Synaptotagmin 1 (syt1) functions as the Ca\(^{2+}\) sensor in neuronal exocytosis, and it has been proposed to act by modulating lipid bilayer curvature. Here we examine the effect of the two C2 domains (C2A and C2B) of syt1 on membrane lipid order and lateral organization. In mixtures of phosphatidylcholine and phosphatidylserine (PS), attenuated total internal reflection Fourier transform infrared spectroscopy indicates that a fragment containing both domains (C2AB) or C2B alone disorders the lipid acyl chains, whereas the C2A domain has little effect upon chain order. Two observations suggest that these changes reflect a demixing of PS. First, the changes in acyl chain order are reversed at higher protein concentration; second, selective lipid demixing demonstrates that the changes in lipid order are associated only with the PS component of the bilayer. Independent evidence for lipid demixing is obtained from fluorescence self-quenching of labeled lipid and from natural abundance \(^{13}\)C NMR, where heteronuclear single quantum correlation spectra reveal Ca\(^{2+}\)-dependent chemical shift changes for PS, but not for phosphatidylcholine, in the presence of the syt1 C2 domains. The ability of syt1 to demix PS is observed in a range of lipid mixtures that includes cholesterol, phosphatidylethanolamine, and varied PS content. These data suggest that syt1 might facilitate SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors)-mediated membrane fusion by phase separating PS, a process that is expected to locally buckle bilayers and disorder lipids due to the curvature tendencies of PS.

Neuronal exocytosis is mediated by a calcium-triggered fusion event that joins the synaptic vesicle and the presynaptic membrane, thereby releasing neurotransmitter into the synaptic cleft. The SNAREs\(^{2}\) (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are the central protein machinery in the fusion process and include vesicle-associated synaptobrevin and plasma membrane-associated syntaxin and SNAP-25 (1). Although a large number of proteins are required to regulate and promote fusion, the SNAREs play a critical role by forming a four-helix bundle that drives the vesicle and plasma membranes together (2, 3). However, the SNAREs are not directly responsible for sensing Ca\(^{2+}\), and in neuronal exocytosis there is compelling evidence that synaptotagmin 1 (syt1) functions as the Ca\(^{2+}\)-sensor (4–6).

Synaptotagmin 1 is composed of two C2 domains, C2A and C2B, that are connected by a flexible linker (7) and attached to the vesicle membrane through a transmembrane helical segment (see Fig. 1). C2A and C2B both bind Ca\(^{2+}\) and bind membranes (8). In the absence of Ca\(^{2+}\), C2A fails to bind phosphatidylcholine (PC):phosphatidylserine (PS) bilayers, and its affinity is so weak that it cannot be measured; however, C2B has a weak affinity for bilayers in the absence of Ca\(^{2+}\) and associates through a polybasic face formed largely by basic residues in \(\beta\)-strand 4 (9). In the presence of Ca\(^{2+}\), the C2 domains of syt1 interact with the bilayer through their first and third Ca\(^{2+}\)-binding loops, which are observed to insert into the membrane bilayer so that the backbone of these loops lies \(-5\) Å below a plane defined by the lipid phosphates (10, 11). This position appears to be slightly deeper when the two domains are examined in tandem (C2AB), an observation that might be explained by an enhanced electrostatic interaction, perhaps due to a demixing of PS by syt1 (12).

The actual mechanism by which syt1 promotes fusion is not understood. Synaptotagmin 1 is known to interact with the SNAREs, and it has been proposed to act by regulating the SNARE complex, perhaps by competing with complexin for a binding site on the SNAREs (13). There is also evidence that syt1 might act by directly modulating the lipid bilayer. For example the C2 domains of syt1 have been reported to promote the demixing or phase separation of PS from the bulk lipid (14), to induce positive bilayer curvature (15), and to alter the integrity of the bilayer (16). Synaptotagmin 1 is also observed to aggregate vesicles (17, 18), and a structure obtained for membrane-associated syt1 (19) indicates that the two C2 domains are oriented so that they bind to opposing bilayer surfaces. This work suggests that syt1 may function to bridge the vesicle and plasma membrane interfaces. However, a number of these studies raise questions. For example, the demixing of PS in an earlier study has only been reported for C2A (14), and it is observed in the absence of Ca\(^{2+}\) under conditions where C2A is not observed to bind bilayers. It is not known whether demixing can be promoted by the C2B domain or the tandem C2
domains. In addition, the connection, if any, between demixing and the induction of bilayer curvature is not clear.

In the present study we used several approaches to examine the effect of C2A, C2B, and the tandem C2AB fragments of syt1 upon the acyl chain dynamics and phase behavior of PC:PS bilayers. Using polarized ATR-FTIR on supported bilayers, we demonstrate that C2B and C2AB increase acyl chain disordering upon membrane binding. These changes in chain order are reversed at higher concentrations of protein, and deuteration indicates the changes are associated with the PS component in the bilayer and not with the PC component. This behavior suggests that C2AB and C2B act to demix PS from a PC:PS mixture and that this demixing is responsible for the induction of positive curvature strain. Further evidence for demixing and for interactions with clusters of PS lipids by the syt1 C2 domains has been obtained by fluorescence and NMR spectroscopy. A qualitatively similar behavior is seen in various lipid mixtures containing PS and suggests mechanisms by which syt1 might facilitate SNARE-mediated membrane fusion.

**EXPERIMENTAL PROCEDURES**

**Lipids**—All lipids including NBD-PC (1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]lauryl]-sn-glycero-3-phosphocholine), NBD-PS (1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]lauryl]-sn-glycero-3-phosphoserine), NBD-PG (1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]lauryl]-sn-glycero-3-phosphoglyceride), D62-DPPC (1,2-dipalmitoyl(d62)-sn-glycero-3-phosphocholine), and D62-DPPS (1,2-dipalmitoyl(d62)-sn-glycero-3-phospho-1-serine) were purchased from Avanti Polar Lipids (Alabaster, AL).

**Protein Expression and Purification**—Synaptotagmin 1 C2A (96–265), C2B (249–421), and C2AB (96–421) from rat were encoded in fusion with the glutathione S-transferase at the N terminus. The plasmids were transformed into BL21 (DE3) Escherichia coli cells. Expression of the recombinant protein was induced with 0.1 mM IPTG when cells had reached an absorbance of ~0.8–1.0 and were allowed to grow for 7 h at 20 °C before being harvested. The proteins were purified by GSTrap affinity chromatography followed by either HiTrap-Q HP (C2A and C2AB) or HiTrap-SP HP (C2B) ion exchange columns as described previously (9–11).

**Preparation of Liposomes**—For liposomes without PI(4,5)P2, desired amounts of lipids from chloroform stock solutions were mixed, and the solvent was evaporated under a stream of nitrogen. The dispersions were then dried at the bottom of glass test tubes overnight under vacuum. For liposomes containing PI(4,5)P2, desired amount of lipids from chloroform stock solutions were mixed and dried for 1 h by rotovaporation followed by drying overnight under vacuum. The lipids were resuspended in a buffer solution containing Ca2+ (CaCl2, 20 mM HEPES, 100 mM NaCl, pH 7.4) to form a dispersion. Small unilamellar vesicles (SUVs) were prepared by sonication of the lipid dispersion in an ice-water bath using a titanium tip ultrasonicator (Branson, Danbury, CT) until the solution was transparent (~15 min). To prepare large unilamellar vesicles (LUVs), the suspensions were freeze-thawed 5 times before being passed through two polycarbonate membranes (100-nm pore size) 19 times using a liposofast extruder (Aves tin, Ottawa, ON).

**Preparation of Planar Lipid Bilayers and Fourier Transform Infrared Spectroscopy**—Planar lipid bilayers were prepared on germanium ATR plates using a Langmuir-Blodget/vesicle fusion method described previously (20, 21). Briefly, a single monolayer of desired pre-mixed lipids was deposited onto a germanium plate (50 × 20 × 1 mm3) from the interface of a Langmuir trough to form the proximal monolayer. SUVs composed of the desired lipids were injected into the cell containing the monolayer-coated germanium plate and allowed to incubate for 2 h at room temperature to form the distal monolayer. Excess SUVs were washed away using deuterated Ca2+ buffer containing 150 mM NaCl, 20 mM HEPES, and 1 mM CaCl2, pH 7.4, prepared in D2O. Desired amounts of syt1 C2AB, C2A, or C2B were injected into the cell containing supported lipid bilayers on the germanium plate. Excess protein was washed with 5 volumes of deuterated Ca2+ buffer after a 30-min incubation of the protein with the bilayer. Both 0° and 90° polarization absorption FTIR spectra were recorded using a Vector 22 FTIR spectrometer (Bruker Optic Inc., Billerica, MA).

Polarization experiments in FTIR can be used to estimate the acyl chain order for phospholipids in bilayers. Here, we describe the lipid acyl chain order in a fluid lipid bilayer by the order parameter $S_L$, which reflects the weighted average tilt angles $\vartheta$ between the main axis of acyl chain and the membrane normal according to

$$S_L = (3\cos^2\vartheta - 1)/2 \quad (\text{Eq. 1})$$

Larger values of $S_L$ reflect more ordered structures. In a perfectly ordered bilayer (i.e. one where the chain tilt angle equals to 0), $S_L$ equals to 1, and in a totally disordered bilayer, $S_L$ equals to 0.

The transition dipole moments for the asymmetric and symmetric lipid acyl CH2 stretching vibrations (around 2920 and 2850 cm$^{-1}$, respectively) are oriented at right angles to the long axis of the acyl chain. The order parameter, $S_L$, may be determined experimentally using polarized ATR-FTIR by mea-
suring the dichroic ratio, $R_{\text{ATR}} = A_0/A_{90}$, where $A_0$ and $A_{90}$ represent the absorption for 0 and 90° polarized beams. $S_i$ is then given by

$$S_i = -\frac{E_x^2 - R_{\text{ATR}}E_y^2 + E_z^2}{E_x^2 - R_{\text{ATR}}E_y^2 - 2E_z^2} \quad (\text{Eq. 2})$$

where $E_x$, $E_y$, and $E_z$ are the components of the electric field (20). Under the conditions used here, the values of $E_x$, $E_y$, and $E_z$ are: $E_x^2 = 1.969$, $E_y^2 = 2.249$, and $E_z^2 = 1.892$ (22). From Equations 1 and 2, an increase in the dichroic ratio $R_{\text{ATR}}$ corresponds to an increasing average tilt angle $\theta$, indicating a less ordered lipid structure.

**Fluorescence Self-quenching Experiments—POPC:POPS (3:1)** LUVs having 1% NBD-PS at a total lipid concentration of 0.1 mM were prepared in Ca$^{2+}$ buffer. A total volume of 300 $\mu$L was used for the measurement, and desired amounts of protein were titrated into the LUV solution and incubated for 15 min before each measurement. After each titration series, EDTA was added to a final concentration of 1 mM. Emission spectra were obtained by scanning from 500 to 600 nm using a Fluoromax-3 fluorimeter (Jobin Yvon, Edison, NJ) with the excitation wavelength set to 460 nm (1-nm excitation and 3-nm emission slit widths). The fluorescence intensity changes due to the volume change were corrected with titration data using buffer as a blank. The fluorescence decreases due to the volume change were subtracted with experimental data of the titration using the same volume of buffer. The percent quenching was calculated according to $1 - F_p/F_0$, where $F_p$ is the fluorescence intensity in the presence of protein, and $F_0$ is the fluorescence intensity in the absence of protein.

**NMR Spectroscopy—SUVs** were prepared by sonication of a lipid dispersion having total lipid concentration of 10 mM and a 3:1 composition of POPC:POPS. These vesicles were prepared in a $\text{D}_2\text{O}$ buffer containing 100 mM NaCl, 20 mM $\text{CD}_3\text{COONa}$, and 1 mM CaCl$_2$, pH = 7.4. Desired amounts of the syn1 C2 domains were dissolved in the same buffer, added to lipid vesicles, and allowed to incubate for 1 h at room temperature before collecting spectra. $^{13}$C,$^1$H HSQC spectra (23) were collected on a Varian Unity INOVA 500 MHz spectrometer (Varian Inc., Palo Alto, CA) at 30 °C. The spectra were processed, assigned, and analyzed with MestReNova (Mestrelab Research, Escondido, CA).

**RESULTS**

**Synaptotagmin 1 C2 Domains Alter the Acyl Chain Order of POPC:POPS Bilayers—** ATIR-FTIR is a well established method of studying the binding, secondary structure, and orientation of proteins at bilayer interfaces. In addition, this method provides information on the acyl chain order of the membrane lipid (24). Shown in Fig. 2a are FTIR spectra obtained on supported lipid bilayers composed of POPC:POPS (3:1), where the 0° and 90° polarization absorption spectra are represented by **solid** and **dotted traces**, respectively. In this figure, the addition of syn1 C2B at a concentration of 100 $\mu$g/ml altered the absorption of polarized symmetric (2850 cm$^{-1}$) and antisymmetric (2920 cm$^{-1}$) stretch vibrations of the lipid acyl chains and increased their dichroic ratios. This increase in dichroic ratio reflects a decrease in the molecular ordering of the acyl chains upon the addition of the domain.

The changes in dichroic ratio are summarized in Fig. 2b for POPC:POPS (3:1) bilayers upon the addition of C2AB, C2B, and C2A in the presence of Ca$^{2+}$. In the absence of protein, dichroic ratios were ~1.32, which yields an acyl chain order parameter, $S_2$, of 0.36. Both C2B and C2AB increase the dichroic ratio when added to the aqueous subphase adjacent to the bilayer; however, the changes plateau and then decrease at higher protein concentrations. This increase in dichroic ratio corresponds to decrease in lipid order, and the largest changes that were seen upon the addition of C2B represent an ~2-fold change in the order parameter. In contrast, C2A produces virtually no change in acyl chain order within experimental error. It should be noted that this change may represent either a decrease in dynamic order (an increase in the gauche-trans isomer ratio of the acyl chains) or a decrease in static order (for example a change in the tilt of the lipids or the lipid interface).

**Addition of PI(4,5)P$_2$ Enhances the Effect of C2B and C2AB upon Lipid Disorder—** We investigated the effect of incorporating 1 mol % of PI(4,5)P$_2$ into POPC:POPS on the changes in dichroic ratio produced by the synaptotagmin 1 C2 domains. Shown in Fig. 2c are the dichroic ratios for bilayers composed of POPC:POPS:PI(4,5)P$_2$ (75:25:1) when either C2B or C2AB are added. Both C2AB and C2B increase the dichroic ratio in these bilayers, corresponding to a decrease in chain order. These effects are similar to those obtained in the absence of PI(4,5)P$_2$; however, the magnitudes of the changes are larger, and the plateau or maximum in the dichroic ratio occurs at much lower protein concentrations. In the presence of 1% PI(4,5)P$_2$, no significant effect of C2A upon lipid order was observed. The effect of PI(4,5)P$_2$ on the concentration dependence of the dichroic ratio was likely due to an enhanced binding of the synaptotagmin C2 domains to bilayers containing PI(4,5)P$_2$. An increased membrane and Ca$^{2+}$ affinity for C2AB in the presence of PI(4,5)P$_2$ has been observed (25–27), and estimates based upon fluorescence correlation spectroscopy indicate that the membrane binding of C2AB was increased by 3–5-fold by the incorporation of 1 mol % PI(4,5)P$_2$ into PC:PS (3:1) bilayers.

The acyl chain disordering upon the addition of syn1C2AB or syn1C2B was also observed in other lipid mixtures. Shown in Fig. 2d are the dichroic ratios as a function of protein concentration for supported lipid bilayers containing a mixture POPC:POPS:cholesterol (55:25:20). The initial dichroic ratios were lower than those without cholesterol, which is expected due to the condensing effect that cholesterol is known to have on lipid bilayers (28). The trends are qualitatively similar to those seen in mixtures without cholesterol (Figs. 2, b and c), where both C2AB and C2B were observed to decrease the molecular ordering. Qualitatively similar changes were also seen in membranes at a lower POPS content (POPC:POPS:5:1) or with the addition of phosphatidylethanolamine (POPE).

It should be noted that changes are also seen in the dichroic ratio upon the addition of either C2B or C2AB in the absence of Ca$^{2+}$ (Fig. 2, e and f). Under Ca$^{2+}$-free conditions, C2B and C2AB increase the dichroic ratio; however, the changes in

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3 W. Kuo and D. S. Cafiso, unpublished information.
dichroic ratio have a weaker concentration dependence and do not go through a maximum. The C2A domain has no effect upon the dichroic ratio in the absence of Ca\textsuperscript{2+}. The observation of a change in dichroic ratio for C2B and C2AB in the absence of Ca\textsuperscript{2+} is consistent with previous membrane binding studies showing that C2AB and C2B, but not C2A, bind to bilayers in the absence of Ca\textsuperscript{2+}.

In summary, the binding of C2AB and C2B initially disorders bilayers composed of POPC:POPS, but this effect is reversed at higher protein concentrations. This behavior is not limited to
Phosphatidylinerse is a negatively charged lipid at neutral pH, whereas PC has no net charge. To determine whether the C2 domain induced disordering seen in POPS:POPC mixtures might take place with another negatively charged lipid, we repeated the ATR-FTIR experiment adding 100 μg/ml C2B in the presence of Ca2+ with supported POPC:POPG (3:1) lipid bilayers. Under these conditions the dichroic ratio, RATR, for the CH2 stretch is unchanged upon protein addition (data not shown), indicating that the effect we see is not universally observed for negatively charged lipids.

**Fluorescence Quenching Indicates That C2AB and C2B Demix or Sequester POPS**—The data presented in Figs. 2 and 3 suggest that syt1C2AB and syt1C2B act to demix POPS from these lipid mixtures. First, as shown in Fig. 2, the dichroic ratio reached a maximum as the concentration of C2B and C2AB was increased and then reversed. If these C2 domains are associating with PS and promoting the formation of domains, the domains would be expected to dissociate when the protein densities saturate and exceed the available PS on the bilayer surface. Lipid domain formation having this type of protein concentration dependence has been observed previously, and it is predicted by simple models that describe electrostatic peptide-lipid interactions on bilayer surfaces (29). Second, as shown in Fig. 3, the changes in the dichroic ratio that occur upon protein binding are associated only with POPS and not with POPC.

To test the idea that demixing or sequestration of POPS is taking place, the fluorescence quenching of PS labeled with NBD in one of its acyl chains was monitored as a function of C2 domain addition using an approach described previously (30). In this experiment, 1 mol % of the fluorescent analog of PS was incorporated into LUVs. NBD is a self-quenching fluorophore, with a Förster radius of about 35 Å (31). The extent of self-quenching will be strongly dependent upon the average distance between labeled lipids, particularly near the Förster radius, and self-quenching will be sensitive to the phase separation of PS. If the syt1 C2 domains reduce the average distance between NBD-labeled PS lipids, a decrease in NBD fluorescence will be observed.

Shown in Fig. 4a are fluorescence emission spectra upon the addition of increasing concentrations of C2AB, titrated into 0.1 mM POPC:POPG (3:1) LUVs. The addition of C2AB produced a quenching in the fluorescence and at 4 μM protein; the percent quenching approached 36%. This effect was Ca2+-dependent, and the addition of 1 mM EDTA reversed the changes.

Similar effects were seen for C2B (Fig. 4b), and the addition of 1 mM EDTA reversed the quenching. In contrast to C2AB and C2B, C2A did not alter the fluorescence of NBD-PS (Fig. 4c). Small light scattering changes in the LUV suspensions were detected upon the addition of C2AB; however, at the lipid concentrations used here, this scattering did not significantly affect the NBD fluorescence. Thus, the observed quenching apparently reflects an increase in the local concentration of the PS component upon the binding of C2AB and C2B.

The data shown in Fig. 4 are summarized in Fig. 5a, which shows fluorescence changes induced by C2AB, C2B, or C2A in NBD-PS-labeled POPC:POPS LUVs. The fluorescence quenching was not observed when the experiment was run
using NBD-labeled PG in POPC:POPG (3:1). Thus, the demixing seen for PS is not universally seen for all acidic lipids.

**Evidence for PS Sequestration Is Obtained in a Range of Lipid Mixtures**—The experiments shown in Fig. 4 were repeated with LUVs formed from mixtures of POPC and POPS along with combinations of PI(4,5)P2, POPE, and cholesterol. The results of fluorescence measurements using LUVs formed from POPC:POPS:NBD-PS:PI(4,5)P2 (74:25:1:1) are summarized in Fig. 5b. The results are qualitatively similar to those obtained in the absence of PI(4,5)P2, except that the maximum quenching is slightly less, and the quenching approaches a maximum at lower protein concentrations.

Cholesterol is a component in the presynaptic and synaptic vesicle membrane, and it has been reported to promote demixing of PS in PC:PS bilayers (32–35). To examine the effect of cholesterol, C2B, C2AB, and C2A were added to vesicles containing POPC:PS:cholesterol (55:25:20) along with 1% NBD-PS. The data, which are summarized in Fig. 5c, indicate that C2AB and C2B produce fluorescence quenching in this mixture that is Ca2+-dependent. The levels of quenching for C2AB were similar to those seen in the absence of cholesterol, whereas C2B alone appeared to be less effective at quenching in the presence of cholesterol. In contrast to cases where cholesterol was absent, C2A was also found to induce a measureable fluorescence quenching that was Ca2+-dependent.

Shown in Figs. 5, d–f, are fluorescence quenching changes produced upon C2 domain addition to membranes with a lower POPS content to membranes containing POPE or to membranes with both POPE and cholesterol, respectively. Quenching changes were still observed at a lower POPS content but were not as large. The incorporation of POPE also suppressed the quenching changes, but the magnitude of the changes was enhanced with cholesterol addition. As discussed below, these trends are consistent with the known behavior of PS.

In addition to exploring a range of lipid components, we tested the effect Mg2+ ion on the fluorescence quenching observed here. Mg2+ concentrations of 100 and 500 μM produced no qualitative change in these data but slightly reduced the quenching changes (by about 20–30%) at a particular lipid composition. This is likely due to a slight reduction in membrane affinity for the C2 domains in the presence of Mg2+ (data not shown). This is expected due to a small reduction in membrane surface charge density that will result from absorption of Mg2+ to the membrane surface (36).

**NMR Spectroscopy Indicates That syt1 C2 Domains Alter the PS Environment in a PC:PS Mixture**—The FTIR and fluorescence results described above suggest that the addition of syt1 C2B and C2AB change the environment of PS and demix PS
from the bulk lipid. This lipid demixing might be promoted by specific C2 domain-PS interactions, and NMR was used here to determine whether the syt1 C2 domains alter the environment or interact with the PS headgroup. NMR chemical shifts are highly sensitive to changes in local environment, and high resolution spectra (for both $^1$H and $^{13}$C nuclei) can be obtained for lipids in small unilamellar vesicles produced by sonication (37).

Shown in Fig. 6 is a natural abundance $^{13}$C HSQC NMR spectrum of 10 mM POPC:POPS (3:1) SUVs showing resonances for the lipid headgroups. The peak assignments were determined using previous assignments for these lipids (38) and by comparing the HSQC spectra of pure POPC SUVs and pure POPS SUVs.

When C2AB is bound to small vesicles in the presence of Ca$^{2+}$, the PS-β peak underwent a dramatic upfield shift in the $^{13}$C dimension of ~14 ppm (Fig. 6b), whereas the other headgroup resonances remained unchanged. In particular, there are no indications for shifts in the resonances of PC, and the PS-α peak did not undergo a significant shift. The movement of the PS-β peak to a higher field indicates that this headgroup $^{13}$C nucleus became more shielded. The source of this shift is not entirely clear, but it might result from a ring current effect due to a nearby aromatic side chain on syt1 (39). Identical chemical shift changes were seen in solution for short chain PS (1,2-dihexanoyl-sn-glycero-3-phospho-L-serine) in the presence of C2AB, indicating that this chemical shift change was due to a lipid-protein interaction.

As the concentration of protein was increased, the intensity of the shifted PS-β peak increased relative to the unshifted peak. These changes were C2 domain and Ca$^{2+}$-dependent, and removal of Ca$^{2+}$ reversed the chemical shift change. The altered PS resonance seen in the $^{13}$C,$^1$H HSQC NMR spectra indicates that the addition of syt1 C2AB alters the environment of the PS headgroup and produces two populations of PS. The peak intensity rather than the chemical shift was altered as the C2AB concentration was increased, indicating that the two populations are in slow exchange on the NMR timescale. The ratio of the peak intensities (shifted:unshifted) increased linearly with C2AB concentration and was ~4.0 at a protein:lipid ratio of 1:200. Because the resonances at 38 and 52 ppm have approximately the same line widths, the ratio between shifted and unshifted populations can be used to estimate the number of PS lipids that are altered as a result of the C2AB membrane binding. At the highest concentrations of C2AB used, this estimate yielded ~15–20 PS lipids per C2AB.

Identical experiments were also performed using both C2B and C2A, and the results are shown in Fig. 7. At a 1:200 protein:lipid ratio of C2B, the PS-β peak was also shifted, and the ratio between shifted and unshifted peaks was 3.54. In the absence of Ca$^{2+}$, the effect was largely reversed, although a small popula-
Synaptotagmin 1 Demixes and Disorders Phosphatidylserine

It has been reported that synt1 prefers to bind to membranes with positive curvature and tubulate liposomes (15); moreover, these observations suggest that membrane binding by synt1 induces a strain energy in the bilayer, sometimes referred to as spontaneous curvature (40). Although the curvature tendencies of synt1 have been proposed to be a mechanism that mediates Ca$^{2+}$-triggered neuronal exocytosis, the mechanism by which strain is induced through synt1 binding is not understood.

The measurements made here indicate that there is a connection between the demixing of PS, induced by synt1 membrane binding, and the induction of membrane curvature strain. The C2AB and C2B domains of synt1 were both observed to increase the molecular disorder of the acyl chains of PS and to promote a demixing of PS from PC:PS bilayers. Moreover, this capacity to disorder bilayers and demix PS was observed in a range of lipid mixtures, including mixtures containing PI(4,5)P$_2$, cholesterol, and PE. The ability to disorder or demix PS appears to be largely associated with the C2B domain, as little evidence for a C2A induced change in molecular order or demixing was found either by FTIR or by fluorescence quenching in PC:PS.

Previous work has shown that the Ca$^{2+}$ binding loops of the C2 domains of synt1 penetrate the bilayer interface (12). This insertion into the interface is expected to result in an area expansion of the hydrocarbon region and would be expected to produce a disordering of the lipid acyl chains. However, several findings presented here indicate that this simple packing mechanism cannot account for the effects of synt1 on lipid chain order. First, the C2A domain produces little effect on lipid acyl chain order, even though it penetrates PC:PS bilayers to approximately the same extent as seen for C2B. Second, the synt1 C2 domains bind membranes containing the negatively charged lipid PG but are not observed to produce disorder in these bilayers.

How could the demixing of PS and the decrease in lipid chain order be connected? Phosphatidylserine is a lipid that tends to induce a positive curvature strain in bilayers, a process that is pH-dependent and associated with the PS ionization state (41). As a result, in a membrane of well mixed PC and PS, PS should not have the same curvature tendencies as it does in a demixed state. When well mixed, the charge due to PS will be diffuse on the bilayer interface, and PS will tend to adopt the chain packing of the surrounding PC. However, when demixed or phase-separated, PS lipids will experience an additional repulsive force in the headgroup region due to neighboring lipids. The hydrocarbon region will respond to this repulsive interaction by increasing its disorder and its area per lipid. If this is not sufficient to relieve all the strain, the bilayer may assume positive curvature locally and buckle where PS is concentrated.

The ability of the synt1 C2 domains to demix PS from PC when other lipids are present follows the expected behavior. It is well known that PC and PS do not form ideal mixtures (42, 43) and are, therefore, prone to demix. However, PS and PE are reported to form nearly ideal lipid mixtures (44). The addition of PE to a PC:PS mixture resulted in a lower level of fluorescence quenching when the synt1 C2 domains were added and by inference an incomplete level of demixing or phase separation (Fig. 5e). Cholesterol, which is reported to promote PS demixing (35), enhanced the level of fluorescence quenching when added to a PC:PS:PE mixture (Fig. 5f).

Highly basic peptides derived from the myristoylated ala-nine-rich protein kinase C substrate (MARCKS) as well as a number of synthetic basic peptides have been shown to sequester PI(4,5)P$_2$ (45) but not PS (30). Because PI(4,5)P$_2$ is multivalent, the strength of the electrostatic interaction between this lipid and a charged peptide is enhanced relative to that of PS, and the weaker peptide-PS interaction is apparently insufficient to overcome the entropic loss associated with demixing. Here, the observation of PS demixing suggests that the interaction of the synt1 is not entirely analogous to that of these charged peptides and that there must either be a reduction in the entropy loss associated with demixing or additional favorable C2 domain/PS interactions, such as the lipid–protein interactions detected here by NMR.

Phosphatidylserine appears to be required for membrane fusion, and depletion of PS severely impairs membrane fusion in vivo (46). There are several ways in which PS might play a role in membrane fusion. In the context of the current work, Ca$^{2+}$-dependent PS demixing, which is induced by C2AB, might act as the trigger for exocytosis. The precise lipid composition or lipid phase structure at the focal site of fusion is not known. Nonetheless, the data shown here indicate that PS can be demixed by synt1 from mixtures of lipids commonly found in the inner leaflet of the plasma membrane. In the membrane fusion “stalk” model, the vesicle and plasma membranes are brought into proximity and the membrane structure is perturbed to form a hemifusion intermediate (47). This is followed by fusion of the inner layers and the generation and enlargement of a fusion pore (48, 49). The two C2 domains of synt1, C2AB, have the ability to bridge membrane bilayers, which may help bring...
the in vivo role of syt1 in fusion will require careful attention to bilayer composition.

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