Membrane domain formation by calcium-dependent, lipid-binding proteins: insights from the C2 motif

Anne K. Hinderliter a,b, Paulo F.F. Almeida c, Rodney L. Biltonen a,b, Carl E. Creutz a,⁎

a Department of Pharmacology, University of Virginia Health Science Center, Jordan Hall 5036, Box 448, Charlottesville, VA 22908, USA
b Department of Biochemistry and Molecular Genetics, University of Virginia Health Science Center, Charlottesville, VA 22908, USA
c Department Quimica, Universidade de Coimbra, 3049 Coimbra Codex, Portugal

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Abstract

We propose a novel role in cellular function for some membrane-binding proteins and, specifically, the C2 motif. The C2 motif binds phospholipid in a manner that is modulated by Ca²⁺ and is thought to confer membrane-binding ability on a wide variety of proteins, primarily proteins involved in signal transduction and membrane trafficking events. We hypothesize that in the absence of Ca²⁺ the C2 motif couples the free energy of binding to a bilayer membrane comprised of zwitterionic and negatively charged lipids to the formation of a domain enriched in the negative lipids. This in turn leads to the dynamic clustering of bound homologous or heterologous proteins incorporating the C2 motif, or other acidic lipid-binding motifs. In the presence of Ca²⁺, the protein clusters may be further stabilized. In support of this hypothesis we present evidence for membrane domain formation by the first C2 domain of synaptotagmin in the absence of Ca²⁺. Fluid state phospholipid mixtures incorporating a pyrene-labeled phospholipid probe exhibited a change in pyrene excimer/monomer fluorescence ratio consistent with domain formation upon binding the C2 domain. In addition, we present the results of simulations of the interaction of the C2 domain with the membrane that indicate that protein clusters and lipid domains form in concert. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The C2 motif is a structural motif lacking enzymatic function found in a wide variety of proteins implicated in eukaryotic signal transduction and cellular membrane trafficking processes. The C2 motif has a highly conserved structure, an eight-stranded, antiparallel β sandwich comprised of four strands superimposed upon each other [1,2]. The Ca²⁺-binding site is nestled in the short linker loops connecting the β-strands [1]. A string of basic residues found on the edges of the β sandwich are a feature common to most C2 motifs. This strand is suggested to promote binding to acidic phospholipids and does not lie in the vicinity of the Ca²⁺-binding loops. An additional or alternative membrane-binding site found adjacent to the Ca²⁺-binding loops may partially penetrate the membrane [3–5]. This putative lipid-binding site is dominated by hydrophobic and anionic residues in the C2 motif of cytosolic phospholipase A₂ (cPLA₂),...
a zwitterionic lipid-specific C2 motif, as opposed to the cationic residues that dominate this region in most other C2 motifs. These variations suggest a means to regulate the phospholipid selectivity of proteins which incorporate the C2 motif.

Currently, the C2 motif is viewed as a Ca\(^{2+}\)-sensitive trigger that regulates membrane localization of proteins in which it is found, a trigger which is primed by the postulated cooperative binding of multiple calcium ions to the C2 domain. The two models commonly invoked amongst a plethora of variations to explain the ability of the C2 domain to be a Ca\(^{2+}\)- and phospholipid-binding motif are that the C2 domain simultaneously binds to Ca\(^{2+}\) and phospholipid and exists as a ternary complex, or that the cooperative binding of Ca\(^{2+}\) provides the impetus for the protein to undergo a conformation change exposing hydrophobic residues for membrane binding (for a review see [6]). Both models are consistent with the decrease in the apparent dissociation constant of the Ca\(^{2+}\)-C2 motif complex in the presence of phospholipid vesicles compared to that obtained in the absence of lipids.

There is very little evidence for a change in structure of the C2 motif upon Ca\(^{2+}\)-binding. The binding of a single calcium ion did not disrupt the crystal structure of the first C2 domain of synaptotagmin I [1]. The destruction of the protein crystals upon soaking them in very high Ca\(^{2+}\) concentration solutions has been the basis for a proposed conformational change in the C2 motif [6]. However, this may simply represent the disruption of lattice contacts in the crystal with the added salt. A Ca\(^{2+}\)-induced conformational change of the C2 domain in the presence and absence of lipid has also served as the explanation for the observed decrease in protease sensitivity of synaptotagmin upon Ca\(^{2+}\)-induced binding to membrane [7]. However, binding of ligand increases the stability of proteins; proteins do not become less stable with ligation. Indeed, the C2 motifs from PKC-\(\beta\)II, synaptotagmin and cPLA\(_2\) are more stable to thermal denaturation when bound to Ca\(^{2+}\) [3,8]. The increased stability of the protein upon binding of additional ligand may lead to the increased resistance to partial proteolysis. Thus, a major conformational change in the C2 motif upon binding Ca\(^{2+}\), while an interesting possibility, is not well supported.

We suggest that the energy available upon membrane binding, rather than simply leading to a major change in protein conformation, is coupled to a change in membrane organization, specifically lipid demixing and formation of ‘domains’ in the plane of the membrane. The studies with the C2 motif of synaptotagmin and model lipid systems presented here are consistent with this hypothesis.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) were from Avanti Polar Lipids (Birmingham, AL). The pyrene-acyl chain-labeled phospholipid, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol, ammonium salt (Pyr-PG) was from Molecular Probes (Eugene, OR). All were greater than 99% pure as determined by chromatographing 0.1 mg on Adsorbosil-Plus thin-layer chromatography (TLC) plates (Alltech Associates, Deerfield, IL) using the following solvent systems: for acidic lipids (chloroform/methanol/acetic acid/water, 50:30:8:4 v/v/v/v) and for neutral lipids (chloroform/methanol/water, 65:25:4 v/v/v). Hydrated lipid mixtures were periodically extracted for analysis by TLC after fluorescence experiments and lipid breakdown did not exceed 1–5%. Ethylene glycol-\(O\cdot O\cdot bis(2-aminoethyl)\cdot N\cdot N\cdot N\cdot N\cdot tetraacetic acid (EGTA) and potassium chloride were puriss-grade and 3-morpholinopropanesulfonic acid (MOPS) was Biochemika grade from Fluka (Ronkonkoma, NY). Chelex-100 ion-exchange resin was from Bio-Rad (Rockville Center, NY). Aprotinin, leupeptin trifluoroacetate, phenylmethylsulfonyl fluoride (PMSF), thrombin and glutathione agarose beads were from Sigma (St. Louis, MO). Chloroform, methanol and benzene were HPLC-grade (Fisher Scientific, Pittsburgh, PA) or Baker Analyzed (J.B. Baker, Bricktown, NJ). All other chemicals were reagent grade.

2.2. Preparation of solutions

Water was double-distilled through glass. The primary standard phosphate solution in water was pre-
pared from analytical concentrate (J.T. Baker). The concentration of phospholipid stock solutions in chloroform was periodically determined by phosphorus analysis [9]. All lipid stock solutions were stored in the dark, under an argon atmosphere, at −20°C. All buffers used in fluorescence spectroscopy studies were decalcified by passage of the 2× buffer, 40 mM MOPS, 200 mM KCl, pH 7.5, over a Chelex column before dilution with ddH2O. The calcium ion standard in decalcified buffer was prepared from analytical concentrate (J.T. Baker). Lyophilized protein was weighed out on Mettler H31AR balance and concentration was confirmed by Bradford assay.

2.3. Expression and purification of recombinant proteins

Recombinant proteins were purified by glutathione agarose affinity essentially as documented in Damer and Creutz [10,11]. A pGEX-KG plasmid encoding glutathione S-transferase in frame with the C2 motif nearest the transmembrane sequence of rat synaptotagmin I was transformed into the AB1899 strain of *Escherichia coli* and kindly provided by Dr. Lisa Elferink. A bacterial expression culture in 2×TY with ampicillin was grown for a doubling time of 69 min at 37°C with vigorous shaking before expression of the fusion protein was induced by the addition of 100 μM isopropyl-β-D-thiogalactopyranoside. Bacteria were collected by centrifugation at 7500 rpm for 10 min at 4°C before resuspending in phosphate-buffered saline (8.1 mM Na2HPO4, 1.9 mM NaH2PO4, 150 mM NaCl, pH 7.3) in the presence of 5 mM EGTA and a protease inhibitor cocktail (100 μg/ml PMSF, 19 μg/ml aprotinin and 25 μg/ml leupeptin) and 1% Triton X-100. The resuspended pellet was lysed while on ice by a probe sonicator set to half-max with a 50% pulse for 30 s. Following sonication the lysate was repeatedly passed through a Dounce homogenizer before centrifugation at 9500 rpm for 10 min at 4°C. The supernatant was removed and incubated with a 75% slurry of 200 mg of glutathione agarose beads (Sigma) for 30 min at room temperature while being continuously mixed by gentle rotation. The beads were hydrated and washed with 1×PBS prior to addition. The protein-bound beads were pelletted by centrifugation in a table top centrifuge at room temperature (RT) and washed 5× with a buffer comprised of 1×PBS, 1% Triton and the protease cocktail with gentle rotation between each wash. Prior to cleavage of the fusion protein, the beads were washed 3× with 50 mM Tris-HCl, 150 mM NaCl, 2.5 mM CaCl2, 0.1% β-mercaptoethanol, pH 8.0. To cleave the C2 motif from the fusion protein 1 unit of thrombin/ml of bead slurry resuspended in 1.5 ml of the CaCl2-containing buffer was added to the bead slurry and incubated for 45 min at RT with rotation. Released C2 motif was recovered upon washing the beads 6× with 1.5 ml per wash of 50 mM Tris, 150 mM NaCl, 10 mM EGTA, pH 8.0; the washes were pooled and dialyzed overnight at 4°C in 1 l of 10 mM Tris-HCl, pH 7.4 in the presence of 2 mM EGTA and the protease cocktail.

The dialyzed recombinant protein was further purified on a FPLC Mono Q column (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated in 40 mM HEPES, pH 7.0 at RT and eluted with a linear gradient of 0–500 mM KCl in 40 mM HEPES. The C2 motif eluted at approx. 250 mM KCl. The purified C2 motif was desalted on a P-10 G-25 column (Pharmacia LKB Biotechnology) equilibrated with 0.5 mM ammonium bicarbonate, pH 7.8 and lyophilized. Lyophilized protein was hydrated in decalcified 2 mM MOPS, 100 mM KCl, pH 7.5. Fourier transform infrared spectroscopy (FTIR) indicated that Ca2+ ligation did not alter the secondary structure of the protein (data not shown). Binding enthalpy of C2 motif for Ca2+ was also measured by isothermal titration calorimetry (ITC) and was comparable to previously reported data [12].

2.4. Preparation of large unilamellar vesicles (LUV)

Mixtures of PC and PS were prepared by aliquoting stock solutions of lipid in chloroform into borosilicate culture tubes using gastight syringes (Hamilton, Reno, NV). Samples were dried to a thin film under a gentle stream of argon and dried briefly under a vacuum of less than 20 mT before being lyophilized from benzene/methanol (19:1, v/v) or left on the vacuum line overnight. The two methods of lipid preparation, thin film or lyophilization, yielded essentially identical results. Lipid was hydrated in the dark above its phase transition temperature with 2 mM MOPS, 100 mM KCl, pH 7.5.
under argon. LUV were prepared by extruding 250 μl of a 4 mM multilamellar vesicle (MLV) solution through a 0.1 mm pore size polycarbonate filter (Costar Scientific, Cambridge, MA) 30 times above its phase transition temperature using a hand-held extruder (Avanti Polar Lipids, Birmingham, AL). LUV incorporating fluorescence probe were used within 36 h of preparation, otherwise lipid samples were used within a week. All lipid samples were stored in the dark under an argon atmosphere at room temperature.

2.5. Fluorescence spectroscopy experiments

All fluorescence measurements were made on a SLM Aminco 8100. All stock solutions were titrated into an approx. 300 μl mini-fluorimeter cell using a 10 μl gastight Hamilton syringe. The sample was continuously stirred throughout the experiment and the cell was vortexed after each addition for non-pyrene experiments. For fluorescence experiments monitoring the change in the intrinsic fluorescence of the C2 motif, excitation wavelength was 285 nm and emission spectra were recorded, excitation and emission slit widths were 4 nm and 16 nm, respectively; time traces were taken of the emission at 340 nm after each addition to determine equilibrium before emission scans were collected and shutters were closed when data were not accumulating. The excitation wavelength of 285 nm was the maximum in the excitation spectra where the emission wavelength was 340 nm. Collection of spectra after an incubation time of 15 min without time traces gave essentially the same results. Incubation times were selected based on equilibration of the fluorescence emission for Ca^{2+} binding and recovery of initial fluorescence with EGTA addition.

For experiments employing the fluorescent probe pyrene the excitation wavelength was 344 nm and emission spectra were recorded, excitation and emission slit widths were 1 nm and 8 nm, respectively. Changes in the E/M ratio, emission at 396 nm/470 nm, were also examined by collecting 10 s time traces using dual monochromators. Changes in the excimer/monomer (E/M) ratio with addition of protein were essentially the same by either data collection method, indicating spectral changes were not due to sample degradation. Lipid samples were also extracted and analyzed for degradation by thin-layer chromatography and pyrene containing samples were used within 36 h of preparation. To avoid photobleaching of the pyrene, time traces were not taken to follow equilibration. There was an incubation time of 15 min between each addition. Total lipid concentration was 200 μM for all pyrene experiments. A gentle stream of argon continuously flowed into the bottom of the fluorimeter cell to minimize O₂ quenching of excimer emission and samples were stirred continuously. Samples were not vortexed to avoid introduction of O₂ nor degassed to avoid variable concentrations of O₂ and argon in sample.

3. Results

These studies were initiated to investigate the possibility that a protein with affinity for anionic lipid could promote clustering of anionic lipid in an anionic-zwitterionic lipid system. For this we selected the first C2 motif of synaptotagmin, produced as a recombinant protein in E. coli and a defined lipid system comprised of a mixture of anionic lipid and zwitterionic lipid. The first step was to determine the thermodynamics of interaction of the various components of the system. To characterize the binding of C2 motif to its ligands, Ca^{2+} and anionic lipid, an anionic lipid content of 50% was selected to minimize the total lipid needed to determine binding isotherms. Decreasing the total lipid content minimized potential light scattering which can be pronounced in the presence of lipid and Ca^{2+}. Analysis of the binding isotherms of Ca^{2+} binding to the C2 motif, determined by measuring fluorescence changes associated with Ca^{2+} binding, yielded an estimate of the dissociation constants of K_{d1} = 97 μM in the absence of lipid and a value of K_{d2} = 17 μM in the presence of excess POPC/POPS (1:1) LUV. The binding of calcium ion appeared to be non-cooperative in both cases. Characterization of the ligation of Ca^{2+} and lipid by the C2 motif found binding behavior consistent with previously reported work [8,10,13].

There was no detectable change in the intrinsic fluorescence of this C2 domain upon binding to lipid in the absence of Ca^{2+}. However, it is possible to determine the binding constant of the C2 domain for lipid by taking advantage of the difference of
Ca^{2+} binding to the C2 domain in the absence and presence of lipid and determining the thermodynamic linkage between cation binding and the presence of lipid (Fig. 1). The apparent value of \( K_{d4} \) was determined by titration of a solution of protein containing 6.7 \( \mu \)M Ca\(^{2+}\) with lipid and monitoring the increase in Ca\(^{2+}\) binding. Using values for \( K_{d1} \) and \( K_{d3} \) to correct the apparent value (31 \( \mu \)M) yielded an estimate of \( K_{d4} = 6.1 \) \( \mu \)M. The \( K_{d2} \) of C2 binding to POPC/POPS (1:1) LUV in the absence of Ca\(^{2+}\) was estimated to be 43.5 \( \mu \)M POPC/POPS (1:1) by completing the thermodynamic cycle. Our hypothesis is that binding of C2 motif to the membrane couples its binding energy to rearrangement of the lipid, leading to membrane domain formation. The \( K_{d2} \) of binding of C2 motif to POPC/POPS (1:1) provided an estimate of the energy available upon binding lipid alone, from \( \Delta G = -RT \ln(1/K_{d2}) \) an estimated \(-6\) kcal/mol energy may be released upon binding. However, the interaction energy between unlike nearest neighbor lipids is only on the order of hundreds of cal/mol. As the coupling of binding energy to a conformational change of the protein is not readily apparent, an interesting and obvious possibility was that upon binding of protein, the lipids were demixed into domains enriched in anionic lipids. The possible formation of acidic lipid-enriched domains upon C2 motif binding to the membrane was investigated using pyrene-labeled phosphatidylglycerol (Pyr-PG).

Domain formation was investigated using 20% anionic lipid and 80% zwitterionic lipid because the chance of lipid domain formation enriched in anionic lipid in the absence of protein is small in comparison to the induction of a lipid domain upon protein binding with this low concentration of anionic lipid. Changes in excimer to monomer (\( E/M \)) ratio of the pyrene-labeled lipid are indicative of changes in the local concentration of anionic lipid or in changes in the lateral mobility of the probe upon protein binding [14–16]. Binding the C2 fragment to POPC/POPS/Pyr-PG (80:16:4) LUV in the absence of Ca\(^{2+}\) produces an increase in the \( E/M \) ratio that reaches a maximum and then decreases (Fig. 2). This is consistent with an increase in excimer formation resulting from domain formation upon C2 binding until at a sufficiently high level of saturation further binding causes disruption of the domains because the number of anionic lipids bound per protein is reduced. A protein-induced change in the lateral mobility of the probe would not be expected to cause both an increase and a decrease in \( E/M \). This result is similar to that found by Junker and Creutz [17] for the binding of annexin IV to PC/PG bilayers. The results of Monte Carlo computer simulations using a simple model of lipid clustering in a binary mixture provide a conceptual basis for understanding these phenomena. The general approach for developing and running Monte Carlo simulations of membrane dynamics is described in detail elsewhere [18–20]. In the present case, the thermodynamic coupling of lipid clustering to protein binding can be described in terms of four transition probabilities: Diffusion of a randomly selected lipid in the two-dimensional lattice of the membrane, the probability of a protein in solution binding to the membrane, the probability that a bound protein will dissociate from the membrane, and the probability that a bound protein will diffuse on the surface of the membrane. These probabilities are related to the energy change associated with lipid diffusion, the protein association and dissociation and protein diffusion. The transition probability is given by Eq. 1:

\[
P_1 = \exp(-\Delta G/RT)/(1 + \exp(-\Delta G/RT))
\]

where

\[
\Delta G = \Delta N_{ab} \omega_{ab} + \lambda_a \Delta G_a + \Delta N_b \delta G_{ab}
\]
The parameters in Eqs. 1 and 2 are defined as:

$\Delta N_{ab} = \text{change in the number of unlike nearest neighbor contacts.}$

$\delta g_{ab} = \text{difference in interaction energy between an unlike nearest neighbor lipid-lipid contact and the mean of the interaction energy between like nearest neighbor lipids.}$

$V_a = 0 \text{ if a bound protein is selected to dissociate, } 3 \text{ if a bound protein is selected to diffuse, } -1 \text{ if a bound protein is selected to bind to the membrane.}$

$\delta G_{ab} = \text{the Gibbs energy change associated with protein binding to a membrane composed only of 'A'-type lipids.}$

$N_{G_{ab}} = \text{the change in the number of 'B'-type lipids to which the protein binds.}$

$\delta G_{ab} = \text{Gibbs energy difference between the protein binding 1 mole of type B lipid and one mole type A.}$

The results of Monte Carlo computer simulations using this simple model of lipid clustering are in qualitative agreement with the experimental results using the pyrene probe described above. The average number of clusters, representing ‘domains’, and the average cluster size as a function of proteins bound to a lattice of $10^5$ lipids composed of PC/PS (4:1) is shown in Fig. 3. As can be seen, the average number of clusters exhibits a minimum and the average size exhibits a maximum. Assuming that Pyr-PG accurately reflects the behavior of PS and $E/M$ reflects the ‘local’ concentration, these results indicate that the Pyr-PG ‘local’ concentration could vary from mole-fraction = 0.04 to a maximum of about 0.20 and then decrease as the clusters disperse. Other results obtained from these simulations are the cluster distribution functions shown in Fig. 4, where the relative frequency of finding a PS lipid in a cluster of size $N$ is plotted versus $N$. The distribution function calculated for 700 proteins bound on the average (Fig. 4, bottom) exhibits clear maxima at about 25, 50, 75, 100 and 125 lipid molecules per cluster. These maxima likely correspond to assemblies of one, two, three, four or five proteins associated with the lipid clusters. This distribution function should be compared to that obtained in the absence of protein which is a monotonically decreasing function of size (see Fig. 4, top).
4. Discussion

Membranes are a mosaic of lipids and proteins. The lipids differ in charge, acyl-chain length and degree of saturation, hydration and hydrogen-bonding capability while the proteins associated with membranes may be integral or peripheral, with wide variation in size and charge. A membrane ‘domain’ is a region of lipid and protein that differs on average from its surrounding milieu. The membrane, by virtue of its collection of diverse molecules of various degrees of immiscibility, exists on a threshold primed for domain formation (for a review see [21]). The present study has focused on the induction of membrane domains upon peripheral protein binding, using a simplified system involving a single lipid-binding protein and lipid bilayers of defined composition. The ‘domains’ formed in this case are regions of increased negatively charged lipid in a background of neutral lipid. The promotion of such domains by a C2 motif is suggested by the behavior of the pyrene-labeled probe and supported by the theoretical simulation of the binding event and has potential implications for the regulation of signal transduction events at the inner leaflet of the plasma membrane or on organelle surfaces.

Annexins, Ca\(^{2+}\)- and lipid-binding proteins of a distinct class from the C2 motif, are likely to act in a manner analogous to the C2 motif and to promote formation of membrane domains [17,22,23]. The annexins are composed of a conserved core and a variable N-terminal region that may impart specific roles in cellular function to each of the annexins. The conserved core is thought to contain the lipid-and Ca\(^{2+}\)-binding sites in the annexin and crystal structures have been solved for many of the annexin cores [24–26]. The conserved core is defined by a four domain repeat consisting of five homologous amphiphilic \(\alpha\)-helices folded into a right-handed superhelix where the domains are splayed out (reviewed in [27]). The binding of calcium ion does not appear to cause major changes in annexin conformation as monitored by crystallographic structure determination [25,28] or circular dichroism studies [29]. We suspect that membrane binding by the annexins, similar to the C2 motif, is coupled not to major changes in annexin conformation, but to membrane reorganization.

In general, membrane-binding proteins that induce the formation of similar lipid domains, such as domains rich in anionic lipids, would be predicted to colocalize on membranes. This is a natural consequence of the domain formed by one protein having a higher anionic lipid concentration and therefore higher affinity for other anionic lipid-binding proteins. This effect may be partly responsible for the self-association of annexins that occurs on membrane surfaces [30] and for the association of PKC, which contains a C2 motif, with annexins I, II and IV, an association dependent on the presence of PS-containing membranes [31,32]. Once proteins are colocalized on a membrane surface, specific protein-protein interactions may come into play to stabilize the assembly or to organize interacting proteins into functional units. A peptide sequence in annexin I resembling a portion of RACK-1, a receptor for activated protein kinase C, may act to enhance protein aggregates of annexin-PKC-\(\beta\) [33,34], a colocalization that we suggest may be initiated by lipid domain formation.

Annexins have been postulated to be ion channel proteins that conduct calcium, barium, lithium and cesium ions (reviewed in [27,35,36]). Ion permeability of membranes can be a consequence of domain formation or phase separation in the membrane. The permeability of phospholipid bilayers to small water-soluble molecules is increased at the gel-to-liquid crystalline phase transitions of lipid ([37], reviewed in [38]). This permeability is attributed to defects between the lipid phases and theoretical models suggest that the interfacial regions between the domains are poorly ordered due to an abundance of partially disordered acyl chain conformations [39,40]. Such interfacial regions are present at the boundaries of lipid phases or domains induced by temperature or cation binding to lipids and may form in single component or lipid mixtures. We hypothesize that the binding of annexin to a membrane may induce the formation of a domain enriched in anionic phospholipid. The formation of such domains could then lead to increased permeability of the phospholipid bilayer around the edges of the domain that form an interfacial region. Since this should be a general consequence of membrane domain formation we would also predict that increased ion permeability would be detected with the association of the C2 motif with membranes.
The domains in membranes formed by the C2 motif or annexins may influence the association of other proteins with membranes or the activation of membrane-associated proteins. For example, PKC activity was observed to be influenced by the coexistence of a diacylglycerol (DAG)-enriched and DAG-poor phase. A maximum in activity was observed at mole fractions of DAG that approximate the midpoint of the phase coexistence region of the lipid gel state. Possible explanations are: (1) if PKC preferentially associates with one type of lipid domain, the enzyme will cluster on the membrane surface. And if PKC activity is related to the extent of oligomerization, enzyme activity will exhibit a maximum in the region of lipid domain coexistence. (2) If PKC is activated by binding of the enzyme to the interfacial region between coexisting DAG-enriched and DAG-poor phases, enzymatic activity would exhibit a maximum near the midpoint of the lipid phase coexistence region or percolation threshold [41,42]. (3) The effect of a percolation threshold on the kinetics of a reaction in which the components are clustered within the same lipid domain could also influence enzymatic activity [49].

Isoprenoid and myristoyl-derivatized proteins may be preferentially bound to interdomain boundaries. Experimental measurement of the partitioning ability conferred by myristoylation or farnesylation on proteins have indicated only a weak effect [43–45]. However, myristoylation of Src, for example, is necessary for Src binding to membranes in vivo and ultimately for Src transforming ability [43,46]. The induction of a lipid domain by protein binding to membrane will create an interfacial region prone to defects at the edges of the domain. The interface is a sink for impurities which partition favorably into such regions [38,40]. Farnesyl and myristoyl groups on proteins may be similarly attracted to these interfaces. Therefore, we postulate that proteins such as annexins or synaptotagmin might enhance the partitioning of myristoylated or farnesylated proteins from the cytosol to their vicinity and potentially lead to their activation. Some proteins such as Src [45,47] or K-Ras [48] further enhance their membrane-binding ability with a string of basic residues. We suggest that binding of proteins such as Src and K-Ras to membrane is thermodynamically coupled to domain formation.

In this report we have focused on the first C2 domain of synaptotagmin as a model for general phenomena associated with lipid-binding proteins. However, it is interesting to speculate that the properties of the C2 domain of synaptotagmin described here could have specific relevance to the role of synaptotagmin in neurotransmission. The C2 domain could promote specialized domains in the synaptic vesicle membrane and/or the plasma membrane that recruit other components of the exocytotic machinery. The boundaries of such domains might be prime locations for the lipid rearrangements associated with membrane fusion. Although these changes in membrane organization may occur even in the absence of Ca$^{2+}$, as shown in this report, it is likely that they would be stabilized by Ca$^{2+}$ binding to the C2 domain, providing precise control of exocytosis.

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