for POPS (Fig. 2 *A*), POPA (Fig. 2 *B*), or POPG (Fig. 2 *C*). In this assay, the effect of increasing the relative percentage of acidic phospholipid on C1B membrane binding was also studied. The data were analyzed and best fitted to the Hill model.

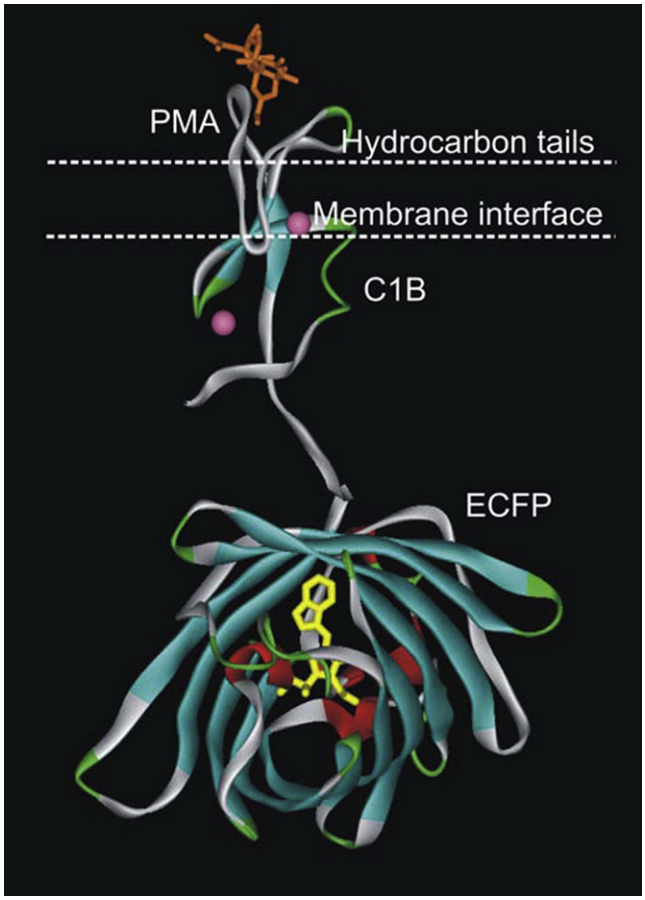

FIGURE 1 Structural model of the C1-CFP construction used to measure the interaction of C1 domains from PKCs to membranes by FRET. C1Bγ domain is depicted in the upper part (PDB code 1CVZ) anchored to the membrane through the polar part of a PMA molecule (phorbol 12-myristate-13-acetate). Zn^{2+} ions appear as spheres. The cyan fluorescent protein (CFP) (PDB code 1HUY) appears fused to the C1B domain. Side chains of the residues from CFP involved in fluorescence are shown.

In the case of C1Bγ, its membrane binding affinity increased when the mol % of acidic phospholipid was increased in the lipid vesicles. Of the three acidic phospholipids studied, POPA promoted the highest binding affinity (Fig. 2 *B*, Table 1), it being ~4-fold higher than the affinity observed in the presence of POPS or POPG (at 40 mol % phospholipid, the K_D was 7.5 μM for POPA compared with 29 μM for POPS and 30 μM for POPG).

C1Bδ (Fig. 3, Table 1) showed the highest binding affinity for membranes when POPS was present, although all the values were within the same order of magnitude (at 40 mol % of anionic phospholipids, the K_D was 11.5 μM for POPS compared with 19.5 μM for POPA and 37 μM for POPG).

In the case of C1Bε (Fig. 4, Table 1), the highest binding affinity corresponded to membranes incorporating POPG, followed by those with POPA, and finally those with

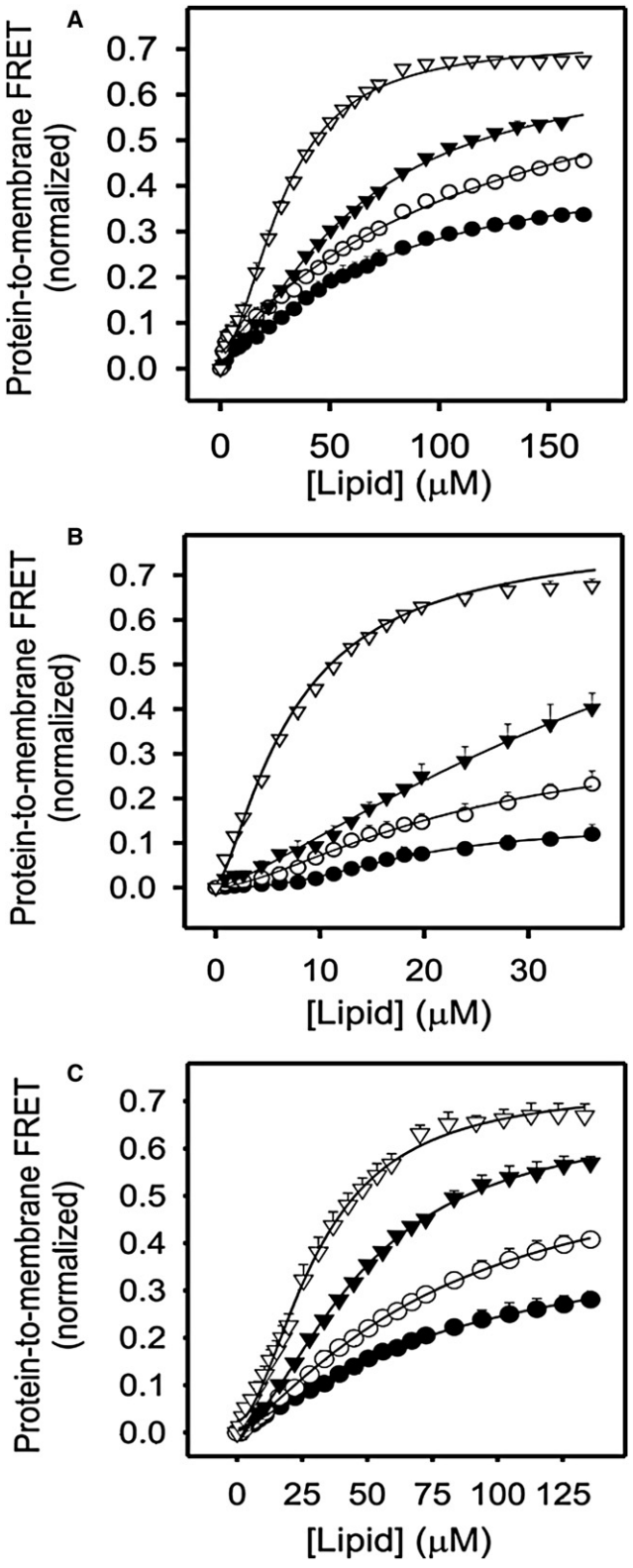

FIGURE 2 Binding of C1Bγ-CFP domains to lipid vesicles, which contained different types of anionic phospholipids. Vesicles contained POPC/POPX/OGPE in molar ratios 90:5:5 (●), 85:10:5 (○), 75:20:5 (▼), and 55:40:5 (∇). POPX was POPS (*A*), POPA (*B*), or POPG (*C*). Five mol % of DOG were present in all cases. Normalized FRET values are depicted versus total lipid concentration.

POPS, although all of them were in the same order of magnitude (at 40 mol % of the anionic phospholipids, the K_D was 0.96 μM for POPG μM, 1.7 for POPA, and 3.2 μM for POPS).

An interesting finding was the important role played by anionic phospholipids in the binding of C1B domains to the membranes. As the contents of the anionic phospholipids increased from 5 mol % to 40 mol %, the membrane binding affinity increased up to 20-fold. See, for example, the case of C1Bε with POPG (K_D 20 μM at 5 mol % and 0.96 at 40 mol %) or that of C1Bδ with POPA, when the increase was 47-fold (K_D of 920 μM at 5 mol % and 19.5 μM at 40 mol %). These results confirm that not only is diacylglycerol important for the binding of C1 domains to the membrane, but that anionic phospholipids are as well.

Another interesting result was that there were substantial differences in the binding affinity of the domains studied. The highest binding affinities were observed in C1Bε (Fig. 4) for the three anionic phospholipids, the increase being ~30-fold for 40 mol % of POPG (K_D values of 0.96 μM for C1Bε compared with 30 μM for C1Bγ and 37 μM for C1Bδ). Increases of up to 10-fold were also observed in the presence of POPA (K_D values of 1.7 μM for C1Bε compared with 7.5 μM for C1Bγ and 19.5 μM for C1Bδ) and up to ninefold when POPS was present in the membranes (K_D values of 3.2 μM for C1Bε compared with 29 μM for C1Bγ and 11.5 μM for C1Bδ).

On the other hand, when the results obtained in the presence of C1Bγ (Fig. 2) and C1Bδ (Fig. 3) were compared, a higher binding affinity at 40 mol % of the anionic phospholipids was observed for the C1Bγ domain when using POPA (K_D of 7.5 μM for C1Bγ versus 19.5 μM for C1Bδ) and POPG (K_D of 30 μM for C1Bγ compared with 37 μM for C1Bδ), but a higher binding affinity was observed for POPS in the case of C1Bδ isoenzyme (K_D of 11.5 μM for C1Bδ compared with 29 μM for C1Bγ). Note that binding affinities are within the same range for these two domains.

The effect of diacylglycerol fatty acid composition on membrane binding of C1B domains

The binding dependency of the C1B domains to lipid vesicles was also studied by increasing the concentrations of three different diacylglycerols, namely DOG, SAG, and DPG, at a fixed concentration of anionic phospholipid (Table 2). When C1Bγ was studied, binding affinities were observed to increase as the concentrations of either DOG or SAG were increased, this holding true for any of the three anionic phospholipids used. The binding affinities were in the same range in all these cases, and no clear specificity was observed for either one of these two diacylglycerols. However, in the presence of DOG, the binding affinities were lower (higher K_D values) than those obtained with the other DAGs for all three anionic phospholipids. In addition, no clear increase or decrease in binding affinities were

TABLE 1 Binding parameters of C1B-ECFP domains to phospholipid vesicles

		C1Bγ		C1Bδ		C1Bε	
	Mol (%)	ΔF max (%)	K_D (μM)	ΔF max (%)	K_D (μM)	ΔF max (%)	K_D (μM)
POPS	5	0.34 ± 0.05	130 ± 1.4	0.12 ± 0.02	40 ± 0.4	0.42 ± 0.01	17 ± 0.1
	10	0.45 ± 0.04	68 ± 1.2	0.2 ± 0.01	28 ± 0.2	0.43 ± 0.05	9 ± 0.08
	20	0.54 ± 0.04	58 ± 0.6	0.24 ± 0.02	22 ± 0.2	0.43 ± 0.02	10 ± 0.1
	40	0.68 ± 0.03	29 ± 1.3	0.30 ± 0.01	11.5 ± 0.2	0.44 ± 0.01	3.2 ± 0.2
POPA	5	0.12 ± 0.02	32 ± 0.2	0.11 ± 0.03	920 ± 0.4	0.1 ± 0.01	17 ± 0.02
	10	0.23 ± 0.02	22 ± 0.6	0.16 ± 0.02	30 ± 0.4	0.2 ± 0.03	17 ± 0.06
	20	0.4 ± 0.04	18 ± 0.8	0.21 ± 0.05	20 ± 0.8	0.33 ± 0.02	10 ± 0.04
	40	0.74 ± 0.02	7.5 ± 0.2	0.3 ± 0.01	19.5 ± 0.4	0.4 ± 0.01	1.7 ± 0.05
POPG	5	0.28 ± 0.01	78 ± 0.6	0.43 ± 0.01	64 ± 0.2	0.48 ± 0.01	20 ± 0.04
	10	0.4 ± 0.02	76 ± 0.4	0.43 ± 0.02	52 ± 0.2	0.48 ± 0.04	16 ± 0.08
	20	0.57 ± 0.01	46 ± 0.4	0.52 ± 0.01	48 ± 0.2	0.48 ± 0.02	11 ± 0.1
	40	0.71 ± 0.03	30 ± 0.2	0.53 ± 0.01	37 ± 0.7	0.52 ± 0.02	0.96 ± 0.04

Phospholipid vesicles contained POPC/DOG/POPX/OG-PE (X:5:X:2 mol %), whereas anionic and POPC phospholipids varied as indicated in the table. FRET data were fitted to a Hill equation as described in Materials and Methods. Mean values ± SE are given.

observed when the concentration of DPG was increased, probably as a result of changes in membrane organization, as will be discussed later.

The effects of increasing the diacylglycerol mol % in the membrane were studied as a function of the anionic phospholipids incorporated in the system (Table 2). We will discuss first the case of C1Bγ. Only modest differences were observed when DOG was the diacylglycerol used, whereas POPA showed slightly higher binding affinities with a less than twofold increase with respect to POPS and POPG (at 15 mol % of DOG, K_D was 0.33 for POPA compared with 0.46 for POPS and 0.6 μM for POPG). When SAG was the diacylglycerol studied, again the highest binding affinities were observed in the presence of POPA; and at 15 mol % of SAG, a four- to sixfold increase was found with respect to the other anionic phospholipids (K_D values of 0.23 for POPA versus 1.3 for POPS and 0.85 μM for POPG). Again, for 15 mol % DPG, a higher binding affinity was observed for POPA with a fivefold increase with respect to POPS (K_D values of 0.5 μM for POPA versus 2.5 for POPS and 1.6 for POPG).

In the case of C1Bδ (Table 2), binding affinities also increased as the concentration of either DOG or SAG was increased, with binding affinities in the same range for the three anionic phospholipids. Again, when DPG was used, the binding affinities were lower than for the other two diacylglycerols, and no increase was observed when the concentration of DPG was increased (Table 2).

When C1Bε was studied (Table 2), binding affinities were observed to increase as the concentration of either DOG or SAG was increased, for all three anionic phospholipids studied. Consistent with the results obtained for the other two domains, when DPG was added, lower binding affinities were observed, and either no increase in binding affinity or even a decrease in affinity was observed at increasing concentrations of this diacylglycerol. The possible role of membrane effects in this observation is discussed below.

We have described above that for C1Bε the highest binding affinity in the presence of 5 mol % of DOG was observed in the presence of POPG (Table 1). It can be seen in Table 2, however, that when the mol % of the DAGs in the membranes was increased up to 15 mol %, the binding affinity was higher in the presence of POPA and even POPS than in the presence of POPG. At 15 mol % of DAGs, the binding affinity was highest in the presence of POPA, with an ~4-fold increase with respect to POPG (K_D of 0.006 for POPA versus 0.025 μM for POPG) and 12-fold with respect to POPS (0.006 for POPA compared with 0.075 μM for POPS).

Similar observations can be made with respect to the three domains in the presence of 15 mol % SAG, with the highest binding affinity corresponding to POPA (K_D 0.005 μM), which was almost two orders of magnitude higher than with POPS (K_D of 0.16 μM) or POPG (K_D of 0.15 μM). However, no clear binding preference for a given phospholipid was observed in the presence of DPG.

Comparison of the C1Bγ and C1Bδ binding affinities (Table 2) showed that they were within the same range for the same pairs of anionic phospholipids and diacylglycerols. When the three domains were compared, binding affinities were always higher (lower K_D) for C1Bε, and this was true for all the combinations of anionic phospholipids and diacylglycerols studied. The highest binding affinities observed were always for this C1Bε domain, and at 15 mol % of each diacylglycerol, the highest binding affinity was observed for DOG in the presence of POPA (K_D 0.006 μM), and for SAG with POPA (K_D 0.005 μM). Even in the presence of DPG, higher binding affinities were observed for C1Bε, the K_D of C1Bγ and C1Bδ being within the same range.

DSC of the different membrane systems

To explain the lower binding levels observed in the presence of DPG, the physical state of membranes was examined using DSC. Fig. 5 depicts the thermograms obtained in the presence of the different lipid mixtures containing 5% or

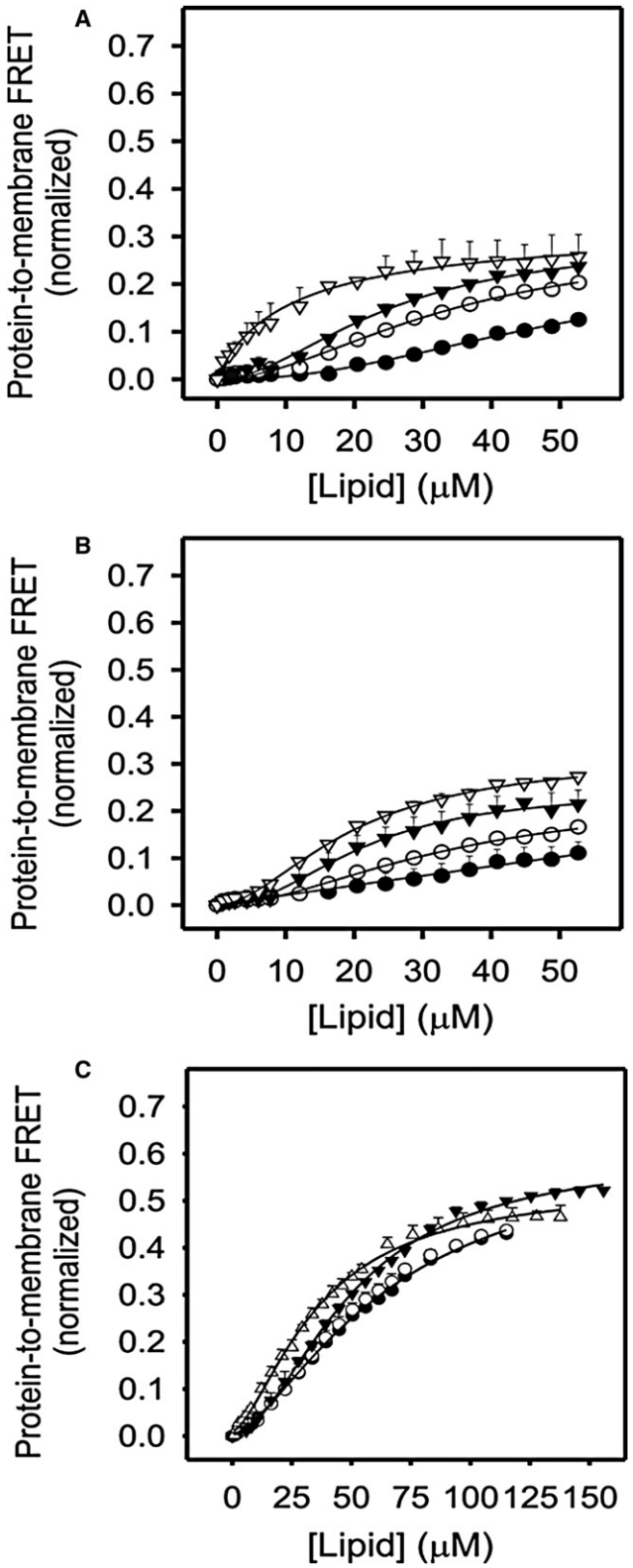

FIGURE 3 Binding of C1Bδ-CFP domains to lipid vesicles, which contained different types of anionic phospholipids. Vesicles contained POPC/POPX/OGPE in molar ratios 90:5:5 (●), 85:10:5 (○), 75:20:5 (▼), and 55:40:5 (∇). POPX was POPS (*A*), POPA (*B*), or POPG (*C*). Five mol % of DOG were present in all cases. Normalized FRET values are depicted versus total lipid concentration.

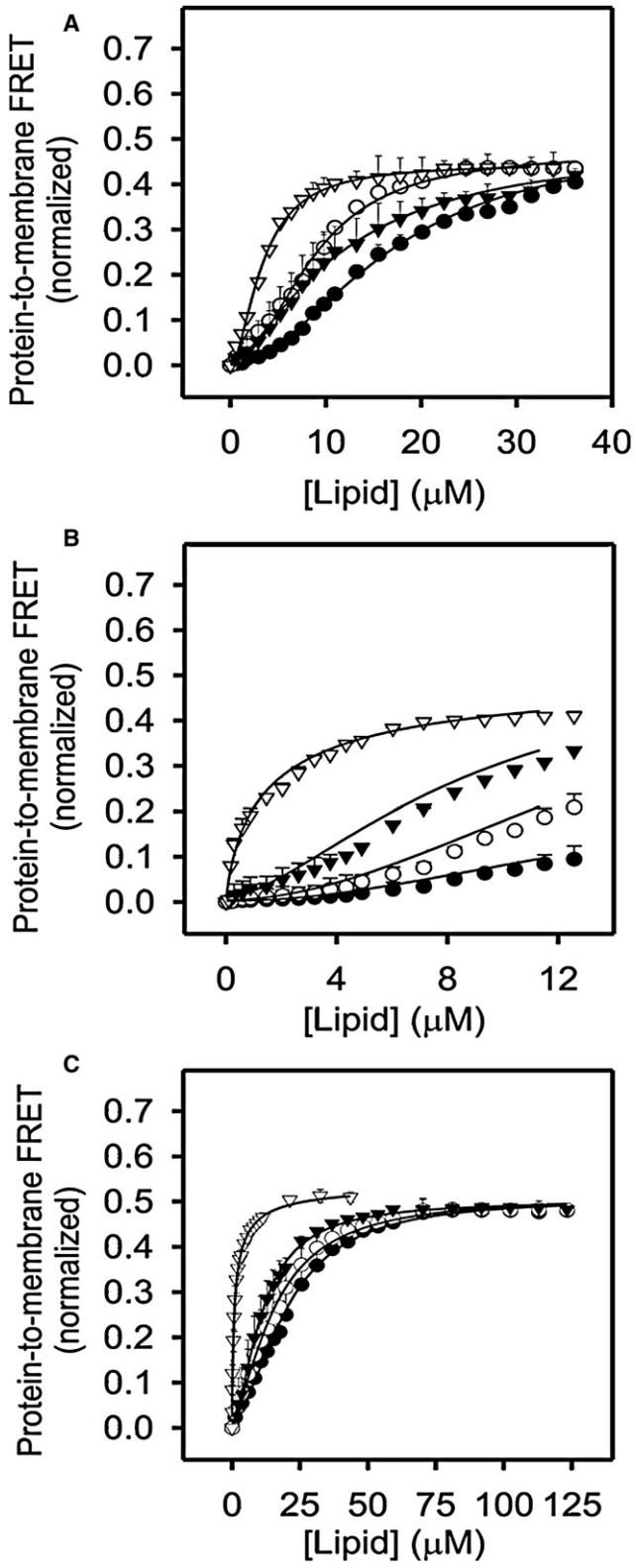

FIGURE 4 Binding of C1Bε-CFP domains to lipid vesicles, which contained different types of anionic phospholipids. Vesicles contained POPC/POPX/OGPE in molar ratios 90:5:5 (●), 85:10:5 (○), 75:20:5 (▼), and 55:40:5 (∇). POPX was POPS (*A*), POPA (*B*), or POPG (*C*). Five mol % of DOG were present in all cases. Normalized FRET values are depicted versus total lipid concentration.

TABLE 2 Binding parameters of C1B-ECFP domains to phospholipid vesicles

		C1Bγ	C1Bδ	C1Bε
	DOG (%)	K_D (μM)	K_D (μM)	K_D (μM)
POPS	5	1.4 ± 0.04	0.57 ± 0.04	0.16 ± 0.01
	10	1.0 ± 0.05	0.36 ± 0.02	0.055 ± 0.02
	15	0.46 ± 0.01	0.25 ± 0.005	0.075 ± 0.03
POPA	5	0.37 ± 0.01	1.0 ± 0.005	0.085 ± 0.005
	10	0.38 ± 0.1	0.43 ± 0.006	0.006 ± 0.001
	15	0.33 ± 0.005	0.35 ± 0.007	0.006 ± 0.001
POPG	5	1.5 ± 0.01	1.8 ± 0.03	0.048 ± 0.002
	10	1.0 ± 0.008	0.92 ± 0.02	0.035 ± 0.005
	15	0.6 ± 0.02	0.6 ± 0.01	0.025 ± 0.003
	SAG (%)			
POPS	5	1.9 ± 0.04	0.7 ± 0.01	0.5 ± 0.1
	10	1.4 ± 0.02	0.5 ± 0.006	0.35 ± 0.08
	15	1.3 ± 0.04	0.48 ± 0.01	0.16 ± 0.05
POPA	5	0.5 ± 0.008	1.25 ± 0.02	0.04 ± 0.001
	10	0.29 ± 0.01	0.75 ± 0.01	0.012 ± 0.001
	15	0.23 ± 0.01	0.4 ± 0.02	0.005 ± 0.0005
POPG	5	1.7 ± 0.01	1.6 ± 0.02	0.4 ± 0.01
	10	1.2 ± 0.01	1.0 ± 0.03	0.24 ± 0.008
	15	0.85 ± 0.03	0.66 ± 0.02	0.15 ± 0.01
	DPG (%)			
POPS	5	2.7 ± 0.07	1.0 ± 0.02	0.26 ± 0.02
	15	2.5 ± 0.06	1.0 ± 0.02	0.38 ± 0.01
POPA	5	0.9 ± 0.01	0.27 ± 0.02	0.28 ± 0.01
	15	0.5 ± 0.02	1.0 ± 0.02	0.5 ± 0.01
POPG	5	1.6 ± 0.03	1.9 ± 0.05	0.11 ± 0.02
	15	1.6 ± 0.02	2.3 ± 0.04	0.23 ± 0.01

Phospholipid vesicles contained 53 mol % POPC, X mol % of the indicated diacylglycerol, 2 mol % OG-PE, and 40 mol % of the indicated anionic phospholipid. The increase in DAG concentration was balanced by a decrease in the POPC concentration. FRET data were fitted to a Hill equation as described in Materials and Methods. Mean values ± SE are given.

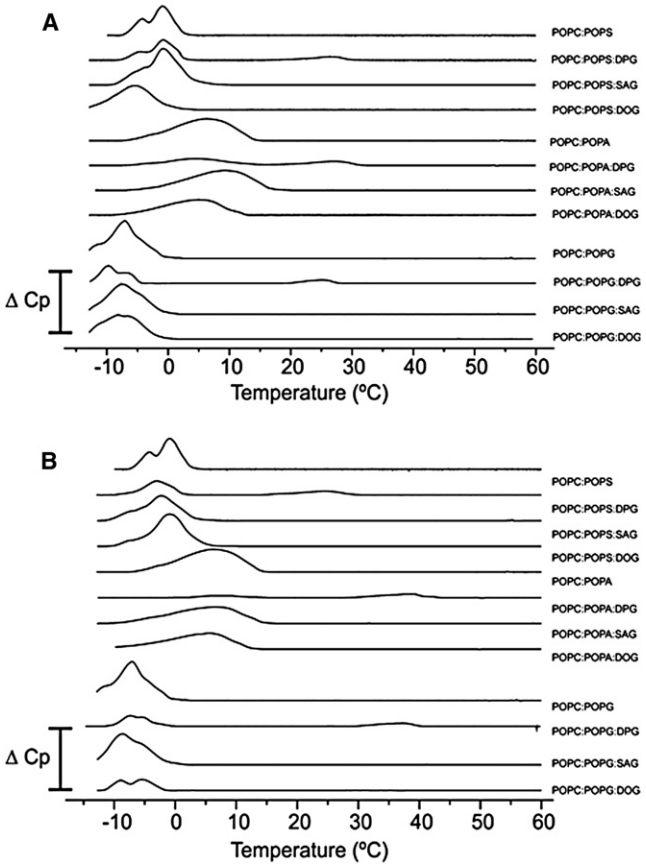

FIGURE 5 DSC thermograms of lipid vesicles containing POPC/POPX in a molar ratio 60:40, whereby POPX stands for an anionic phospholipid (POPS, POPA, or POPG as stated in each case). Five mol % (*A*) or 15 mol % (*B*) of DAG was present (DOG, SAG, or DPG, as stated in each case). The increment of Cp units (*Δ*) was 1000 cal/mol/°C. Mixtures used were: (*A*) POPC/POPS (60:40 mol/mol); POPC/POPS/DPG (55:40:5 mol/mol/mol); POPC/POPS/SAG (55:40:5 mol/mol/mol); POPC/POPS/DOG (55:40:5 mol/mol/mol); POPC/POPA (60:40 mol/mol); POPC/POPA/DPG (55:40:5 mol/mol/mol); POPC/POPA/SAG (55:40:5 mol/mol/mol); POPC/POPA/DOG (55:40:5 mol/mol/mol); POPC/POPG (60:40 mol/mol); POPC/POPG/DPG (55:40:5 mol/mol/mol); POPC/POPG/SAG (55:40:5 mol/mol/mol); POPC/POPG/DOG (55:40:5 mol/mol/mol); (*B*) POPC/POPS (60:40 mol/mol); POPC/POPS/DPG (45:40:15 mol/mol/mol); POPC/POPS/SAG (45:40:15 mol/mol/mol); POPC/POPS/DOG (45:40:15 mol/mol/mol); POPC/POPA (60:40 mol/mol); POPC/POPA/DPG (45:40:15 mol/mol/mol); POPC/POPA/SAG (45:40:15 mol/mol/mol); POPC/POPA/DOG (45:40:15 mol/mol/mol); POPC/POPG (60:40 mol/mol); POPC/POPG/DPG (45:40:15 mol/mol/mol); POPC/POPG/SAG (45:40:15 mol/mol/mol); POPC/POPG/DOG (45:40:15 mol/mol/mol).

15% of different DAGs. In Fig. 5 *A* it can be seen that in the presence of 5 mol % DAGs, phase transitions occurred at temperatures below 25°C, that is, the temperature at which the binding measurements were carried out, except in the three mixtures containing DPG. In POPC/POPS/DPG, a transition centered at 27°C was observed. In POPC/POPA/DPG, this was observed at 27.5°C. Finally, in POPC/POPG/DPG, a broad transition centered at 27°C can be observed.

In the samples containing 15 mol % DAG (Fig. 5 *B*), again the only samples with thermal transitions were POPC/POPS/DPG (at ~26°C), POPC/POPA/DPG (at ~38°C, and POPC/POPG/DPG (at ~37°C).

DISCUSSION

Structural studies have established that all C1 domains have a similar fold (24–29) based on a compact α/β structural unit, which tightly binds two zinc ions. This folding contains an unzipped β-sheet that forms a single ligand binding site for diacylglycerol or phorbol esters (Thr12, Gly23, and Leu21) in its top, surrounded by hydrophobic residues involved in membrane insertion (26). Membrane interaction is also facilitated by a ring of positive charges located around the middle of the domain that potentially interact with phosphatidylserine and other anionic lipids (30–32).

Conventional and novel PKCs contain two C1 subdomains, C1A and C1B; although the exact function of each of these domains and the reason for the existence of this double domain are not yet clear. Much research effort has focused on trying to understand the specific function of these subdomains, which we have reviewed in detail elsewhere (1).

Study of the C1A domains is hampered by the difficulty in obtaining them in soluble form from transfected cell cultures.

C1B, however, can be obtained in soluble form and in sufficient amounts to carry out studies like the one reported here. The objective of this study was to use the physiological activator DAGs and not the artificial phorbol esters. Therefore, of the classical isoenzymes, we have used C1Bγ, which is the only one that shows a certain binding affinity to DAGs (12). This same approach was used in this work to test the C1Bα, and the results have shown that the extent of binding is approximately one order of magnitude lower than for the other domains studied here, i.e., too low to permit accurate measurement (data not shown). We have also used the C1Bδ and C1Bε (from the novel PKCs), which were produced in soluble form and have good binding affinity to membranes containing DAGs.

From the binding affinity results (the reciprocal of the apparent K_D values) for the three domains, it can be concluded that C1Bε has the highest binding affinity. However, C1Bγ and C1Bδ showed different binding affinities, depending on the phospholipids and the DAG used for the assays, although their membrane binding affinities were generally within the same order of magnitude, at approximately a 3- to 10-fold lower binding affinity than C1Bε.

A comparison of the binding observed for the three DAGs led to the conclusion that they show similar membrane binding affinities in the presence of DOG and SAG, and generally lower values in the presence of DPG. DSC experiments showed that this is a consequence of the DPG-containing systems not being in a fluid state at the measuring temperature of 25°C. This demonstrates the importance of the fluidity of the membrane to modulate the binding of the C1 domain. The fluidity of the membrane has been shown to be important in determining the activation energy of PKCα (33); in certain circumstances, at least, membrane fluidity is important for determining the functioning of the enzyme.

Previous studies showed that 1,2-DAG-*sn*-isomers have a similar capacity to activate PKCα, regardless of the chain length or the saturation/unsaturation level of their fatty acyl chains, as long as the membrane is in a fluid state (18).

In another study, PKCα activity was measured in the presence of 200 μM Ca^{2+} and 5 mM Mg^{2+}; and, with POPS present in the membranes, the enzyme is able to bind maximally, even in the total absence of diacylglycerol, probably through the C2 domain, which is known to interact with Ca^{2+}/phosphatidylserine and PIP_2 (5). However, the binding was not accompanied by maximal activation, which was only achieved in the presence of a sufficiently high concentration of DAG, suggesting that the binding of the C1 domain is involved (34). Moreover, it was also found that when PKCα was mutated in residues essential for the binding of Ca^{2+} to the C2 domain, thus inducing binding through the C1 domain, POG was much more efficient than DPG (34), and the binding was closely correlated with the activating capacity. In these experiments in which POG was a better activator than DPG, the membrane system was in a fluid state in the presence of POG and in a nonfluid state in the presence of DPG. An explanation could be that it is more difficult for the enzyme to insert its C1 domain in a rigid than in a fluid membrane because unsaturated DAGs produce an increase in the hydrocarbon volume and also an increase in the headgroup spacing, which may facilitate the insertion of the enzyme in the membrane (35). However previous studies have claimed that membrane fluidity is not important when cholesterol is used to modulate this fluidity (35), although cholesterol will only give rise to an intermediate state of fluidity. It should also be stated that cholesterol will modulate fluidity but will not produce the type of immiscibility that appears when the concentration of diacylglycerol is changed. In this work, we observe immiscibility of this type when DPG is present, so that at the temperature of the assays (25°C) all the added DPG is concentrated in a gel phase, forming a DPG/phospholipid complex phase separate from the excess phospholipid (34). It can therefore be concluded that the lateral membrane organization is not suitable for C1 domain binding, although it was not seen to affect C2 domain binding (34). This can easily be explained if the C1 domain needs to penetrate the membrane, whereas the C2 domain only needs to bind superficially.

The C1 domain binds to the membrane by interacting with DAGs (or phorbol esters), but electrostatic interactions provided by anionic phospholipids also make an important contribution. These interactions have been found to be important for the binding of many extrinsic proteins, such as cytochrome *c* (36–39), myelin basic protein (40), phospholipases (41), K-Ras (42), charybdotoxin (43), cardiotoxin (44), and Aβ peptides (45,46).

In regard to anionic phospholipids, it has been shown in this work that when their concentration in the membrane was increased from 5 mol % to 40 mol %, increases in membrane binding affinity of up to 40-fold were observed. Whereas at 5 mol % of DOG each domain exhibited maximum binding affinity with a different anionic phospholipid (C1Bγ with POPA, C1Bδ with POPS, and C1Bε with POPG), at higher concentration (15 mol %) of DOG or SAG, both C1Bγ and C1Bε showed a veryclear preference for POPA. Especially noticeable was the case of C1Bε, which showed an increase of up to more than one order of magnitude with respect to POPS and POPG.

PA is produced in membranes by enzymatic activity through two routes, the action of phospholipase D and that of diacylglycerol kinases. PA constitutes ~1%–5% of total cellular lipids (47,48) and is found in plasma and inner membranes like the Golgi network (49–51). Nevertheless, it should be taken into account that in the immediate vicinity of the points where PA is being synthesized, local concentrations will be considerably higher. It is interesting that PA has been found to be important in the activation of PKCε (7,52), and, although it has been shown that PA binds to the C2 domain of PKCε (7,52–54), the findings described here suggest that lipid microdomains containing PA and

DOG/SAG also play an important role in inducing the C1 domain anchorage in membranes.

Taken together, our results show that 1), C1B domains have different binding affinities for membranes; 2), membrane fluidity is important for activity; 3), the dependency on anionic phospholipids is complex; and 4), PS is not the only anionic phospholipid capable of facilitating the binding of C1B domains.

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