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# Calcium-dependent Membrane Penetration Is a Hallmark of the C2 Domain of Cytosolic Phospholipase A2 Whereas the C2A Domain of Synaptotagmin Binds Membranes Electrostatically*

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**C2 domains have been identified in a wide range of intracellular proteins, including lipid modifying enzymes, protein kinases, GTPases, and proteins involved in membrane trafficking. Many C2 domains bind membranes in a calcium-dependent manner. The first C2 domain from synaptotagmin I (SytIC2A) and the C2 domain from cytosolic phospholipase A2 (cPLA2C2) are among the best characterized C2 domains in terms of their structures and calcium binding. Here we demonstrate that the protein-lipid interaction is dramatically different for these two domains. Photolabeling with 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine ([$^{125}$I]TID) in the presence of phospholipid vesicles indicates that cPLA2C2 penetrates into the hydrophobic region of the membrane. Hydrophobic surfaces on cPLA2C2 are exposed even in the absence of calcium, but only in its presence does the domain penetrate into the nonpolar core of the membrane. The interaction of SytIC2A with phospholipid membranes is primarily electrostatic with binding being abolished in 500 mM NaCl. Because soluble phospholipid head group analogues do not compete with binding of either SytIC2A or cPLA2C2 to vesicles, it is likely that membrane binding by these domains involves multiple interactions.**

C2 domains, named for homology to the second conserved domain in protein kinase C, have been identified in a variety of proteins involved in membrane interactions (for reviews, see Refs. 1 and 2). Calcium-dependent phospholipid binding has been observed for many of these domains, and it has been suggested that they are responsible for calcium-dependent protein translocation to membranes. The three-dimensional structures have been reported for the C2 domains from synaptotagmin I (SytIC2A)[1] (3, 4), phosphoinositide-specific phospholipase Cδ1 (5–7), and 85 kDa cytosolic phospholipase A2 (cPLA2C2) (8). Despite great sequence diversity, the domains are structurally quite similar and consist of an eight-stranded anti-parallel β-sandwich. The most notable structural differences occur in three loops at the calcium-binding end of the domain. The domains bind multiple calcium ions in adjacent sites using the side chains of several aspartate or asparagine residues at the bases of these three loops. Phosphoinositide-specific phospholipase Cδ1 binds three calcium ions in this region, whereas SytIC2A and cPLA2C2 bind two (4, 6, 8–10). In contrast to calcium binding, the nature of membrane binding by C2 domains is not fully understood. Studies of lipid specificity indicate that SytIC2A binds acidic phospholipids, particularly phosphatidylserine (PS) (11), whereas cPLA2C2 shows a preference for binding zwitterionic phosphatidylcholine (PC) (12). We have examined in parallel the nature of the membrane interactions for SytIC2A and cPLA2C2. Our results suggest that these two types of C2 domains have very different membrane binding characteristics: calcium-dependent phospholipid binding by cPLA2C2 is accompanied by penetration into the hydrophobic core of membranes, whereas electrostatic interactions with membranes predominate for SytIC2A.

## EXPERIMENTAL PROCEDURES

*Expression and Purification of cPLA2 and Synaptotagmin C2 Domains*—Expression vectors encoding the first C2 domain from rat synaptotagmin I (residues 140–267) fused to glutathione *S*-transferase and the C2 domain from 85-kDa human cytosolic phospholipase A2 (residues 17–141) fused to a hexahistidine tag are described elsewhere (8, 11). After expression and isolation, recombinant proteins were treated with thrombin to remove the affinity tags (3, 8). The resulting SytIC2A was used directly in all described experiments whereas cPLA2C2 was first refolded (8) and then dialyzed against 150 mM NaCl, 2 mM $Na_2EDTA$, 20 mM Tris-HCl, pH 7.5 (buffer A). Protein concentration was estimated using the BCA assay kit from Pierce.

*Phase Separation of Proteins In Triton X-114*—Phase separation of proteins in Triton X-114 was performed essentially as described previously (13). An aliquot of 55 μl of 10% Triton X-114 (Sigma) was added to 500 μl of buffer A containing 15 μg of C2 domain in the presence or absence of 2.2 mM $CaCl_2$. The reaction mixture was incubated on ice for 10 min, mixed, warmed at 37 °C for 5 min, and then centrifuged at 5000 × *g* for 2 min at 25 °C. The upper aqueous phase was withdrawn and mixed with 50 μl of 10% Triton X-114. After incubation and centrifugation as described above, the lower detergent phases from the two extractions were combined, and buffer A was added to bring the volume to 450 μl. To 450 μl aliquots of the detergent phase and the aqueous phase, 1 ml of a 1:1 chloroform:methanol mixture was added. After mixing and centrifugation at 5000 × *g* for 2 min at 25 °C, the protein pellet was recovered from the interphase, washed with 1 ml of methanol, and dissolved in 100 μl of SDS sample buffer. Aliquots (20 μl) were analyzed by SDS-PAGE on 14% polyacrylamide gels.

*Protein Binding to Large Multilamellar Vesicles*—Brain phospholipids from Avanti Polar Lipids Co. were used for vesicle preparation. A sample of 10 mg of pure PC or a PS:PE:PC mixture (molar ratio 35:50:15) in chloroform were dried under a stream of nitrogen in a glass vial and resuspended in 10 ml of buffer A by vigorous vortexing. This procedure results in formation of large multilamellar vesicles (LMVs) that can be sedimented in a microcentrifuge by centrifugation at 11,600 × *g* for 15 min at 4 °C. An aliquot of 5 μg of recombinant C2 domain in 300 μl of buffer A was mixed with 100 μl of LMVs either in the presence or absence of 2.2 mM $CaCl_2$. After 5 min of incubation at 25 °C, the mixture was centrifuged. The supernatant was carefully

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[1] The abbreviations used are: SytIC2A, C2A domain of synaptotagmin I; cPLA2, cytosolic phospholipase A2; [$^{125}$I]TID, 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine; PC, L-α-phosphatidylcholine; PS, L-α-phosphatidylserine; PE, L-α-phosphatidylethanolamine; LMVs, large multilamellar vesicles; bis-ANS, 4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; cPLA2C2, C2 domain of cytosolic phospholipase A2; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C.

withdrawn by aspirating with a long gel-loading tip, and pellet was dissolved in 100 $\mu$l of SDS sample buffer. A 40-$\mu$l sample was analyzed by SDS-PAGE on 12 or 14% polyacrylamide gels.

*Radiolabeling of Proteins with the Hydrophobic Probe [$^{125}$I]TID*—A 2-$\mu$g sample of recombinant protein was incubated with or without 50 $\mu$g of vesicles in 150 $\mu$l of buffer A for 10 min at 25 °C and then mixed with 1 $\mu$l of [$^{125}$I]TID (9.25MBq in 65 $\mu$l, Amersham Pharmacia Biotech). After a further 10-min incubation to allow the hydrophobic probe to partition into membranes, the sample was transferred into a quartz cuvette and irradiated for 2 min with a 100 watt, 360 nm UV lamp. The reaction mixture was added to 50 $\mu$l of 4× SDS sample buffer, and 40 $\mu$l of the sample was analyzed by SDS-PAGE on a 14% polyacrylamide gel. The gel was dried and placed on X-OMAT AR film (Kodak) for 48 h at −70 °C with an intensifying screen. The radioactive bands were excised and counted in a Wallac $\gamma$-counter (1261 Multigamma).

*Fluorescence Study with Bis-ANS*—A stock solution of 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) (Molecular Probes) was prepared by dissolving 10 mg in 5 ml of methanol (final concentration of 3 mM). Bis-ANS stock was added (final concentration of 1 $\mu$M) to a 1-ml solution of 0.5 $\mu$M C2 domain in buffer A either in the presence or absence of 2.2 mM $CaCl_2$. The solution was incubated for 5 min at 25 °C and then placed in a cuvette in Fluoromax-2 fluorimeter (Jobin Yvon-Spex, I.S.A. Inc.), and the 500-nm emission was measured with an excitation wavelength of 399 nm. The fluorescence of bis-ANS bound to protein was calculated by subtracting a blank prepared identically but without protein.

## RESULTS

*Hydrophobicity of the C2 Domains of cPLA2 and Synaptotagmin I*—To determine whether calcium has an effect on the overall polarity of the C2 domains, we examined the partitioning of the proteins between the aqueous and detergent phases using Triton X-114. A solution of Triton X-114 is homogeneous at temperatures below 20 °C. Above this temperature, the solution separates into an aqueous phase and a detergent phase. It has been shown that when hydrophobic portions of a protein are exposed, the protein partitions into the detergent phase (12). As illustrated in Fig. 1*A*, both SytIC2A and cPLA2C2 partition into the aqueous phase in the absence of calcium. Remarkably, in the presence of calcium, cPLA2C2 partitions into the detergent phase, whereas distribution of SytIC2A is unaffected by calcium. This suggests that cPLA2C2 becomes much less polar upon calcium binding.

*Labeling of the C2 Domains with a Membrane-sequestered Photolabeling Reagent*—To determine the nature of the interaction between bilayer membranes and these prototypic C2 domains, protein binding to phospholipid vesicles was first tested by co-sedimentation. In agreement with previous studies (10, 11), SytIC2A binds PS-containing vesicles but not vesicles made of PC only, whereas the C2 domain from cPLA2 preferentially binds PC-rich vesicles (Fig. 1*B*). We carried out photolabeling with a reagent that has a strong tendency to partition into the hydrophobic core of lipid bilayers. The proteins were incubated with lipid vesicles in the presence of the hydrophobic photolabeling reagent 3-(trifluoromethyl)-3-(*m*-[$^{125}$I]iodophenyl)diazirine ([$^{125}$I]TID) (14). When PC vesicles are incubated with cPLA2C2 in the presence of [$^{125}$I]TID, there is a calcium-dependent photo-labeling of the protein (Fig. 1*C*). In contrast, SytIC2A shows only trace labeling in the presence of calcium despite its strong binding to PS:PE:PC vesicles.

It has been demonstrated by NMR studies that calcium binding to SytIC2A does not cause a gross conformational change (4, 15). A separate work on cPLA2C2 indicated that small changes in the domain conformation occur upon calcium binding (10). Because cPLA2C2 exhibited strong hydrophobic properties in a calcium-dependent manner in our experiments, we looked at whether calcium would trigger exposure of hydrophobic surfaces in cPLA2C2. In a number of studies, [$^{125}$I]TID has been used in the absence of lipid membranes as a tool to determine changes in exposure of hydrophobic surfaces (14). Fig. 2*A* shows that there is no calcium-dependent increase in labeling by [$^{125}$I]TID in the absence of lipids, suggesting no changes in exposure of hydrophobic surfaces upon calcium

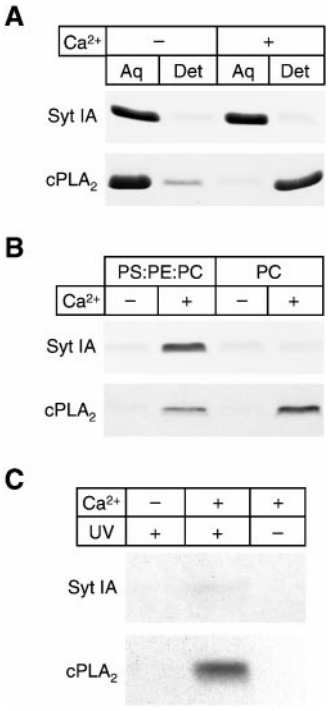


FIG. 1. **Hydrophobic properties of cPLA2C2 and SytIC2A.** *A,* Triton X-114 extraction of cPLA2C2 and SytIC2A. Protein in the absence or presence of 0.2 mM free $CaCl_2$ was incubated with Triton X-114 as described under "Experimental Procedures." The aqueous (*Aq*) and detergent (*Det*) phases were adjusted to the same volume, and equal aliquotes were subjected to SDS-PAGE followed by Coomassie Blue staining. *B,* binding of cPLA2C2 and SytIC2A to phospholipid vesicles. Vesicles were prepared as described under "Experimental Procedures" with either a mixture of PS, PE, and PC (35:50:15 mol fraction) or just PC. Proteins were incubated with vesicles in the presence or absence of 0.2 mM free $CaCl_2$ for 5 min, and the vesicles were then sedimented by centrifugation. Protein bound to the sedimented vesicles was analyzed by SDS-PAGE followed by Coomassie Blue staining. *C,* photolabeling of protein bound to vesicles. SytIC2A (0.5 $\mu$g) was incubated with PS:PE:PC vesicles and cPLA2C2 (0.5 $\mu$g) with PC vesicles in the presence of [$^{125}$I]TID. Samples were irradiated with UV light to cross-link [$^{125}$I]TID to the protein and analyzed by SDS-PAGE and autoradiography as described under "Experimental Procedures." The last *lane* represents a control for the photochemical reaction where calcium-containing samples were not subjected to UV light.

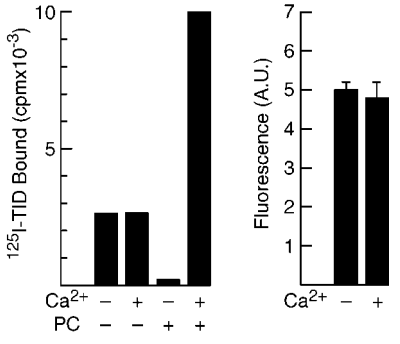


FIG. 2. **Hydrophobic labeling displayed by cPLA2C2.** *Left panel,* [$^{125}$I]TID photolabeling of cPLA2C2 was performed as in Fig. 1*C* but either in the absence or presence of vesicles. Radioactive bands were excised and counted in a Wallac $\gamma$-counter. *Right panel,* binding of the hydrophobic probe bis-ANS to cPLA2C2. Bis-ANS (1 $\mu$M) was added to the solution of 0.5 $\mu$M cPLA2C2 in the absence or presence of 0.2 mM free calcium. Samples were excited at 399 nm, and fluorescent emission was measured at 500 nm. The fluorescence of bis-ANS bound to protein is plotted in arbitrary units (*A.U.*). *Bars* indicate the mean ± S.E. from triplicate determinations.

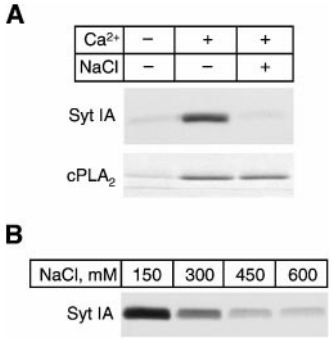


FIG. 3. **The effect of ionic strength on protein binding to vesicles.** *A,* C2 domains were incubated with PS:PE:PC vesicles as in Fig. 1*B* except that the incubation solution contained 0.5 M (*last lane*) instead of 0.15 M NaCl. *B,* the effect of increasing NaCl concentration on SytIC2A vesicle binding.

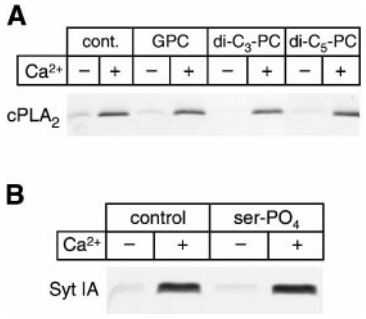


FIG. 4. **Competition of vesicle binding with soluble head group analogues.** Proteins were incubated with vesicles as in Fig. 1*B* except that the incubation solution contained, in addition, head group analogues. *A,* the soluble PC head group analogues L-α-glycerophosphorylcholine (*GPC*) (Sigma), 1,2-dipropinoyl-*sn*-glycero-3-phosphocholine (*di$C_3$PC*) (Avanti), and 1,2-divaleroyl-*sn*-glycero-3-phosphocholine (*di$C_5$*PC) (Avanti) used at 20 mM, have no effect on cPLA2C2 binding to PC vesicles. *B, O*-phospho-L-serine (Sigma) at 20 mM has no influence on PC:PE:PS vesicle binding by SytIC2A.

binding. As also seen in Fig. 2*A*, the [$^{125}$I]TID photolabeling in the absence of calcium is more pronounced without vesicles than in their presence because [$^{125}$I]TID partitions into vesicular membranes making it not available for protein labeling. Another useful tool to measure conformational changes in proteins is the hydrophobic probe bis-ANS which strongly increases its fluorescence upon binding to hydrophobic surfaces. Consistent with the [$^{125}$I]TID photolabeling in the absence of lipids, there is no calcium-induced increase in bis-ANS binding to cPLA2C2 (Fig. 2*B*).

*The Effect of Ionic Strength on Vesicle Binding*—In both Triton X-114 and [$^{125}$I]TID labeling experiments SytIC2A does not display hydrophobic properties. Two observations suggest that, for SytIC2A, electrostatic forces may predominate in membrane binding. First, SytIC2A binds strictly negatively charged phospholipids. Second, the calcium-binding site in the SytIC2A structure is surrounded by a cluster of positively charged residues, and it was proposed that the calcium-binding part of the domain may serve as an electrostatic switch (15). Thus, we sought to find out whether high salt would prevent either of the two C2 domains from binding to vesicles. As Fig. 3*A* shows, 0.5 M NaCl has a profound inhibitory effect on binding of SytIC2A to PS:PE:PC vesicles, whereas cPLA2C2 binding to the same vesicles is unaffected. Titration of binding with NaCl shows that salt significantly inhibits binding of SytIC2A at concentrations as low as 0.3 M (Fig. 3*B*).

*The Interaction with Phospholipid Vesicles Is Not Strictly a Property of the Lipid Head Groups*—To determine whether the lipid presentation in the lipid bilayer is important for membrane binding by C2 domains, we sought to compete vesicle binding with soluble phospholipids or their headgroup analogues. As shown in Fig. 4*A*, neither glycerophosphorylcholine nor soluble short acyl-chain PCs at a concentration of 20 mM (20 times the bulk vesicular lipid concentration) were able to compete with cPLA2C2 binding to vesicles. Phosphoserine, a soluble analogue of the charged part of the PS headgroup, was not able to prevent SytIC2A/vesicle binding (Fig. 4*B*).

## DISCUSSION

The three-dimensional structure of SytIC2A suggested that clusters of basic residues around the calcium-binding site were responsible for SytIC2A binding acidic phospholipids (3), whereas clusters of hydrophobic residues present in cPLA2C2 might be important for neutral lipid specificity (8). Thus we sought to find out whether these differences would be reflected in the hydrophobic properties of these domains. Our results for Triton X-114 partitioning show that cPLA2C2, unlike SytIC2A, acquires an overall nonpolar character in the presence of calcium. This acquisition of a nonpolar character for cPLA2C2 is not the result of a large conformational change that exposes a hydrophobic surface in a calcium-dependent manner because both bis-ANS binding and [$^{125}$I]TID labeling in the absence of vesicles are unaffected by calcium. A previous observation that high salt concentration (*e.g.* 1.5 M $Na_2SO_4$) promotes binding of cPLA2C2 to vesicles even in the absence of calcium (12) suggests the presence of constitutively exposed hydrophobic surfaces in cPLA2C2. A possible role of the calcium binding is to neutralize the patch of acidic calcium-binding residues that are seen in the crystal structure. This would thereby decrease the polarity of cPLA2C2, enabling a hydrophobic part of the domain to penetrate the membrane. The third calcium-binding loop that has three exposed hydrophobic residues ($^{96}$YVM$^{98}$) might be a plausible candidate for penetration into the hydrophobic core of the lipid bilayer (8). Because SytIC2A has a single exposed phenylalanine residue in the middle of this loop ($^{233}$R*F*SK$^{236}$), trace labeling of SytIC2A in the presence of calcium might be because of a much reduced degree of membrane penetration. Site-directed mutagenesis shall provide us with a direct answer as to which residues penetrate into the hydrophobic layer of membranes.

It has been demonstrated that cPLA2 requires the C2 domain for calcium-dependent translocation to internal membranes where the enzyme catalyzes the release of free arachidonic acid (16, 17). The precise calcium-dependent function for the C2 domains of synaptotagmin is not known. SytIC2A has been shown to bind *in vitro* both proteins and membranes as a function of calcium (18, 19). It has been suggested that calcium-triggered electrostatic changes in SytIC2A (15) are responsible for binding syntaxin, a presynaptic protein with a function in neurotransmitter release. Our present results indicate that electrostatic interactions also play an important role in membrane binding by SytIC2A. Similarly, loss of membrane binding by PKCβII at increased ionic strength suggests that the C2 domain of PKC binds PS-containing membranes primarily electrostatically (20). The calcium-dependent interaction of the C2 domain of PKCβII with phospholipids does not seem to be simply because of the change in the balance of negative and positive charges at the calcium-binding site because mutations that introduced positive charges at this site did not lead to calcium-independent membrane binding (21). Calcium may play an additional role of directly bridging C2 domains and the phospholipids as it does for annexin V (22). Our competition study with headgroup analogs suggests that the C2 domains binding requires a certain display of phospholipid headgroups on the membrane and occurs via multiple interactions. In addition, cPLA2C2 may also require the intact hydrophobic core

of the membrane.

In summary, using several complementary approaches, we have demonstrated that the mode of calcium-dependent membrane interaction may vary for different C2 domains. Our data suggest a model for cPLA2C2 binding to membranes with constitutively exposed hydrophobic surfaces that partially penetrate into the nonpolar regions of lipid membranes whereas SytIC2A has a predominantly electrostatic interaction with membrane surfaces. The C2 domains described here may serve as valuable prototypes for understanding calcium-dependent membrane binding by these modules in a broad range of proteins.

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