

# The $\text{Ca}^{2+}$ Affinity of Synaptotagmin 1 Is Markedly Increased by a Specific Interaction of Its C2B Domain with Phosphatidylinositol 4,5-Bisphosphate<sup>§</sup>

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The synaptic vesicle protein synaptotagmin 1 is thought to convey the calcium signal onto the core secretory machinery. Its cytosolic portion mainly consists of two C2 domains, which upon calcium binding are enabled to bind to acidic lipid bilayers. Despite major advances in recent years, it is still debated how synaptotagmin controls the process of neurotransmitter release. In particular, there is disagreement with respect to its calcium binding properties and lipid preferences. To investigate how the presence of membranes influences the calcium affinity of synaptotagmin, we have now measured these properties under equilibrium conditions using isothermal titration calorimetry and fluorescence resonance energy transfer. Our data demonstrate that the acidic phospholipid phosphatidylinositol 4,5-bisphosphate ( $\text{PI}(4,5)\text{P}_2$ ), but not phosphatidylserine, markedly increases the calcium sensitivity of synaptotagmin.  $\text{PI}(4,5)\text{P}_2$  binding is confined to the C2B domain but is not affected significantly by mutations of a lysine-rich patch. Together, our findings lend support to the view that synaptotagmin functions by binding in a *trans* configuration whereby the C2A domain binds to the synaptic vesicle and the C2B binds to the  $\text{PI}(4,5)\text{P}_2$ -enriched plasma membrane.

Calcium-dependent secretion of neurotransmitter-loaded synaptic vesicles is at the heart of synaptic transmission. The underlying membrane fusion reaction between vesicle and plasma membrane has been intensively studied and found to be promoted by both protein-protein as well as protein-lipid interactions. From the multitude of proteins involved in this membrane fusion event, the  $\text{Ca}^{2+}$ -binding protein synaptotagmin 1 is one of its central regulating factors (for review, see Refs. 1–6). Synaptotagmin 1 is anchored in the membrane of synaptic vesicles via a single transmembrane region. Its N-terminal region comprises a short luminal domain, whereas the larger cytoplasmic C-terminal region consists of tandem C2 domains, termed C2A and C2B, tethered to each other via a short linker (7) (a schematic outline of the structural features of synaptotagmin 1 is given in Fig. 1A). Several isoforms with similar domain structure have been identified (8).

C2 domains are  $\text{Ca}^{2+}$  binding modules of ~130 amino acids, first described as the second conserved region of protein kinase

C (PKC)<sup>2</sup> (9). The C2A domain of synaptotagmin 1 was the first C2 domain structure to be determined (10). In subsequent studies other C2 domains, including the C2B domain of synaptotagmin, were shown to exhibit very similar three-dimensional structures. They have a conserved eight-stranded anti-parallel  $\beta$ -sandwich connected by surface loops. C2 modules are most commonly found in enzymes involved in lipid modifications and signal transduction (PKC, phospholipases, phosphatidylinositol 3-kinases, etc.) and proteins involved in membrane trafficking (synaptotagmins, rabphilin, DOC2, etc.) (11).

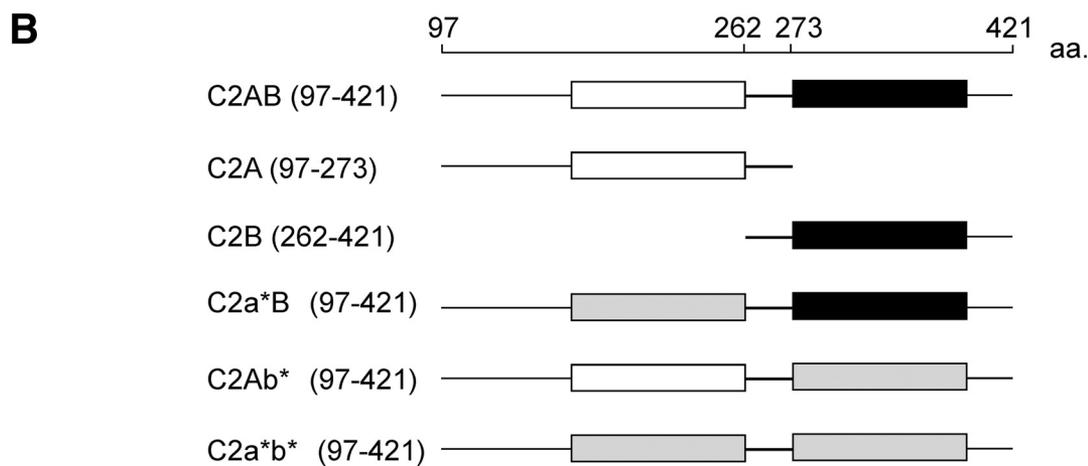
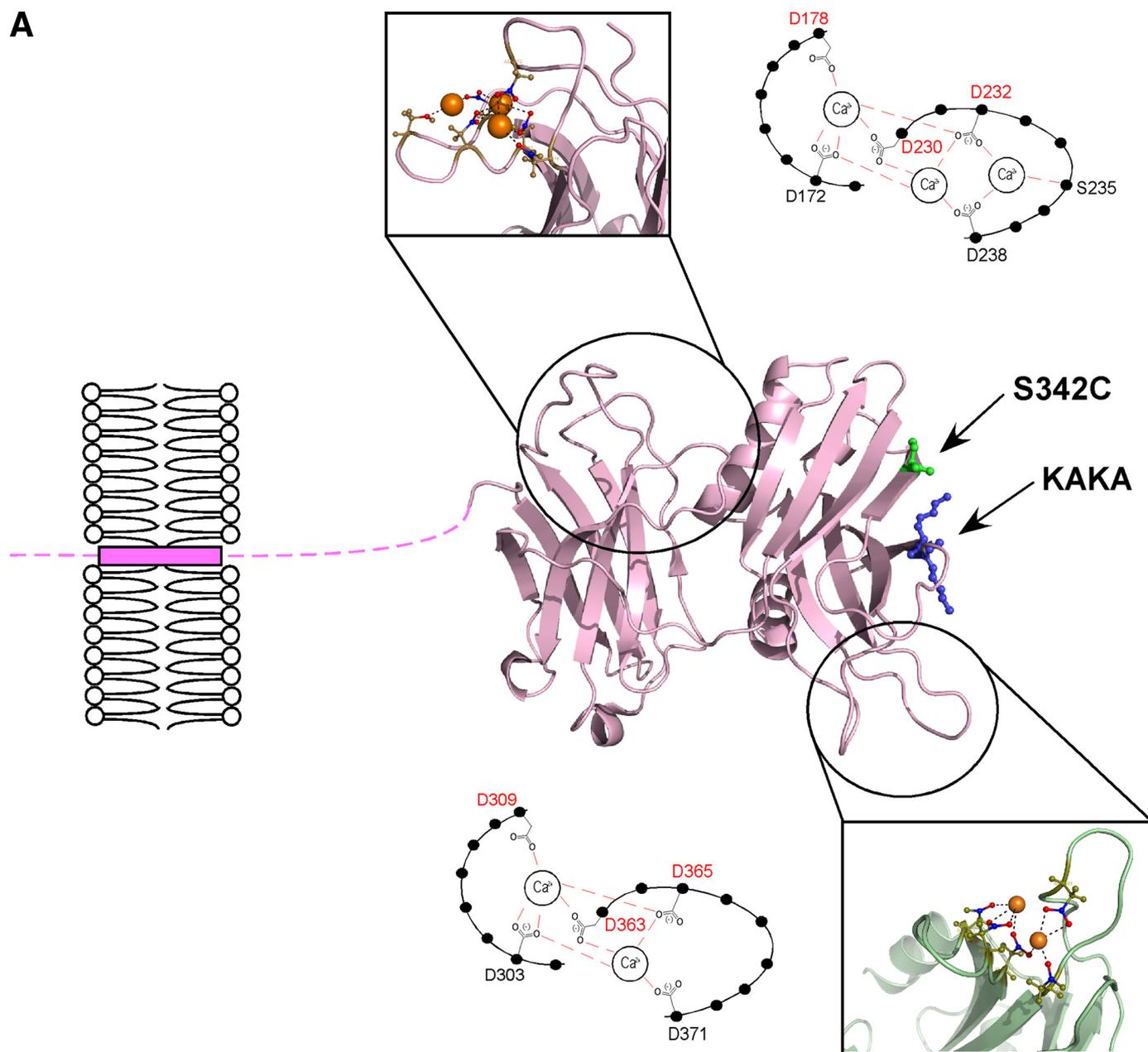
Calcium ions bind in a cup-shaped depression formed by the N- and C-terminal loops of the C2 key motifs of C2 domains. Notably, the coordination spheres for the  $\text{Ca}^{2+}$  ions are incomplete (12, 13). In canonical C2 domains, this incomplete coordination sphere can be occupied by anionic and neutral (14, 15) phospholipids, enabling the C2 domain to be attached to the membrane. Hence, it is thought that the general function of C2 domains is to mediate  $\text{Ca}^{2+}$ -triggered binding of the protein to a membrane. In fact, upon rise of the intracellular calcium level, C2 domain-containing enzymes are translocated to the membrane so that the catalytic domains can interact with lipids or membrane-anchored protein substrates (11). Yet synaptotagmin 1 does not contain such a catalytic domain, suggesting that the properties of its tandem C2 domains are the sole key to understanding its molecular function. In neurotransmission, synaptotagmin is thought to transmit the  $\text{Ca}^{2+}$  signal onto the core membrane fusion machinery, composed of the three SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor) proteins syntaxin 1, SNAP-25 (Q-SNAREs, residing on the plasma membrane), and synaptobrevin 2 (also referred to as VAMP2 (vesicle-associated membrane protein) (R-SNARE, residing on the synaptic vesicle)). So far the multifarious interplay between the SNARE machinery, the two fusing membranes, and synaptotagmin 1 is not well understood. The crystal structure of the entire cytosolic domain of synaptotagmin in the absence of  $\text{Ca}^{2+}$  has revealed an interesting domain arrangement with the two C2 domains facing in opposite directions (16), hinting at the possibility that the mol-

<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. 1–4.

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<sup>2</sup> The abbreviations used are: PKC, protein kinase C;  $\text{PI}(4,5)\text{P}_2$ , phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; DPTA, diethylenetriaminepentaacetic acid; SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, SNAP receptor; SNAP-25, synaptosomal-associated protein of 25 kDa; ITC, isothermal titration calorimetry; FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance; TRPE, phosphatidylethanolamine; aa, amino acids; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.

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ecule might interact with two opposing membranes upon rise of intracellular Ca<sup>2+</sup>.

Although the underlying processes of Ca<sup>2+</sup> binding and Ca<sup>2+</sup>-dependent membrane binding of synaptotagmin 1 have been studied by a multitude of structural and biochemical investigations, they have not revealed features of synaptotagmin C2 domains that are different from those of other C2 domain-containing proteins. Calcium binding to synaptotagmin in the absence of membranes has been studied by NMR. These studies showed that the isolated C2A domain of synaptotagmin 1 binds three calcium ions with an apparent affinity of ~60–75 μM, ~400–500 μM, and more than 1 mM (17). The isolated C2B domain binds two calcium ions with similar calcium affinities in the range of ~300–600 μM (18). The relatively low intrinsic Ca<sup>2+</sup> affinities of both C2 domains are difficult to reconcile with the role of synaptotagmin 1 as the Ca<sup>2+</sup> sensor for fast and synchronous neurotransmitter release, suggesting that interaction with phospholipids contributes to its Ca<sup>2+</sup> sensitivity. Indeed, Ca<sup>2+</sup>-triggered binding of isolated C2 domains to lipid membranes was first shown in an *in vitro* study of synaptotagmin 1 using a fluorescence-based approach (19). Subsequent equilibrium fluorescence studies have shed more light on the molecular process underlying membrane binding of synaptotagmin 1, for example by demonstrating that the isolated C2A domain dips into the membrane bilayer upon Ca<sup>2+</sup> binding (20). This penetration was corroborated by electro-paramagnetic resonance (EPR) spectroscopy studies, which also showed that the penetration depth increased when both C2 domains of synaptotagmin 1 were attached to each other (21) as compared with the single domains (22, 23). However, a variety of different Ca<sup>2+</sup> and lipid preferences for the individual C2 domains of synaptotagmin has been reported (3, 5, 6).

To resolve these discrepancies and to shed more light on the molecular interactions of synaptotagmin 1, we have now used quantitative approaches to study the Ca<sup>2+</sup> concentration and the lipid composition needed for synaptotagmin to bind to membranes. We employed isothermal titration calorimetry (ITC) to measure the intrinsic calcium binding affinities of synaptotagmin 1 C2 domains both as isolated domains as well as in the context of the tandem C2AB protein. Then, we investigated whether the intrinsic calcium affinity is modulated in the presence of lipids using a newly developed fluorescence resonance energy transfer (FRET) approach. In addition, we investigated how Ca<sup>2+</sup> and phospholipid binding of synaptotagmin is affected when the Ca<sup>2+</sup> binding sites in both C2 domains and the putative phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>)-interacting site in the C2 domain are inactivated. We found that the two C2 domains bind calcium largely independently but cooperate in membrane binding. Furthermore, we confirmed that the C2B domain interacts specifically with PI(4,5)P<sub>2</sub>. Remarkably, in the presence of PI(4,5)P<sub>2</sub>, drastically lower amounts of calcium were needed for membrane binding.

## EXPERIMENTAL PROCEDURES

**Protein Constructs**—All protein constructs used were from *Rattus norvegicus* and cloned into the expression vector pET28a. Expression constructs of the isolated C2A domain (aa 97–273), the C2B domain (aa 262–421), the soluble domain of synaptotagmin (aa 97–421), and of the full-length protein (aa 1–421) have been described before (24). Also the following calcium mutants of the full-length protein and of the soluble domain have been described earlier (24): C2a\*B (D178A, D230A, and D232A), C2Ab\* (D309A, D363A, and D365A), and C2a\*b\* (D178A, D230A, D232A, D309A, D363A, and D365A). The constructs for the neuronal SNAREs were the SNARE motif of syntaxin 1A with its transmembrane domain (aa 183–288), a cysteine-free variant of SNAP-25A (aa 1–206), and full-length synaptobrevin 2 (aa 1–116). The synaptotagmin 1 (aa 97–421) KAKA mutant (K326A, K327A) was generated. The single cysteine variant (S342C) was obtained after first removing the single native cysteine (C278S) and then introducing a point mutation at position 342.

**Protein Purification and Labeling**—All proteins were expressed in *Escherichia coli* strain BL21 (DE3) and purified using Ni<sup>2+</sup>-nitrilotriacetic acid beads (GE Healthcare) followed by ion exchange chromatography on the Äkta system (GE Healthcare). The protein concentrations were determined using either the Bradford assay or UV absorption. The single cysteine variant was further labeled with Alexa Fluor 488 C<sub>5</sub> maleimide. This was done by first dialyzing the proteins against the labeling buffer (50 mM HEPES, pH 7.4, 500 mM NaCl, 100 μM Tris(2-carboxyethyl)phosphine). The dialyzed protein solution was then incubated with the fluorophore for 2 h at room temperature and separated from the free dye using a Sephadex G50 superfine column. The transmembrane region containing proteins syntaxin 1A (183–288) and synaptobrevin 2 (1–116) were purified by ion-exchange chromatography in the presence of 15 mM CHAPS. The binary complex containing syntaxin 1A (183–288) and SNAP-25A was assembled from purified monomers and subsequently purified by ion-exchange chromatography in the presence of 1% CHAPS. Full-length synaptotagmin was purified in the presence of 0.03% (w/v) *n*-dodecyl-β-D-maltoside using ion exchange and size exclusion chromatography essentially as described before (24).

**Preparation of Liposomes**—All lipids were purchased from Avanti Polar Lipids except for the Texas Red-labeled phosphatidylethanolamine (TRPE), which was purchased from Invitrogen. Liposomes were prepared as previously described (24), with a few modifications. Briefly, lipid mixes with either 0 mol % (phosphatidylcholine (PC):phosphatidylethanolamine (PE):TRPE:phosphatidylserine (PS):cholesterol = 70:17:3:0:10) or 30 mol % (PC:PE:TRPE:PS:cholesterol = 40:17:3:30:10) PS stocks were first prepared. These stocks were then mixed in appropriate amounts to obtain the desired PS concentrations. In the case where PI(4,5)P<sub>2</sub> was used, 1 mol % PI(4,5)P<sub>2</sub> was

FIGURE 1. **Structure of synaptotagmin 1.** Synaptotagmin 1 protein consists of two C2 domains, C2A and C2B, that coordinate three and two calcium ions, respectively (16). The acidic residues that coordinate calcium binding is shown schematically, with the residues mutated in the calcium binding mutants (*i.e.* C2Ab\*, C2a\*B, and C2a\*b\*) shown in red. The Lys-rich patch is represented as a ball-and-stick model colored blue with the single cysteine site for the FRET assay (S342C) colored in green (A). The different mutants and constructs used in the study are schematically depicted (B).

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added to the lipid stock solutions with the PC amount corrected accordingly. The liposomes were formed by detergent removal using the Fast Desalting PC 3.2/10 column on the SMART system (GE Healthcare). The PS concentration was calculated from the total phospholipid concentration, which was determined using the total phosphate determination method (25).

**ITC**—The protein solutions were dialyzed twice against the ITC buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM  $\beta$ -mercaptoethanol). The buffer was pretreated with Chelex-100 (Bio-Rad; calcium binding constant  $\approx 4.6 \times 10^3 \text{ M}^{-1}$ ) to remove residual calcium ions bound with moderate affinity. This was done by first washing the Chelex-100 beads with water and then adding the beads directly to the dialysis buffer for 2 h. The Chelex-100 beads were removed by filtration through a glass filter. The buffer pH was adjusted and filtered through a 0.2- $\mu\text{m}$  filter. The ITC experiment was done as previously described (26). The protein solution was loaded into the sample cell, and the calcium chloride solution was loaded in the syringe. The calcium chloride solution, in the syringe, was prepared by diluting a 1 M stock solution with ITC buffer to the appropriate concentration. The synaptotagmin 1 protein concentration ranged from 50 to 600  $\mu\text{M}$ , and the calcium chloride concentration was between 8 and 20 mM. Calcium chloride was injected at 3- $\mu\text{l}$  injections 100 times, and the heat evolved per injection was measured. To obtain the effective heat of binding, the heat of dilution, measured by injecting the calcium chloride solution into buffer, was subtracted. All ITC data were analyzed using the Microcal Origin ITC software packet.

**Assay for Synaptotagmin 1-Liposome Binding**—All measurements were carried out in a Fluorolog 3 spectrometer (Horiba Jobin Yvon) in the assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl) at 25 °C in 1-cm quartz cuvettes. Labeled synaptotagmin 1 (C342 Alexa 488) was used to a final concentration of 0.2  $\mu\text{M}$  per reaction, and the spectra upon the addition of Texas Red-labeled liposomes was recorded from 500 to 700 nm with the slit widths set at 2 nm for both the excitation and emission channels. To compare the different experiments with different lipid mixes, the maximum donor fluorescence intensity ( $\lambda = 520 \text{ nm}$ ) at different points of the experiments ( $F$ ) was normalized to a base-line value before the addition of liposomes ( $F_0$ ) to obtain the relative changes in the fluorescence intensities ( $F_0/F$ ). The liposome titrations were done by measuring the donor fluorescence intensity ( $F$ ) upon the addition of the labeled liposomes (Texas Red phosphatidylethanolamine). These intensity counts ( $F$ ) are then normalized similar to above to a base-line value ( $F_0$ ) before the first titration of liposomes ( $F_0/F$ ). The normalized intensity values are plotted against the PS concentration. The calcium titration was done by mixing 0.2  $\mu\text{M}$  labeled synaptotagmin with saturating amounts of liposomes ( $\sim 0.4 \text{ mM}$ ) in the assay buffer containing 10 mM 1,3-diamino-2-propanol-*N,N,N',N'*-tetraacetic acid (DPTA,  $K_D = 80 \mu\text{M}$ ) used to buffer the free calcium. Calcium chloride stock solution was then titrated at a number of steps with the donor signal recorded at each of these steps. Using a similar normalization method as described above (in this case,  $F_0$  represents the donor intensity before calcium addition), the donor intensity was then plotted against the free calcium concentrations, which were

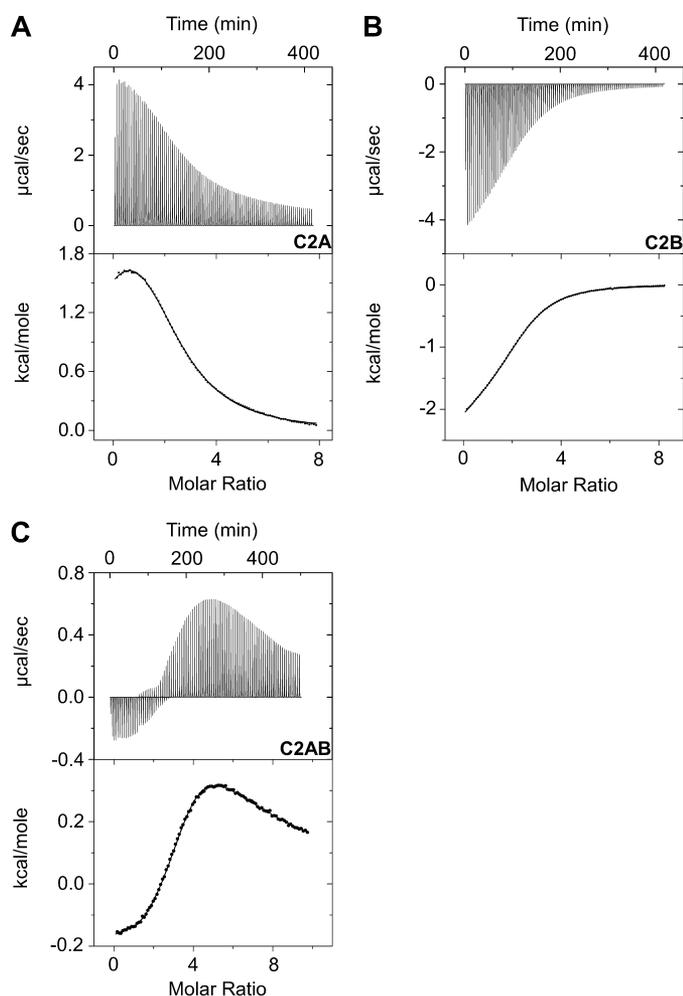
calculated from the total calcium concentrations using the Igor Pro software.

**Liposome Fusion Assay**—Liposome preparation and fusion experiments were done as previously described (24). Briefly, liposome fusion reactions were performed at 30 °C and were followed by FRET between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl), the energy donor, and rhodamine, the energy acceptor. For proteoliposome containing the binary complex of syntaxin 1a and SNAP-25, lipids were mixed in the following molar ratio (in mole %): PC:PE:PS:cholesterol (60:20:10:10). To compensate for the lack of PS in synaptobrevin liposomes, a higher ratio of PC (70) was used. Fluorescence dequenching was measured ( $\lambda_{\text{ex}} = 460 \text{ nm}$ ;  $\lambda_{\text{em}} = 538 \text{ nm}$ ). For each reaction, 10  $\mu\text{l}$  of labeled liposomes and 15  $\mu\text{l}$  of unlabeled liposomes were mixed in 1.2 ml of buffer containing 20 mM MOPS, pH 7.4, 150 KCl, 10 mM DPTA and the appropriate amounts of calcium chloride. For experiments with precise Ca<sup>2+</sup> conditions, the free Ca<sup>2+</sup> concentrations were determined using the fluorescent dye Mag-Fura2 and a Ca<sup>2+</sup> calibration kit (Invitrogen).

## RESULTS

**Calcium Binding of Synaptotagmin 1 in the Absence of Membranes**—To study the intrinsic calcium binding properties of synaptotagmin 1, we employed ITC, adapting an approach previously used for the C2 domains of classical PKCs (26, 27) and phospholipases (14, 28). The ITC approach allows for measuring the heat change associated with binding by simply titrating the ligand to the macromolecule. The heat changes are then integrated and fitted to obtain the entire set of thermodynamic parameters of the interaction. To test whether binding constants determined by ITC agree with earlier NMR studies (17, 18), we initially performed the titration on the isolated C2A (aa 97–273; see Fig. 2A) and C2B (aa 262–421; Fig. 2B) domains of synaptotagmin 1 (an overview of the constructs used for ITC measurements is given in Fig. 1B). Typically, injections of CaCl<sub>2</sub> into solution containing the individual C2 domains of synaptotagmin produced strong heat changes. With progressive injections the heat signal diminished as the Ca<sup>2+</sup> binding sites of synaptotagmin became gradually saturated, and eventually only background heat of dilution was observed. The integrals of the heat changes were then fitted according to the number of Ca<sup>2+</sup> binding sites in the individual C2 domains of synaptotagmin 1 as previously determined by NMR (17, 18). The C2A domain had been described to contain three interdependent Ca<sup>2+</sup> binding sites comprising different affinity ranges. In fact, the best fit of our ITC data for the C2A domain was obtained with a three-site sequential binding model. The C2B domain was shown by NMR to independently bind two Ca<sup>2+</sup> ions with similar affinities. The best fit of the ITC data for the C2B domain was obtained using a one-site binding model that assumes that one or more ligands can bind independently.

Our ITC titrations demonstrated that the C2A domain has three binding sites which bound calcium with the  $K_D$  of  $\sim 120 \mu\text{M}$ , 465  $\mu\text{M}$ , and 1.7 mM, and the C2B domain has two binding sites, both with a  $K_D$  of  $\sim 200 \mu\text{M}$ . The thermodynamic parameters of calcium binding to the different synaptotagmin constructs are listed in Table 1. These affinities are very similar to the values obtained by earlier NMR work. We next titrated



**FIGURE 2. Calcium binding to the C2 domain of synaptotagmin 1 measured by ITC.** Calcium chloride was titrated to 594  $\mu\text{M}$  C2A domain (20 mM  $\text{CaCl}_2$ ) (A), 508  $\mu\text{M}$  C2B domain (18 mM  $\text{CaCl}_2$ ) (B), and 500  $\mu\text{M}$  wild-type C2AB (20 mM  $\text{CaCl}_2$ ) (C) at 25 °C in 50 mM HEPES, pH 7.4, 250 mM NaCl, 5 mM  $\beta$ -mercaptoethanol. The upper panels show the raw titration data, and the lower panels show the integrated heat changes after subtracting the heat of dilution. Interestingly, we observed that the two C2 domains of synaptotagmin adopt a thermodynamically divergent mechanism in calcium binding. The C2A domain bound calcium via an endothermic reaction, whereas the C2B domain exhibited an exothermic profile. The thermodynamic parameters of calcium binding are summarized in Table 1.

**TABLE 1**  
Thermodynamic parameters of calcium binding to different synaptotagmin constructs assessed by ITC

Construct	$K_D$	$\Delta H$	$\Delta S$	$\Delta G$
	$\mu\text{M}$	$\text{cal mol}^{-1}$	$\text{cal K}^{-1} \text{mol}^{-1}$	$\text{kcal mol}^{-1}$
C2A	$K_1 = 119 \pm 2$	$\Delta H_1 = 1841 \pm 7.83$	$\Delta S_1 = 24.1$	$\Delta G_1 = -5.34$
	$K_2 = 465 \pm 10$	$\Delta H_2 = 3309 \pm 37.5$	$\Delta S_2 = 26.3$	$\Delta G_2 = -4.53$
	$K_3 = 1663 \pm 20$	$\Delta H_3 = 471.1 \pm 51.0$	$\Delta S_3 = 14.3$	$\Delta G_3 = -3.79$
C2B	$K = 199 \pm 4$	$\Delta H = -2286 \pm 12.2$	$\Delta S = 9.27$	$\Delta G = -5.05$
C2AB	$K_1 = 48.8 \pm 1.3$	$\Delta H_1 = -178.7 \pm 2.63$	$\Delta S_1 = 19.1$	$\Delta G_1 = -5.87$
	$K_2 = 488 \pm 10$	$\Delta H_2 = -131.7 \pm 15.1$	$\Delta S_2 = 14.7$	$\Delta G_2 = -4.51$
	$K_3 = 142 \pm 2$	$\Delta H_3 = -420.3 \pm 21.1$	$\Delta S_3 = 16.2$	$\Delta G_3 = -5.25$
	$K_4 = 3120 \pm 60$	$\Delta H_4 = 5086 \pm 30.7$	$\Delta S_4 = 28.5$	$\Delta G_4 = -3.41$
C2Ab*	$K_1 = 122 \pm 2$	$\Delta H_1 = 40.46 \pm 17.6$	$\Delta S_1 = 18.0$	$\Delta G_1 = -5.32$
	$K_2 = 427 \pm 14$	$\Delta H_2 = 4329 \pm 47.7$	$\Delta S_2 = 29.9$	$\Delta G_2 = -4.58$
	$K_3 = 3440 \pm 110$	$\Delta H_3 = 1176 \pm 114$	$\Delta S_3 = 15.2$	$\Delta G_3 = -3.35$
C2a*B	$K = 134 \pm 3$	$\Delta H = -1316 \pm 12.6$	$\Delta S = 8.45$	$\Delta G = -5.28$

calcium to the wild-type C2AB fragment of synaptotagmin 1 (aa 97–421) to test whether calcium binding is cooperative between the two C2 domains. Notably, because of the opposing enthalpic changes observed for the two C2 domains, the overall

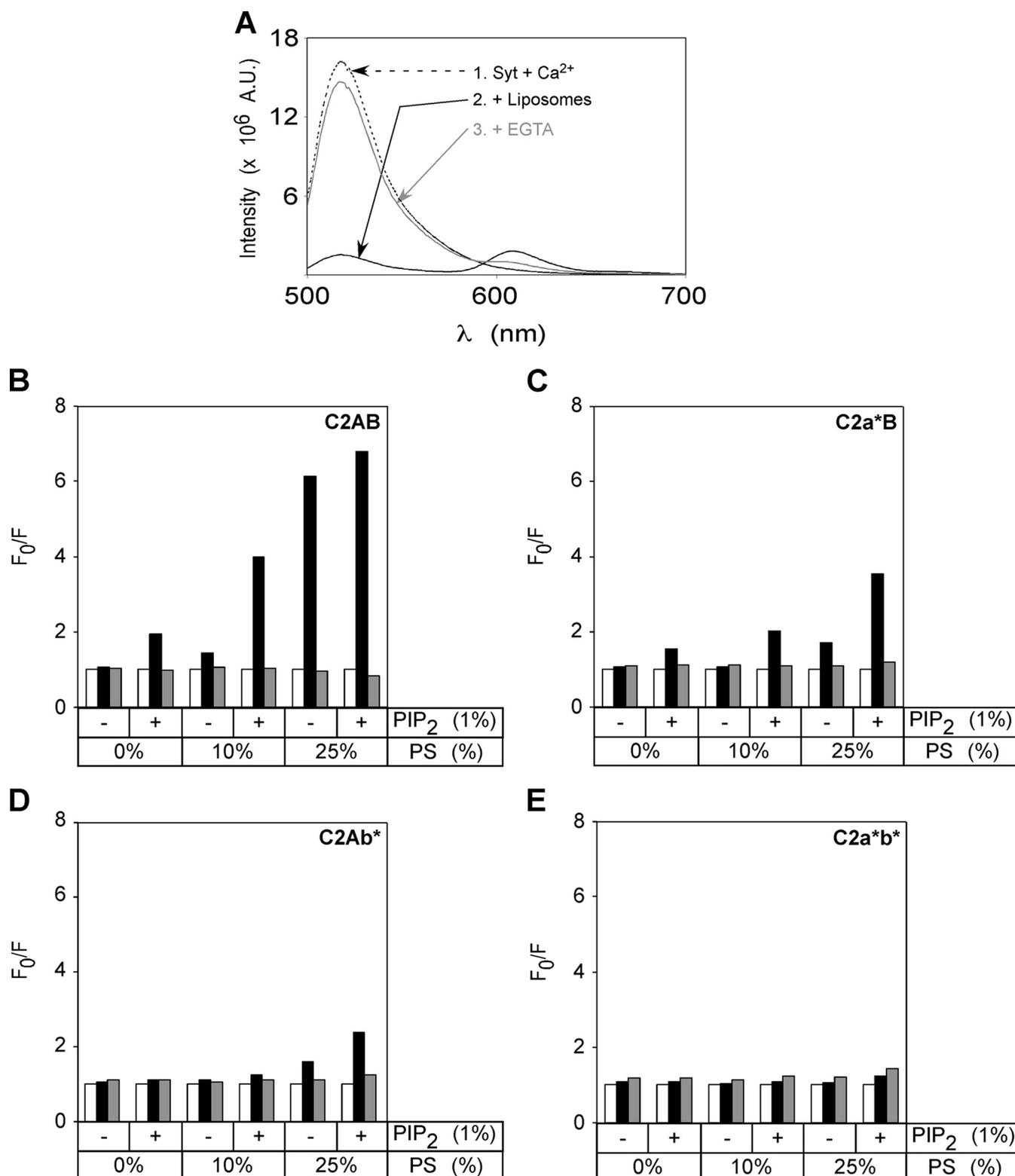
recorded heat changes were much smaller for the wild-type C2AB protein compared with the individual C2 domains (Fig. 2C). The binding isotherm for the C2AB was fitted using a four-site sequential model, again assuming that the two calcium binding sites of the C2B domain bound with similar affinities. According to this model, the calcium affinities were 50  $\mu\text{M}$ , 140  $\mu\text{M}$ , 490  $\mu\text{M}$ , and 3.1 mM. These values only slightly deviate from the results obtained for the individual domains, suggesting that no major cross-talk between the calcium binding sites of the two C2 domains exists.

We also made use of previously described calcium binding mutants for either of the two domains as well as a double mutant that abolishes calcium binding in both C2 domains (24, 29). The calcium mutants are denoted as C2a\*B for the C2A domain mutations, C2Ab\* for the C2B domain mutation, and C2a\*b\* for the double mutant (see “Experimental Procedures” for more details). As expected, the ITC experiment of the double calcium mutant, C2a\*b\*, exhibited no detectable calcium binding activity (supplemental Fig. 1C). The two  $\text{Ca}^{2+}$  binding mutants, in which only one of the two individual C2 domains was mutated, C2a\*B and C2Ab\*, showed ITC profiles comparable with the respective single domain (supplemental Fig. 1, A and B, respectively). Together, these data confirm that the introduced point mutations completely abolish  $\text{Ca}^{2+}$  binding to the mutated C2 domains of synaptotagmin and no auxiliary calcium binding sites are present in this protein.

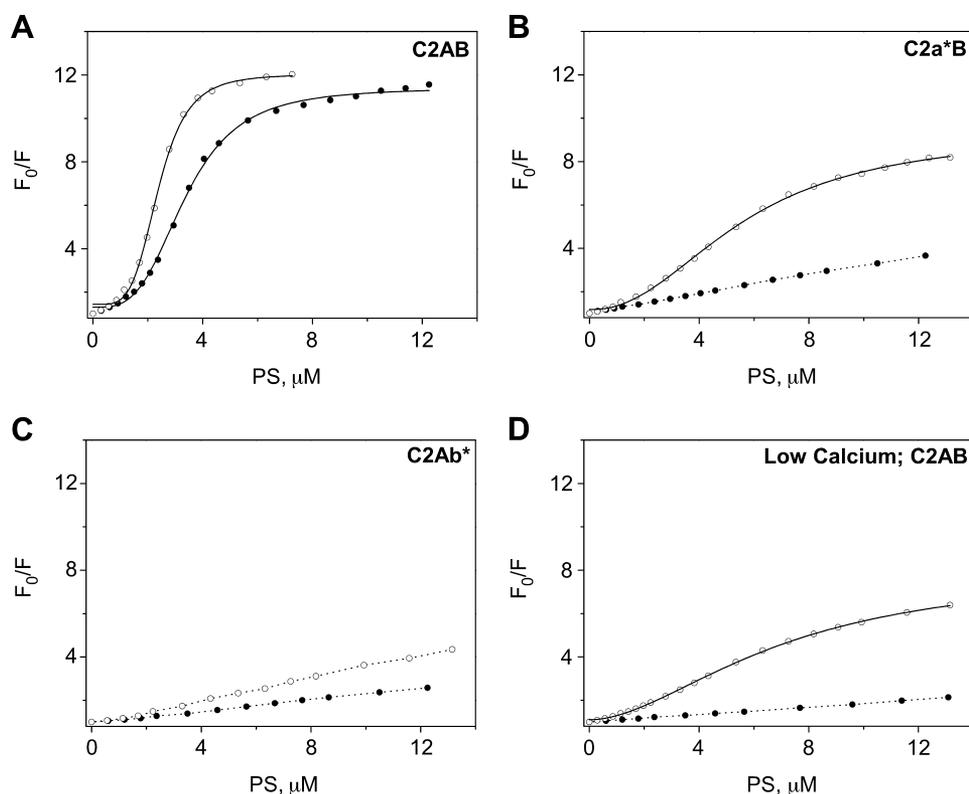
**Synaptotagmin 1 Binding to Liposomes**—The calcium binding experiments enabled us to establish the intrinsic binding properties of the two C2 domains of synaptotagmin 1 for calcium in solution. The next question we had was how the intrinsic calcium binding properties are modulated when lipids are present. We attempted to carry out ITC titrations of calcium to synaptotagmin in the presence of liposomes. However, because of technical difficulties, possibly caused by aggregation, the data we obtained were not sufficiently reliable to be fitted. We, therefore, developed a robust FRET-based assay for the interaction of the soluble portion of synaptotagmin with membranes *in vitro*. For this assay, a variety of single cysteine variants was generated and tested for liposome binding (data not shown). Each single cysteine variant was specifically labeled with the donor fluorophore Alexa 488. Liposomes containing acceptor fluorophores were prepared by incorporating Texas Red-labeled TRPE (3 mol %). Eventually, position 342 (S342C), located in C2B domain of synaptotagmin (Fig. 1A), was chosen for further experiments due to the large and robust signal change associated with this labeling position. As shown in Fig. 3A, the addition of liposomes containing TRPE to fluorescence-labeled synaptotagmin in the presence of 2 mM calcium chloride led to a strong FRET signal change, visible as a decrease in donor fluorescence intensity and an increase in acceptor fluorescence intensity, which was reversible upon chelating  $\text{Ca}^{2+}$  with EGTA, verifying that synaptotagmin is driven onto the membrane solely upon binding of  $\text{Ca}^{2+}$ .

To compare membrane binding at different conditions, quenching of donor fluorescence intensity was normalized as described in the legend to Fig. 3. In the initial set of liposome binding experiments we tested three sets of liposomes containing increasing density of phosphatidylserine (0, 10, and 25%),

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**FIGURE 3. A novel FRET assay allows the monitoring of synaptotagmin 1 binding to liposomes.** Binding was studied using FRET between synaptotagmin 1 labeled with the donor dye, Alexa 488, at position 342 on the C2B domain and liposomes containing phosphatidylethanolamine labeled with Texas Red as acceptor dye. Initially the spectrum was determined for the labeled synaptotagmin (0.2  $\mu$ M) in the presence of 2 mM calcium ( $F_0$ ) (black dotted line). Upon the addition of liposomes (black solid line) and EGTA (gray), subsequent spectra are measured ( $F$ ) (A). To compare the FRET changes for the different liposome samples, the fluorescence at 518 nm is normalized to the baseline value ( $F_0/F$ ). This normalization was done for the different synaptotagmin mutants with liposomes containing different compositions of lipids (i.e. 0, 10, and 25% phosphatidylserine in the absence (denoted as PS) or presence (denoted as PSP) of 1% PI(4,5)P<sub>2</sub> (PIP<sub>2</sub>)) (B–E) (the color scheme is as in A). A.U., absorbance units. For all different liposomes tested, wild-type C2AB (B) exhibits a much stronger FRET signal than the calcium mutants C2a\*B (C), C2Ab\* (D), and C2a\*b\* (E). Note that the C2Ab\* mutant appears to bind somewhat more efficiently to PI(4,5)P<sub>2</sub>-containing membranes but only at higher PS concentrations. It seems, therefore, possible that the mutated C2B domain of the C2Ab\* mutant might still be able to contribute to membrane binding by interacting to some extent with PI(4,5)P<sub>2</sub>, hinting at a cooperative binding mechanism of calcium and PI(4,5)P<sub>2</sub>. The C2a\*b\* variant, which does not bind calcium (supplemental Fig. 1), did not exhibit any detectable binding to the different liposomes.



**FIGURE 4. The two C2 domains of synaptotagmin 1 cooperate for membrane binding.** Texas Red-labeled liposomes containing 25% phosphatidylserine were titrated to 0.2  $\mu\text{M}$  Alexa 488-labeled synaptotagmin in the presence of saturating amounts of calcium (2 mM). Titrations were done with liposomes with (○) and without (●) 1% PI(4,5)P<sub>2</sub> for the wild-type C2AB (A) and the calcium mutants C2a\*B (B) and C2Ab\* (C). When the titration was carried out the presence of 50  $\mu\text{M}$  calcium chloride, hardly any binding of synaptotagmin to liposomes without PI(4,5)P<sub>2</sub> was observed (D). The PI(4,5)P<sub>2</sub>-containing liposomes on the other hand are able to still bind synaptotagmin, albeit with reduced affinity ( $EC_{50} = 7.0 \mu\text{M}$ ), compared with saturating calcium concentrations (2 mM;  $EC_{50} = 2.4 \mu\text{M}$ ). The fits to the Hill function are shown by continuous lines. It is possible that the higher FRET signal observed for the intact protein compared with the Ca<sup>2+</sup> mutants in part arises from its deeper penetration into the membrane (as reported by Herrick *et al.* (21) for the C2AB protein), although this is difficult to confirm in our present study. For each titration the relative fluorescence was plotted against the PS concentration. The PS concentration was calculated from the total lipid concentration, which was determined by measuring the total phosphate content of the liposome sample. Notably, the global membrane binding affinity determined for synaptotagmin is in a similar range found for classical PKC C2 domains (26, 27).

both in the absence and presence of 1% PI(4,5)P<sub>2</sub>. We found that wild-type C2AB did not bind in the absence of anionic phospholipids (PS and PI(4,5)P<sub>2</sub>), confirming that negatively charged lipids are essential for the binding of synaptotagmin to the membrane. The binding strength increased in direct proportion with increasing PS concentrations (Fig. 3B). No further increase in binding strength was monitored at PS concentrations higher than 25% (data not shown). Interestingly, when 1% PI(4,5)P<sub>2</sub> is present in the liposome membrane, we detected an increase in binding strength of synaptotagmin in all tested PS concentrations. To study the contribution of the two different C2 domains, we utilized the calcium mutants described above (C2a\*B and C2Ab\*), again containing a single cysteine at residue 342 for labeling. Compared with wild-type C2AB, both calcium mutants exhibited clearly reduced binding to all tested liposome compositions. As expected, no detectable membrane binding was observed in the double calcium mutant C2a\*b\* (Fig. 3E). The C2Ab\* protein, with an active C2A domain, only bound liposomes with 25% PS regardless of whether PI(4,5)P<sub>2</sub> was present (Fig. 3D). Conversely, the C2a\*B protein, with an active C2B domain, bound liposomes containing 0 and 10% PS only if these

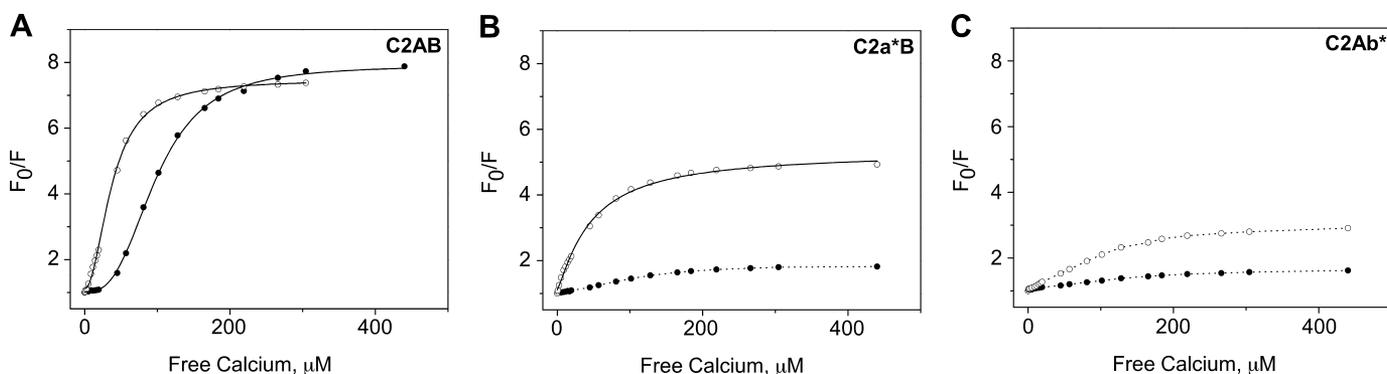
liposomes contained PI(4,5)P<sub>2</sub>. At 25% PS, this mutant was able to bind even in the absence of PI(4,5)P<sub>2</sub> (Fig. 3C).

To gain more insight into the affinity of membrane binding, we titrated labeled liposomes into synaptotagmin-containing solution in the presence of 2 mM calcium chloride. For these experiments we used liposomes containing 25% PS in the absence (denoted as PS) or presence of 1% PI(4,5)P<sub>2</sub> (denoted as PSP) as this concentration of PS resulted in strong Ca<sup>2+</sup>-dependent binding of wild-type synaptotagmin. We found that the affinity of liposomes to the wild-type C2AB was slightly increased when PI(4,5)P<sub>2</sub> was present in the membrane (Fig. 4A;  $EC_{50PS} = 3.4 \mu\text{M}$ ,  $EC_{50PSP} = 2.4 \mu\text{M}$ ). The Hill coefficient in both cases were highly indicative of a cooperative binding ( $n_{PS} = 3.40$ ,  $n_{PSP} = 4.45$ ). In the case of the C2Ab\* mutant, binding to the lipid membrane seemed to be very weak when compared with the wild-type protein both in the absence and presence of PI(4,5)P<sub>2</sub> (Fig. 4C). Even at high concentrations of lipids only marginal binding was detected. Remarkably, binding of C2a\*B mutant to liposomes was also augmented when PI(4,5)P<sub>2</sub> was present in the membrane (Fig. 4B), confirming that PI(4,5)P<sub>2</sub> specifically

operates on the C2B domain of synaptotagmin. Because of much tighter binding of wild-type synaptotagmin, *i.e.* with both Ca<sup>2+</sup> binding domains intact compared with the Ca<sup>2+</sup> mutants, our data corroborate the notion that indeed both C2 domains cooperate in membrane interaction (21).

**Increased Calcium Affinity of Synaptotagmin 1 in the Presence of PI(4,5)P<sub>2</sub>**—To determine whether the calcium affinity of the protein is increased in the presence of PI(4,5)P<sub>2</sub>, we titrated calcium into a mix of labeled synaptotagmin and liposomes containing 25% PS in the absence or presence of 1% PI(4,5)P<sub>2</sub>. The free calcium concentration was buffered with the calcium chelator DPTA. DPTA has a much lower affinity for calcium ( $K_D \approx 80 \mu\text{M}$ ) than EGTA ( $K_D \approx 220 \text{ nM}$  at pH 7.40 (30)) and is, therefore, better suited for buffering the free calcium concentration in a range corresponding to the moderate Ca<sup>2+</sup> affinities of synaptotagmin 1. The data were then fitted using the Hill equation to obtain the apparent affinity for calcium. Remarkably, for wild-type C2AB the apparent calcium affinity increased markedly when PI(4,5)P<sub>2</sub> was present in the membrane ( $EC_{50PS} = 100 \mu\text{M}$ ;  $EC_{50PSP} = 40 \mu\text{M}$ ; Fig. 5A). As a proof

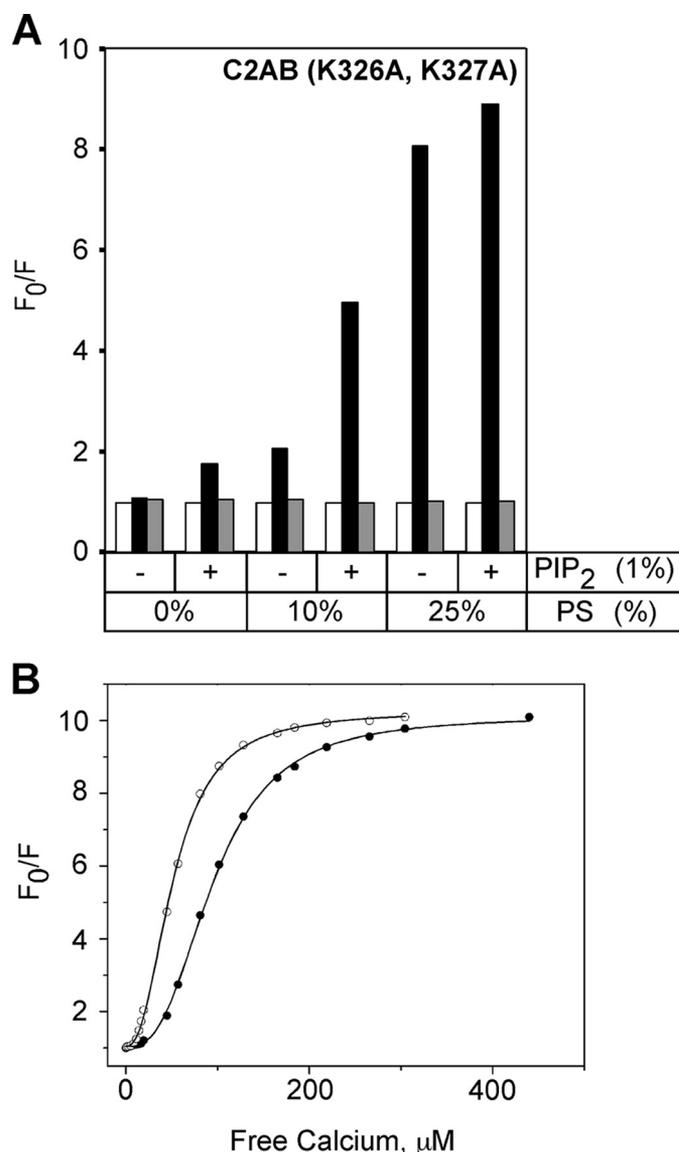
## PI(4,5)P<sub>2</sub> Increases the Ca<sup>2+</sup> Affinity of Synaptotagmin



**FIGURE 5. Calcium affinity of synaptotagmin is augmented by PI(4,5)P<sub>2</sub>.** An excess (~0.4 mM) of Texas Red-labeled liposomes was mixed with 0.2 μM Alexa 488-labeled synaptotagmin in 50 mM HEPES, pH 7.4, 150 mM NaCl containing 10 mM DTPA ( $K_D = 80 \mu\text{M}$ ) as a chelator to ensure accurate free calcium concentrations. Calcium was then added stepwise, and the fluorescence quenching of the donor dye that denotes binding of synaptotagmin to membranes was recorded. The free calcium concentrations were calculated using the Igor Pro software and plotted against the relative fluorescence change. Calcium titrations were done for the wild-type C2AB (A) and the calcium mutants C2a\*B (B) and C2Ab\* (C) using liposomes with (○) and without (●) 1% PI(4,5)P<sub>2</sub>.

of principle, we also performed liposome titrations at lower calcium concentration (50 μM CaCl<sub>2</sub>). At this calcium concentration the binding of liposomes containing only PS to synaptotagmin 1 was extremely weak when compared with the liposomes containing both PS and PI(4,5)P<sub>2</sub> (Fig. 4D). Our data corroborate the earlier report that PI(4,5)P<sub>2</sub> increases the calcium affinity of synaptotagmin (31), although no quantitative information had been provided in the earlier study. When we tested the C2a\*B calcium mutant, we also found an increased Ca<sup>2+</sup>-affinity in the presence of PI(4,5)P<sub>2</sub> (Fig. 5B), although as mentioned above, the overall membrane binding strength of this mutant is much lower than of the intact C2AB. As the C2Ab\* only bound with very low affinity to membranes in the absence and presence of PI(4,5)P<sub>2</sub>, we were unable to accurately estimate the calcium affinity for this variant (Fig. 5C).

A conserved lysine-rich patch in the C2B domain of synaptotagmin 1 is thought to be involved, among a variety of other processes, in binding to PI(4,5)P<sub>2</sub>. This patch consists of four Lys residues in β-strand 4 (Fig. 1A). Mutation of two of the Lys residues (K326A, K327A, dubbed as KAKA mutant) has been reported to abolish the effect of PI(4,5)P<sub>2</sub> on the apparent calcium affinity of synaptotagmin (31). Employing our ITC approach, we first determined that the KAKA mutant exhibits similar Ca<sup>2+</sup> affinities as wild-type synaptotagmin, although the mutant was more prone to precipitate at higher calcium concentrations (data not shown). Hence, the two Lys residues do not contribute significantly to the intrinsic calcium binding properties of the protein. To investigate whether the two Lys residues indeed contribute to the effect of membrane binding via PI(4,5)P<sub>2</sub> interactions, we generated a KAKA mutant containing a single cysteine at position 342 for fluorescence labeling. The mutant also exhibited a strong PI(4,5)P<sub>2</sub> effect at lower PS concentrations, similar to the wild-type protein (Fig. 6A). We then determined the calcium affinity of the KAKA mutant following the FRET approach described above. Surprisingly, in contrast to the earlier study (31), we found that the calcium affinities of the KAKA mutant were almost identical to the ones of the wild-type protein ( $EC_{50PS} = 95 \mu\text{M}$ ;  $EC_{50PSP} = 50 \mu\text{M}$ ; Fig. 6B); that is, the presence of PI(4,5)P<sub>2</sub> was still able to elicit a clear shift in the apparent calcium affinity of the KAKA mutant,



**FIGURE 6. The KAKA mutant behaves similar to the wild-type synaptotagmin protein.** Membrane binding of wild-type or KAKA mutant (K326A, K327A) of synaptotagmin 1 was compared using the FRET assay described in Figs. 3 and 5 (A). Liposomes with different lipid compositions were tested (B) (with similar schemes as described for Fig. 3). For calcium titrations, liposomes with (○) and without (●) 1% PI(4,5)P<sub>2</sub> (PIP<sub>2</sub>) were used.

suggesting that the two mutated Lys residues do not significantly contribute to the PI(4,5)P<sub>2</sub> binding.

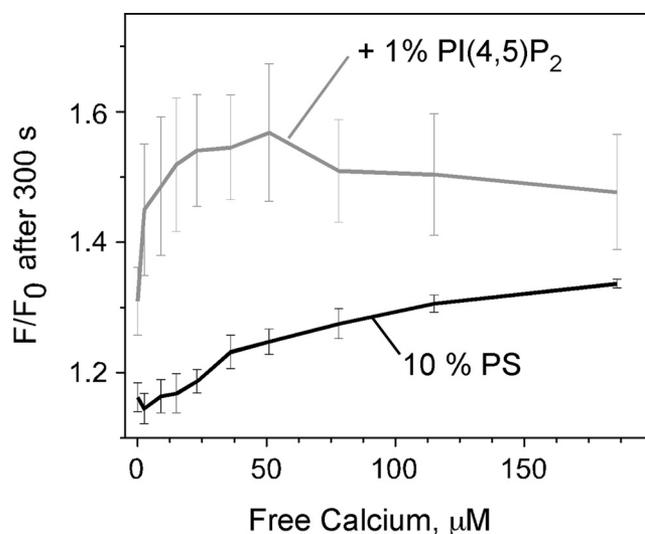
Together our data demonstrate that the two C2 domains of synaptotagmin act as targeting modules that upon Ca<sup>2+</sup> binding drive the protein onto a negatively charged membrane. Both domains exhibit different and mostly independent thermodynamic Ca<sup>2+</sup> binding properties but cooperate during membrane binding. Remarkably, the C2B domain appears to contain a specific PI(4,5)P<sub>2</sub> binding site that increases its ability to bind onto membranes. The affinity for PI(4,5)P<sub>2</sub> is increased by Ca<sup>2+</sup> binding to the C2B domain, suggesting that Ca<sup>2+</sup> not only docks the acidic Ca<sup>2+</sup> binding region to negatively charged phospholipids but also participates in coordinating the head group of the PI(4,5)P<sub>2</sub> molecule.

**In the Presence of PI(4,5)P<sub>2</sub> Less Ca<sup>2+</sup> Is Needed to Stimulate Liposome Fusion**—In the final set of experiments, we tested whether the increased Ca<sup>2+</sup> sensitivity of synaptotagmin 1 has an effect on the process of SNARE protein-mediated membrane fusion. For this approach, we co-reconstituted full-length synaptotagmin 1 together with synaptobrevin into one set of liposomes. This experimental setup avoids the rather unspecific effect of the soluble C2AB domain of synaptotagmin, which probably speeds up SNARE protein-mediated liposome fusion by clustering liposome membranes (for further discussion, see Ref. 24). When synaptotagmin 1 is membrane-bound, fusion is accelerated in the presence of Ca<sup>2+</sup> when only the Q-SNARE liposomes, *i.e.* liposomes containing the co-reconstituted SNARE proteins syntaxin 1 and SNAP-25, contain the negatively charged lipid PS (24). Indeed, in agreement with our observations on the soluble C2AB domain, we found that when PI(4,5)P<sub>2</sub> was added to the membrane of the Q-SNARE liposomes, much less Ca<sup>2+</sup> was needed to accelerate SNARE-mediated liposome fusion in the presence of full-length synaptotagmin (Fig. 7).

## DISCUSSION

The synaptic vesicle protein synaptotagmin 1 is a key factor of the machinery that rapidly catalyzes Ca<sup>2+</sup>-dependent secretion of neurotransmitters. Synaptotagmin is thought to convey the Ca<sup>2+</sup> signal onto the core membrane fusion machinery solely through its tandem C2 domains. A major goal of this work was, therefore, to study Ca<sup>2+</sup> binding and Ca<sup>2+</sup>-mediated interaction of the two C2 domains of synaptotagmin with acidic lipid membranes by employing equilibrium methods. Here we relate these measurements to earlier observations and to structural information and also discuss broader implications of this work for the molecular role of synaptotagmin during exocytosis.

**Calcium Binding to the Synaptotagmin 1 C2 Domains**—Our ITC data on synaptotagmin 1 are largely in concord with earlier NMR results (17, 18). The C2A domain binds three Ca<sup>2+</sup> ions with three different affinities, suggesting that the three binding sites are occupied sequentially and independently. Notably, our ITC titrations revealed a somewhat higher Ca<sup>2+</sup> affinity of the two binding sites of the C2B domain compared with the NMR experiments (NMR = 500–700 μM versus ITC = 200 μM). Furthermore, we confirmed that neutralizing the Ca<sup>2+</sup> binding residues in the two C2 domains abolishes Ca<sup>2+</sup> binding of synap-



**FIGURE 7. Effects of Ca<sup>2+</sup> on the fusion of synaptobrevin liposomes containing full-length synaptotagmin with Q-SNARE liposomes in the presence or absence of PI(4,5)P<sub>2</sub>.** Membrane-inserted synaptotagmin is able to stimulate the process of SNARE protein mediated membrane fusion in the presence of Ca<sup>2+</sup> when only the Q-SNARE liposome membrane contains the acidic lipid phosphatidylserine (24). To test for the effect of Ca<sup>2+</sup> on the fusion process, the Q-SNARE liposome membrane contained 10% PS in the absence or presence of 1% PI(4,5)P<sub>2</sub>, whereas the liposomes containing synaptobrevin did not contain PS. Fusion between syntaxin 1a-SNAP-25-containing liposomes and synaptobrevin-containing liposomes was measured by a standard lipid dequenching assay. Fluorescence values were normalized to the initial fluorescence measured (denoted as F/F<sub>0</sub>). Individual fusion reactions were carried out at different calcium concentrations and repeated three times, each time using freshly prepared liposomes. Selected kinetic traces are shown in supplemental Fig. 4. Although the kinetics were rather complex, *i.e.* composed of at least two different phases, in the presence of 1% PI(4,5)P<sub>2</sub> much less Ca<sup>2+</sup> is needed to increase the efficiency of membrane fusion. To evaluate the stimulating effect, the amount of fusion in each reaction was plotted after 300 s.

totagmin completely. When we measured Ca<sup>2+</sup> binding to the tandem C2AB region of synaptotagmin, we did not observe a major change in the global Ca<sup>2+</sup> affinity, suggesting that both domains bind Ca<sup>2+</sup> largely independently.

ITC provides a direct insight into the thermodynamic processes during binding. Interestingly, the synaptotagmin 1 C2 domains seem to bind calcium with markedly distinctive thermal profiles. The endothermic calcium binding of the C2A domain is coupled with a large favorable entropic change, whereas the profile of the C2B domain is dominated by exothermic enthalpies. We noted that the thermodynamic profiles of both C2 domains of synaptotagmin are different to the ones from classical PKCs, phospholipase A<sub>2</sub> and phospholipase D (26, 28). For classical PKCs, for example, the high affinity binding site is exothermic, and the lower affinity site binds calcium through an endothermic reaction (26). The mechanistic differences in calcium binding between the C2 domains of synaptotagmin remain elusive at the moment, but it is likely that calcium binding might involve divergent forms of solvent reorganizations or conformational changes in the two C2 domains. Previous structural investigations on both C2 domains have suggested that Ca<sup>2+</sup> binding does not induce a significant conformational change but leads to an overall stabilization of the structure, in particular of the C2A domain (17, 32). Hence the large entropic change observed during Ca<sup>2+</sup> binding to the C2A domain might also result from this

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described stabilization of the protein backbone. It should be noted, however, that in another study on the C2 domain of rabphilin, binding of calcium induced a conformational change on one of the calcium binding loops, leading to an enhancement of inositol 1,4,5-trisphosphate (the headgroup of PI(4,5)P<sub>2</sub>) binding (33).

**Influence of the Membrane on Calcium Binding of Synaptotagmin 1**—Remarkably, the Ca<sup>2+</sup> affinities of synaptotagmin 1 are relatively low when compared with that of classical PKC C2 domains. These enzymes are translocated to membranes at relatively low Ca<sup>2+</sup> concentrations, whereas synaptotagmin appears to be activated by a much higher calcium threshold (>10 μM). As synaptotagmin is affixed to the synaptic vesicle membrane *in vivo* by a transmembrane domain, Ca<sup>2+</sup> activation does not lead to a change in its subcellular location but might influence the two C2 domains to interact in a specific spatial orientation to the membranes. It is thought that the Ca<sup>2+</sup> activation step occurs when the synaptic vesicle is already tethered to the plasma membrane. In this framework the C2 domains are already close to two different membranes, the synaptic vesicle membrane (“*cis* interaction”) and the plasma membrane (“*trans* interaction”). Accordingly, the C2 domains do not have to cross a larger distance upon Ca<sup>2+</sup> activation to interact with lipid bilayers.

The exact concentration of calcium required for neurosecretion, *i.e.* the concentration that activates synaptotagmin *in vivo*, is not easy to determine, as it is likely that fusion-competent vesicles are exposed only to a transient and local increase of calcium that enters the cell via close-by voltage-gated Ca<sup>2+</sup> channels. Recent Ca<sup>2+</sup>-uncaging studies in the calyx of Held have revealed a range of 10–25 μM to be sufficient to elicit the release of physiological amounts of neurotransmitter (34), *i.e.* below the intrinsic Ca<sup>2+</sup> affinity of synaptotagmin 1. It is, thus, often assumed that the intrinsic Ca<sup>2+</sup> affinity of synaptotagmin 1 is increased in the presence of acidic phospholipid-containing membranes. Yet, there is so far no strong evidence for such a shift to higher Ca<sup>2+</sup> affinities. Furthermore, the Ca<sup>2+</sup> concentrations determined for binding of synaptotagmin to liposome membranes containing approximately physiological levels of PS vary widely between studies, ranging from 5 to 72 μM (19, 35–37). A reason for these differences may be that in previous studies the free calcium concentration was generally buffered with EGTA. However, due to its high Ca<sup>2+</sup> affinity ( $K_D \approx 220$  nM at pH 7.40 (30)), EGTA effectively buffers free calcium concentrations only at concentrations below 1 μM.

To circumvent these problems, we used the low affinity Ca<sup>2+</sup> chelator DPTA ( $K_D \approx 80$  μM), a chelator that also has been widely used in electrophysiological measurements of neuronal secretion. Using DPTA we determined the free Ca<sup>2+</sup> concentration needed to drive half of the synaptotagmin molecules onto liposomes containing 25% PS to be about 100 μM, close to the affinity range of the higher affinity Ca<sup>2+</sup> sites of synaptotagmin determined by our ITC titrations. It should be noted that the ITC measurements were carried out using a buffer with somewhat higher ionic strength (250 mM NaCl) to prevent protein precipitation compared with the liposome binding studies (150 mM NaCl). The values obtained in both cases are in a similar range, indicating that the change in the salt concentration

from 150 to 250 mM is negligible, although synaptotagmin binds less tightly to membranes at higher salt concentrations (31) (supplemental Fig. 2). Consequently, our results indicate that PS-containing membranes *per se* do not cause a drastic increase in Ca<sup>2+</sup> sensitivity of synaptotagmin. Nevertheless, in contrast to the ITC titrations that confirmed several sequential binding Ca<sup>2+</sup> sites with a broad range of different affinities to be present on the tandem C2 domains, the membrane binding studies can only distinguish between two states, binding and non-binding, and thus cannot reveal an affinity change of individual Ca<sup>2+</sup> sites.

**Role of the Phospholipid PI(4,5)P<sub>2</sub> in the Activation of Synaptotagmin**—The inositol phospholipid PI(4,5)P<sub>2</sub> has long been known to have an important regulatory role in a variety of different cellular processes. PI(4,5)P<sub>2</sub> is known to be enriched in the inner leaflet of the plasma membrane, and it has been demonstrated that PI(4,5)P<sub>2</sub> can influence the calcium affinity of C2 domain-containing proteins (15, 26, 27). In fact, when PI(4,5)P<sub>2</sub> was added to the liposome membrane, we found the Ca<sup>2+</sup> sensitivity of synaptotagmin to be markedly increased to about 40 μM. Moreover, we found that less Ca<sup>2+</sup> is necessary to enhance liposome fusion activity through synaptotagmin when PI(4,5)P<sub>2</sub> is present in the Q-SNARE liposome membrane.

In agreement with previous reports (38–40), our data imply that the site of PI(4,5)P<sub>2</sub> interaction is confined to the C2B domain of synaptotagmin. Interestingly, we observed no significant change in PI(4,5)P<sub>2</sub> binding when two point mutations were introduced in the Lys-rich patch of the C2B domain (KAKA). This finding disagrees with a previous study (31) which reported a minor reduction in affinity of the KAKA mutant (from ~1 μM to 3 μM) using a liposome sedimentation assay. Presently, we have no explanation for this difference. On the other hand, the introduction of the KAKA mutant decreases the Ca<sup>2+</sup> sensitivity of transmitter release (31, 41, 42). Taken together, this might suggest a different activity at the KAKA site, *e.g.* SNARE binding. In fact, this has been shown in an earlier study (43) where, using a fluorescence-based phospholipid binding assay, the KAKA mutation was shown to impair to a large extent SNARE binding but not phospholipid binding. Hence, an obstruction of the interaction with SNAREs might also be a plausible explanation for the observed impairment in release properties seen *in vivo*.

Increases in the calcium affinity of C2 domains in the presence of PI(4,5)P<sub>2</sub> have been reported before for rabphilin-3A (44) and classical PKC (27) C2 domains. For these C2 domains, similar to synaptotagmin, a higher Ca<sup>2+</sup> sensitivity was observed in the presence of PI(4,5)P<sub>2</sub>. It seems possible that that PI(4,5)P<sub>2</sub> and Ca<sup>2+</sup> cooperate in binding of synaptotagmin to membranes such that the head group of the bound PI(4,5)P<sub>2</sub> molecule strengthens the Ca<sup>2+</sup> coordination sphere of the calcium binding site. We noticed that the Lys-rich patch is present in rabphilin and in classical PKC as well. In the case of rabphilin-3A, previous work has shown that four amino acids are involved in binding to PI(4,5)P<sub>2</sub> (Lys-423, His-425, Lys-435, and Arg-437) (44). These residues are found on two of the β-sheets, thereby forming a positively charged patch on the C2 domains. This binding mode was confirmed by the recent crystal struc-

ture of the C2 domain of PKC $\alpha$  bound to PI(4,5)P<sub>2</sub> (45). The homologous residues on the C2A and C2B domains of synaptotagmin 1 are Lys-182, Phe-184, Lys-192, and Glu-194 and Lys-313, His-315, Lys-325, and Lys-327, respectively (supplemental Fig. 3). Based on these residues, the KAKA mutation in the C2B domain only hits one of the four homologous basic residues, *i.e.* Lys-327, found to be involved in binding in rabphilin and PKC $\alpha$ , possibly explaining the absence of a major effect in PI(4,5)P<sub>2</sub> binding. Note that the C2A domain, which does not seem to be influenced by PI(4,5)P<sub>2</sub>, contains one acidic amino acid in this patch. Although these are indeed interesting observations, more detailed experiments would be required to confirm whether this Lys-rich patch is indeed the binding site for PI(4,5)P<sub>2</sub>.

**Cooperativity of Membrane Binding**—Although we observed some binding of synaptotagmin to membranes containing 1% PI(4,5)P<sub>2</sub> in the absence of PS, saturation of binding only occurred in the presence of about 25% PS. Hence, synaptotagmin can bind much more efficiently to membranes containing both PS and PI(4,5)P<sub>2</sub>. In other words, each liposome containing 25% PS and 1% PI(4,5)P<sub>2</sub> is able to sequester many more synaptotagmin molecules onto its surface as compared with liposomes containing only 1% PI(4,5)P<sub>2</sub> and 99% PC. Very likely the higher efficiency is caused by a cooperation of the tandem C2 domains of synaptotagmin. Indeed, recent studies have shown that the C2 domains might cooperate in carrying out its function. Elegant fluorescence approaches monitoring penetration of two C2 domains of synaptotagmin have shown that the C2B domain is heavily influenced in its membrane binding capabilities by the C2A domain and vice versa (40, 46, 47). Furthermore, the tandem protein was shown to penetrate deeper into the bilayer as compared with the single isolated domains by electron paramagnetic resonance spectroscopy (21). Despite the fact that the two different C2 domains of synaptotagmin can discriminate between different lipids, our study now corroborates that they act as a team. Interestingly, our equilibrium titrations suggest that the intact C2AB protein binds to membranes more tightly than the individual domains. When the acidic Ca<sup>2+</sup> ligands of either of the two C2 domains were neutralized, the FRET efficiency was clearly reduced. It must be stressed, however, that it is unclear whether the higher FRET signal for the wild-type protein only reflects an increased binding strength, as a higher FRET efficiency might also arise in part from a deeper penetration into the lipid bilayer.

**Distinct Properties of the Two C2 Domains of Synaptotagmin 1 Might Enable Them to Interact with Opposing Membranes**—The crystal structure of synaptotagmin 1 tandem C2AB protein in the absence of calcium revealed that the two C2 domains calcium binding pockets face away from each other. In the structure, the two domains seem to interact via stabilizing hydrogen bonds (16). Although it is debated whether this configuration of the C2 domains plays a role *in vivo*, the asymmetry and extensive hydrogen bonding between the C2 domains found in the crystal structure make it tempting to speculate that the two C2 domains can readily bind to opposing membranes upon influx of calcium. Calcium binding might lead to repulsion between the two C2 domains that are connected by an eight-amino acid flexible linker, driving them to opposing

membranes during SNARE protein-mediated membrane apposition. Indeed, it has been previously speculated that the C2B domain might interact preferentially with the plasma membrane (24), which is generally enriched in PI(4,5)P<sub>2</sub>, whereas very little PI(4,5)P<sub>2</sub> is present in synaptic vesicle membranes. Interestingly, we now found that less calcium is needed for binding of synaptotagmin, very likely of its C2B domain, to PI(4,5)P<sub>2</sub>-containing membranes. This corroborates the idea that the C2B domain is the first module of synaptotagmin to respond to a rise in intracellular calcium (16). The lipid requirements of the C2A domain appear to be less discriminating, allowing it to interact with the membrane of synaptic vesicle or with the plasma membrane. Also, in a previous study from our laboratory, we showed that the presence of syntaxin-SNAP-25 binary complex in the *trans* membrane tends to drive the synaptotagmin C2B domain to this membrane irrespective of whether calcium is present or not (24). Taken together, these observations evoke the scenario that the C2B domain, in the absence of calcium, might be first tethered on the syntaxin-SNAP-25 complex, located in the plasma membrane. Upon the influx of calcium, the C2B domain is then dislodged from the SNARE proteins and can bind directly to the PI(4,5)P<sub>2</sub>-containing plasma membrane, thereby possibly contributing to membrane fusion.

## REFERENCES

1. Südhof, T. C. (2004) *Annu. Rev. Neurosci.* **27**, 509–547
2. Jahn, R., and Scheller, R. H. (2006) *Nat. Rev. Mol. Cell Biol.* **7**, 631–643
3. Rizo, J., Chen, X., and Araç, D. (2006) *Trends Cell Biol.* **16**, 339–350
4. Wojcik, S. M., and Brose, N. (2007) *Neuron* **55**, 11–24
5. Chapman, E. R. (2008) *Annu. Rev. Biochem.* **77**, 615–641
6. Martens, S., and McMahon, H. T. (2008) *Nat. Rev. Mol. Cell Biol.* **9**, 543–556
7. Perin, M. S., Brose, N., Jahn, R., and Südhof, T. C. (1991) *J. Biol. Chem.* **266**, 623–629
8. Südhof, T. C. (2002) *J. Biol. Chem.* **277**, 7629–7632
9. Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., and Ullrich, A. (1986) *Science* **233**, 859–866
10. Sutton, R. B., Davletov, B. A., Berghuis, A. M., Südhof, T. C., and Sprang, S. R. (1995) *Cell* **80**, 929–938
11. Cho, W., and Stahelin, R. V. (2006) *Biochim. Biophys. Acta* **1761**, 838–849
12. Shao, X., Davletov, B. A., Sutton, R. B., Südhof, T. C., and Rizo, J. (1996) *Science* **273**, 248–251
13. Fernández-Chacón, R., Königstorfer, A., Gerber, S. H., García, J., Matos, M. F., Stevens, C. F., Brose, N., Rizo, J., Rosenmund, C., and Südhof, T. C. (2001) *Nature* **410**, 41–49
14. Xu, G. Y., McDonagh, T., Yu, H. A., Nalefski, E. A., Clark, J. D., and Cumming, D. A. (1998) *J. Mol. Biol.* **280**, 485–500
15. Corbin, J. A., Evans, J. H., Landgraf, K. E., and Falke, J. J. (2007) *Biochemistry* **46**, 4322–4336
16. Fuson, K. L., Montes, M., Robert, J. J., and Sutton, R. B. (2007) *Biochemistry* **46**, 13041–13048
17. Ubach, J., Zhang, X., Shao, X., Südhof, T. C., and Rizo, J. (1998) *EMBO J.* **17**, 3921–3930
18. Fernandez, I., Araç, D., Ubach, J., Gerber, S. H., Shin, O., Gao, Y., Anderson, R. G., Südhof, T. C., and Rizo, J. (2001) *Neuron* **32**, 1057–1069
19. Brose, N., Petrenko, A. G., Südhof, T. C., and Jahn, R. (1992) *Science* **256**, 1021–1025
20. Chapman, E. R., and Davis, A. F. (1998) *J. Biol. Chem.* **273**, 13995–14001
21. Herrick, D. Z., Sterbling, S., Rasch, K. A., Hinderliter, A., and Cafiso, D. S. (2006) *Biochemistry* **45**, 9668–9674
22. Frazier, A. A., Roller, C. R., Havelka, J. J., Hinderliter, A., and Cafiso, D. S. (2003) *Biochemistry* **42**, 96–105
23. Rufener, E., Frazier, A. A., Wieser, C. M., Hinderliter, A., and Cafiso, D. S.

## PI(4,5)P<sub>2</sub> Increases the Ca<sup>2+</sup> Affinity of Synaptotagmin

- (2005) *Biochemistry* **44**, 18–28
24. Stein, A., Radhakrishnan, A., Riedel, D., Fasshauer, D., and Jahn, R. (2007) *Nat. Struct. Mol. Biol.* **14**, 904–911
25. Fiske, C. H., and Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
26. Torrecillas, A., Laynez, J., Menéndez, M., Corbalán-García, S., and Gómez-Fernández, J. C. (2004) *Biochemistry* **43**, 11727–11739
27. Guerrero-Valero, M., Marín-Vicente, C., Gómez-Fernández, J. C., and Corbalán-García, S. (2007) *J. Mol. Biol.* **371**, 608–621
28. Zheng, L., Krishnamoorthi, R., Zolkiewski, M., and Wang, X. (2000) *J. Biol. Chem.* **275**, 19700–19706
29. Tucker, W. C., Weber, T., and Chapman, E. R. (2004) *Science* **304**, 435–438
30. Naraghi, M. (1997) *Cell Calcium* **22**, 255–268
31. Li, L., Shin, O. H., Rhee, J. S., Araç, D., Rah, J. C., Rizo, J., Südhof, T., and Rosenmund, C. (2006) *J. Biol. Chem.* **281**, 15845–15852
32. Shao, X., Fernandez, I., Südhof, T. C., and Rizo, J. (1998) *Biochemistry* **37**, 16106–16115
33. Coudeville, N., Montaville, P., Leonov, A., Zweckstetter, M., and Becker, S. (2008) *J. Biol. Chem.* **283**, 35918–35928
34. Schneggenburger, R., and Neher, E. (2005) *Curr. Opin. Neurobiol.* **15**, 266–274
35. Popoli, M. (1993) *Neuroscience* **54**, 323–328
36. Davletov, B. A., and Südhof, T. C. (1993) *J. Biol. Chem.* **268**, 26386–26390
37. Li, C., Davletov, B. A., and Südhof, T. C. (1995) *J. Biol. Chem.* **270**, 24898–24902
38. Fukuda, M., Kojima, T., Aruga, J., Niinobe, M., and Mikoshiba, K. (1995) *J. Biol. Chem.* **270**, 26523–26527
39. Schiavo, G., Gu, Q. M., Prestwich, G. D., Söllner, T. H., and Rothman, J. E. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 13327–13332
40. Bai, J., Tucker, W. C., and Chapman, E. R. (2004) *Nat. Struct. Mol. Biol.* **11**, 36–44
41. Mackler, J. M., and Reist, N. E. (2001) *J. Comp. Neurol.* **436**, 4–16
42. Borden, C. R., Stevens, C. F., Sullivan, J. M., and Zhu, Y. (2005) *Mol. Cell. Neurosci.* **29**, 462–470
43. Rickman, C., Archer, D. A., Meunier, F. A., Craxton, M., Fukuda, M., Burgoyne, R. D., and Davletov, B. (2004) *J. Biol. Chem.* **279**, 12574–12579
44. Montaville, P., Coudeville, N., Radhakrishnan, A., Leonov, A., Zweckstetter, M., and Becker, S. (2008) *Protein Sci.* **17**, 1025–1034
45. Guerrero-Valero, M., Ferrer-Orta, C., Querol-Audí, J., Marín-Vicente, C., Fita, I., Gómez-Fernández, J. C., Verdaguer, N., and Corbalán-García, S. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 6603–6607
46. Bai, J., Wang, P., and Chapman, E. R. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99**, 1665–1670
47. Hui, E., Bai, J., and Chapman, E. R. (2006) *Biophys J* **91**, 1767–1777

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