Cis- and trans-membrane interactions of synaptotagmin-1

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In neurotransmission synaptotagmin-1 tethers synaptic vesicles to the presynaptic plasma membrane by binding to acidic membrane lipids and SNAREs and promotes rapid SNARE-mediated fusion upon Ca2+-triggering. However, recent studies suggested that upon membrane contact synaptotagmin may not only bind in trans to the target membrane but also cis to its own membrane. Using a sensitive membrane tethering assay we have now dissected the structural requirements and concentration ranges for Ca2+-dependent and -independent cis-binding and trans-tethering in the presence and absence of acidic phospholipids and SNAREs. Using variants of membrane-anchored synaptotagmin in which the Ca2+-binding sites in the C2 domains and a basic cluster involved in membrane binding were disrupted we show that Ca2+-dependent cis-binding prevents trans-interactions if the cis-membrane contains 12–20% anionic phospholipids. Similarly, no trans-interactions were observable using soluble C2AB-domain fragments at comparable concentrations. At saturating concentrations, however, tethering was observed with soluble C2AB domains, probably due to crowding on the vesicle surface and competition for binding sites. We conclude that trans-interactions of synaptotagmin considered to be essential for its function are controlled by a delicate balance between cis- and trans-binding, which may play an important modulatory role in synaptic transmission.

Upon arrival of an action potential, Ca2+ channels in the synaptic membrane open and increase local cytoplasmic Ca2+. This increase is sensed by synaptotagmin-1, a 65-kDa protein anchored to synaptic vesicles (1, 2). Synaptotagmin-1 then triggers fusion of the synaptic vesicles with the plasma membrane resulting in release of neurotransmitter. Fusion itself is mediated by the vesicular R-SNARE synaptobrevin-2 and the plasma membrane Q-SNAREs SNAP-25 and syntaxin-1A. These SNAREs assemble in trans between the membranes and form a tight coiled-coil complex which overcomes the energy barrier of membrane fusion. Synaptotagmin-1 consists of an N-terminal transmembrane helix connected by a long (61-residue) unstructured linker to two C2 domains, called C2A and C2B. The C2A and C2B domains bind three and two Ca2+ ions, respectively (3, 4). They also bind to both individual Q-SNAREs and assembled SNARE complexes (1, 5–7) and to anionic membranes (3, 8–16). Both of these interactions are modulated by Ca2+ and have been implicated in the mechanism of synaptotagmin-1 action (1, 2). In addition, synaptotagmin-1 possesses a polybasic stretch in the C2B domain that is structurally separated from the calcium-binding domain and that mediates calcium-independent binding to acidic phospholipids, particularly phosphatidylinositol-4,5-bisphosphate (PIP2) (8, 9, 15–17).

Despite intense research over the past two decades, it is still unclear by which molecular mechanism synaptotagmin-1 is capable of accelerating exocytosis by more than four orders of magnitude (18). Two types of models are presently discussed that are not necessarily exclusive. The first proposes a direct action of synaptotagmin-1 on the primed state of the fusion apparatus that is established before the arrival of the calcium trigger (3, 4, 19). This state is characterized by partially assembled trans-SNARE complexes in which further zippering is arrested, possibly involving binding of proteins such as complexin or synaptotagmin. Upon activation by calcium ions, synaptotagmin-1 may promote fusion by one of the following mechanisms (20): (i) binding to the SNAREs and thus activating C-terminal zippering, possibly associated with displacement of complexin (activator model); (ii) dissociating from the SNARE complex, thus relieving arrest of SNARE zippering (fusion clamp model); and/or (iii) binding to the lipid bilayer close to the membrane contact site. The latter may destabilize the membrane or induce curvature, thus lowering the energy barrier for fusion. The second model proposes a tethering/docking role of synaptotagmin-1, mediated by “trans” binding to acidic phospholipids in the plasma membrane and/or direct binding to the Q-SNAREs. According to this scenario, calcium activation may result in a closer connection between the vesicle and the plasma membrane that promotes fusion, for instance by facilitating SNARE assembly which is the rate-limiting step in fusion (17).

To shed light on the molecular mechanism of synaptotagmin-1, SNARE-mediated fusion has been reconstituted in liposomes. Both stimulatory and inhibitory effects by synaptotagmin-1 on fusion were reported. In several studies, acceleration was attributed to a tethering/docking function of synaptotagmin-1, which promotes SNARE zippering (19, 21). However, tethering is usually not measured separately, thus a decisive intermediate is not observed. Further complications arise from the observation that membrane-anchored synaptotagmin-1 may bind to its own membrane once activated by calcium (cis-binding). Because cis-binding may compete with membrane tethering (22, 23) it seems likely that this poorly understood phenomenon—which may play an important modulatory role in synaptic transmission—is responsible for the enormous differences in Ca2+ sensitivities of synaptotagmin-1–triggered membrane fusion among various in vitro studies, which can range from as low as 10 μM (24) to higher than 3 mM Ca2+ (25).

In the present study, we have systematically investigated cis- and trans-binding activities of membrane-anchored synaptotagmin using conditions where no fusion occurs. Previous work has shown that membrane binding in trans by synaptotagmin-1 is strong enough to tether membranes. Clustering of liposomes by

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synaptotagmin was observed using dynamic light scattering (DLS) (10, 17) or turbidity measurements (22). However, due to the limited sensitivity of these assays tethering can only be observed when clusters consisting of multiple liposomes are formed. Therefore, we used two-photon fluorescence cross-correlation spectroscopy (TP-FCCS) (23, 26), which is sufficiently sensitive to report tethering between two individual liposomes and can easily be quantified. TP-FCCS is based on analyzing fluorescence fluctuations caused by diffusion of fluorescently labeled liposomes through a two-photon excitation volume (dimension ∼200–500 nm). It is ideally suited to quantify the proportion of free and docked liposomes (for details see Fig. S1 and ref. 23). Autocorrelation analysis of labeled liposomes directly provides their average number in the excitation volume. Cross-correlation of differently labeled liposomes is a direct measure for the proportion of tethered liposomes in the total liposome population. Thus, with TP-FCCS, detailed information about membrane tethering by synaptotagmin-1 can be obtained within a few seconds of measuring time and immediately after initiating tethering by mixing, thus avoiding artifacts such as slow nonspecific aggregation.

**Results**

To analyze the ability of membrane-anchored synaptotagmin-1 to tether membranes, we reconstituted full-length recombinant synaptotagmin-1 into liposomes and measured tethering to protein-free liposomes using TP-FCCS. In addition to wild-type synaptotagmin-1, we used point mutants (C2a*B, C2Ab*, and C2a*b*) in which calcium binding to either one or both C2 domains was disrupted (C2a*B: D178A D230A D232A; C2Ab*: D309A D363A D365A; C2a*b*: D178A D230A D232A, D309A, D363A, and D365A) and mutations in which the polybasic stretch of the C2B domain was inactivated (K326A, D309A D363A D365A; C2a*B: D178A D230A D232A, C2Ab*: D178A D230A D232A, D309A, D363A, and D365A) (3) and mutations in which the synaptotagmin-1 was incorporated at a few seconds of measuring time and immediately after initiating tethering by mixing, thus avoiding artifacts such as slow nonspecific aggregation.

**Fig. 1.** Tethering of liposomes mediated by membrane-bound synaptotagmin-1. The fraction of green acceptor liposomes tethered to red donor liposomes reconstituted with recombinant full-length synaptotagmin-1 was determined with TP-FCCS in the presence (red bars) or absence (black bars) of Ca²⁺ (∼100 μM final concentration, see Fig. S1 for more details). Acceptor liposomes contained 20% PS and (if indicated) 1% PIP2. Donor liposomes contained either no PS (A and B) or 20% PS (C and D). In the control, no synaptotagmin was present in the vesicles. (A) Tethering between donor liposomes reconstituted with synaptotagmin variants [wild type (WT), C2a*B, C2Ab*, C2a*b*, KAKA] and acceptor liposomes. Donor liposomes were free of acidic phospholipids, whereas acceptor liposomes contained 20% phosphatidylserine (PS). (B) Same as A but 1% PIP2, was included in the membrane of the target liposomes. (C and D) Same as in A and B but with 20% PS included in the membrane of the donor liposomes.
native synaptic vesicles (Fig. S3A). When the PS concentration in the synaptotagmin-1–bearing liposomes was reduced to 5% PS, Ca\(^{2+}\)-dependent tethering was restored approximately to the level of liposomes containing no PS (Fig. S3B), whereas Ca\(^{2+}\)-independent tethering, mediated by the polybasic patch, was still inhibited unless PIP\(_2\) was present in the target membrane.

These results were unexpected because soluble C2AB domains were shown previously to cluster liposomes containing acidic phospholipids in the presence of calcium (10, 17, 22). Thus, it is conceivable that membrane anchorage restricts the mobility of the C2 domains in such a way that upon cis-binding there are no free binding sites available that allow for trans-binding. To shed light on this issue, we carried out tethering experiments using a soluble fragment of synaptotagmin-1 containing the C2AB domains (residues 97–421). Intriguingly, Ca\(^{2+}\)-dependent tethering was only observable when the C2AB fragment was added at elevated concentrations (above 200 nM, Fig. 2), whereas virtually no tethering (approximately 4%) was observed at a concentration of 50 nM [comparable to that of the membrane-anchored version (43–120 nM)] even if the incubation time was extended to 30 min. All tethering was reversed upon adding 1–2 mM EGTA (Fig. 2).

It is conceivable that under our experimental conditions a concentration of 50 nM soluble C2AB domain is too low to result in membrane binding upon addition of Ca\(^{2+}\), thus explaining the absence of tethering under these conditions. To find out which C2AB-concentration is necessary for membrane binding, we performed a set of experiments in which Alexa 488-labeled C2AB domains (AF-C2AB) were added to solutions of red liposomes containing 20% PS (Fig. 3A). These experiments revealed that soluble AF-C2AB domains bind with high efficiency at concentrations of 50 nM as well as 215 nM to the membranes even though tethering only began to be observable at concentrations above 215 nM (Fig. 2). Again, binding was reverted by adding 1 mM EGTA.

The discrepancy between Ca\(^{2+}\)-dependent binding and tethering prompted us to investigate whether saturation of binding needs to be achieved for tethering to become apparent. Fluorescence correlation spectroscopy (FCS) is capable of monitoring free and bound AF-C2AB separately, allowing us to address this question directly (Fig. 3B). Whereas at 50 nM AF-C2AB a very large fraction of all protein is bound to the liposomes in the presence of Ca\(^{2+}\), the bound fraction drops significantly at 215 nM, suggesting that binding begins to saturate around this concentration. For further confirmation, we added increasing amounts of unlabeled C2AB domain to the labeled variant AF-C2AB (which was kept at 50 nM for these experiments). Whereas addition of 150 nM only resulted in a slight competition, addition of 400 nM of unlabeled C2AB caused substantial competition, with the fraction of bound labeled AF-C2AB dropping below...
10% (Fig. 3B, columns 5 and 6). We conclude that liposome tethering or clustering is not due to nonspecific electrostatic interactions with Ca2+, because it was not prevented by adding 10 mg l−1 BSA. Addition of Ca2+ did not result in a further enhancement except of a moderate enhancement when only syntaxin was used as target, in agreement with previous reports showing that the interaction between these two proteins is enhanced by calcium. Again, membrane tethering was completely prevented when 20% PS was present in the membrane of the synaptotagmin liposomes (Fig. 4C).

Because most of the observed tethering is Ca2+ independent the question arises whether the polybasic region of the C2B domain is required for such clustering. Therefore, we repeated the experiments using the KAKA mutant in which this region is disrupted (Fig. 4D and E). Intriguingly, both basal and Ca2+ enhancement of tethering was preserved when target liposomes containing free syntaxin were used, whereas binding to both binary and ternary SNARE complexes was reduced to background levels. Again, the observed tethering to free syntaxin was reduced significantly when the synaptotagmin-I liposomes contained 20% PS.

Discussion
Using a sensitive liposome tethering assay based on TP-FCCS we have dissected the contributions of three independent membrane binding sites of synaptotagmin-I, two of which being regulated by Ca2+, to synaptotagmin-I–mediated tethering of membranes. Several conclusions can be drawn from our study (Fig. 5).

Several conclusions can be drawn from our study (Fig. 5). First, membrane-anchored synaptotagmin-I binds to target membranes involving all three binding sites, generally confirming previous reports investigating the C2 domain in recombinant systems (3, 9, 10). In the absence of Ca2+, moderate trans-tethering by the basic cluster occurs. Full tethering by any C2 domain was observed in the presence of 100 μM Ca2+. At around 8.5 μM Ca2+ full tethering

![Fig. 4. Tethering of liposomes mediated by synaptotagmin–SNARE interactions.](image)

![Fig. 5. Diagram summarizing how cis- and trans-membrane interactions of synaptotagmin determine membrane tethering.](image)
was only observed when both C2 domains were intact or when 1% PI-P2 was present in the target membrane. Evidently, membrane anchorage does not interfere with the ability of the C2 domains to interact in trans. Similarly, binding is also observable to membrane-anchored SNAREs, which is (with the exception of binding to isolated syntaxin) not significantly enhanced by calcium, again in agreement with previous studies (1, 5–7). In contrast, all trans interactions were completely abolished when cis binding was enabled by inclusion of 12 or 20% acidic phospholipids (PS) in the resident membrane of synaptotagmin.

This finding is surprising because several previous studies (10, 17, 27) have shown that soluble fragments containing both C2 domains or even only the C2B domain are capable of clustering vesicles. Obviously, clustering can only occur as long as at least two independent binding sites are present. Although we have confirmed this notion, our data show that clustering induced by soluble C2AB domains is only observable when concentrations are used under which binding is saturating, which seems to be the case in most studies. At limiting concentrations soluble C2AB is only capable of interacting with the same membrane. Why membrane cross-linking is only observable under saturating conditions is not clear. If binding sites are limited (as under saturating conditions) the membrane of all liposomes will be similarly crowded. However, cis-binding may be retarded because probably more area is required to position both C2 domains of the same C2AB in the correct orientation on one membrane, whereas less space may still suffice to bind two C2 domains of two different C2AB parallel in trans. Alternatively, it is conceivable that C2AB molecules are capable of trans-interactions that are only sufficiently strong for tethering if the membrane is completely covered with them. We believe that many of the seemingly contradictory findings in the literature (10, 22) can thus be reconciled. In particular, our results confirm and extend previous observations in which reduced fusion efficiency in liposome experiments involving synaptotagmin-1 was attributed to cis-binding of the C2 domains (24, 27), and they may explain some of the conflicting data on synaptotagmin-1 action on fusion in artificial systems (24, 25, 27). While this work was in progress, it was reported that fusion between SNARE and synaptotagmin-containing liposomes in vitro is only stimulated by Ca2+ if there is excess PS in the acceptor membrane, nicely complementing the findings in our study (28). Also, after submission of this manuscript, similar results have been published (29) based on a similar experimental approach as described in Cypionka et al. (23), which largely agrees with the data presented here.

More importantly, the results raise interesting questions concerning the function of cis- vs. trans-interactions of synaptotagmin in the synapse. Synaptic vesicles contain more than 15% anionic phospholipids suggesting that cis-binding may occur under physiological conditions unless prevented by other factors such as charge screening and molecular crowding. On the other hand, in a docked vesicle both the vesicle and the plasma membrane may be sufficiently close to compensate for the preference of cis-binding, thus allowing cross-linking via the C2 domains, with one of them binding to the plasma membrane and the other one to the vesicle membrane (cis-trans) as previously suggested (17, 25). It remains to be clarified whether synaptotagmin action in exocytosis requires such calcium-dependent cross-linking of the C2 domains or whether trans-binding of the C2 domains is sufficient while the protein remains anchored to the vesicle by its transmembrane domain. Also, two recent single-liposome microscopy studies suggested that synaptotagmin-1 massively enhanced membrane fusion even without substantial tethering of the membranes. In these studies tethering was mediated by SNAREs (24, 25) (Fig. 4). Finally, it cannot be excluded (although we consider it as unlikely) that calcium-dependent cis-binding suffices to trigger exocytosis, for instance, by inducing curvature in the vesicle membrane. In any case, membrane tethering by synaptotagmin probably comprises a subtle balance of competing cis- and trans-interactions, which may be modulated by other factors, adding yet another potential mechanism for modulating synaptotagmin-stimulated exocytosis in the synapse.

Methods

Synaptotagmin-1 and SNAREs (rat sequences, bacterial expressed) were purified as described (3, 27). All lipids (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), cholesterol, phosphatidylinositol-4,5-bisphosphate (PI(P)2) were purchased from Avanti Polar Lipids except for the Texas Red-labeled phosphatidylethanolamine (TRPE) and Oregon Green-labeled phosphatidylethanolamine (OGPE), which were purchased from Invitrogen. Lipid mixtures with either 0 mol%, 5 mol%, 12 mol%, or 20 mol% PS, 20% PE (including 1% TRPE or 1.5% OGPE), 10% cholesterol, 0 or 1% PI(P)2, and PC stocks were first prepared by resolving lipid films in 5% sodium cholate HP buffer (20 mM Hepes, 150 mM KCl, 2 mM DTT, 5% sodium cholate, pH 7.4). The final concentration of the lipids was 27 mM. To 16.7 μL of the lipid mixtures, protein was added to achieve a protein/lipid ratio of 1:1,000, except the synaptotagmin-SNAREs experiments (here the synaptotagmin to lipid ratio was 1:500). The liposomes were then formed by detergent removal using a Sephadex G50 superfine column (Bio-Rad). The running buffer for the column was HP150 buffer (20 mM Hepes, 150 mM KCl, 2 mM DTT, pH 7.4). The size of liposomes was about 50 nm. The two-photon confocal microscope has been described in ref. 23, except that we used an UPlanSapo 60x NA 1.2 water immersion objective (Olympus). Membrane tethering was measured at 20 °C by FCCS as described (23) and immediately after mixing 10 nM of each liposome population (approximately 0.09 mg/mL each color) in 20 mM Hepes pH 7.5, 150 mM KCl, 2 mM DTT, 1 mM EGTA with or without 1.1 mM CaCl2 for 100 μM Ca2+. The data presented in Figs. 1–4 and Figs. S2A and S3 represent mean values of at least two independent experiments (bar indicates range of data points) with each experiment representing the average of at least five technical replicates. The Ca2+ titration curves presented in Fig. S2B represent mean values of at least five technical replicates (each of 10 s measuring time) of a single sample batch. The error in the technical replicates was ~10–20%. More details on the sample preparation as well as FCCS analysis can be found in SI Methods.

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**Supporting Information**

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**SI Methods**

**Protein Constructs.** The protein constructs were from *Rattus norvegicus*. They were cloned into the expression vector pET28a. Expression constructs of the full-length protein (amino acids 1–421) and of the soluble domain of synaptotagmin (amino acids 97–421), have been described before (1). In the same publications, the calcium mutants of the full-length protein have also been described (1): C2a+B (D178A, D230A, and D232A), C2Ab+ (D309A, D363A, and D365A), C2a+b+ (D178A, D230A, D232A, D309A, D363A, and D365A), and KAKA mutant (K326A and K327A). The constructs for the neuronal SNAREs were the SNARE motif of syntaxin 1A with its trans-membrane domain (amino acid 183–288), a cysteine-free variant of SNAP-25A (amino acids 1–206), and synaptobrevin 2 without its trans-membrane domain (amino acids 1–96). The synaptotagmin (amino acids 97–421) single cysteine variant (S342C) was obtained after first removing the single native cysteine (C278S) and then introducing a point mutation at position 342 (1, 2).

**Protein Purification and Labeling.** All proteins were expressed in *Escherichia coli* strain BL21 (DE3) and purified using Ni2+-nitrilotriacetic acid beads (GE Healthcare), followed by further purification using ion exchange chromatography as described (1) with a few modifications. The protein concentrations were determined by a Bradford assay or UV absorption (2). Labeling of the synaptotagmin-1 (amino acids 97–421) single cysteine variant (S342C) with Alexa Fluor 488 C5 maleimide was done as follows. First the proteins were dialyzed against the labeling buffer (50 mm Hepes, pH 7.4, 500 mm NaCl, 100 μm Tris(2-carboxyethyl) phosphine). The dialyzed protein solution was incubated with the fluorophore for 2 h at room temperature. Thereafter, the labeled protein was separated from the unreacted dye using a Sephadex G50 superfine column. The labeling efficiency was ~40%. Syntaxin 1A (183–288) and synaptobrevin 2 (1–96) were purified by ion-exchange chromatography (2) 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 (2). The ternary SNARE complex syntaxin 1A (183–288), SNAP-25A, and synaptobrevin 2 (1–96) was generated by incubation of the binary complex and synaptobrevin 2 (1–96) in a ratio of 1:2 overnight at 4 °C. The excess synaptobrevin 2 was removed with Sephadex G50 superfine column during liposome reconstitution. Full-length synaptotagmin was purified in the presence of 1% CHAPS using ion exchange chromatography (as described in ref. 2).

**Liposome Reconstitution.** All lipids were purchased from Avanti Polar Lipids except for the Texas Red-labeled phosphatidylethanolamine (TRPE) and Oregon Green-labeled phosphatidylethanolamine (OGPE), which were purchased from Invitrogen. Lipid mixtures according to Table S1 were first prepared by resolving lipid films in HP buffer (20 mM Hepes, 150 mM KCl, 2 mM DTT, pH 7.4) containing 5% sodium cholate (mass fraction). The final concentration of the lipids was 27 mM. To 16.7 μL of the lipids mixtures protein was added to achieve a protein:lipid ratio of 1:10,000, expect the synaptotagmin-SNAREs experiments (here the synaptotagmin-to-lipid ratio was 1:750). The lipid protein mixtures were adjusted with HP buffer containing 1.5% sodium cholate (mass fraction) to a final volume of 50 μL. The liposomes were formed by detergent removal using a Sephadex G50 superfine column (Sigma; Bio-Rad). The running buffer for the column was HP150 buffer (20 mM Hepes, 150 mM KCl, 2 mM DTT, pH 7.4). The collected liposome volume was about 250 μL. The size of liposomes was about 50 nm. For all liposomes used in this study the average lipid number per liposome was ~12,000. Also, in all experiments the lipid concentration was 10 nm liposomes corresponding to a lipid concentration of 0.09 mg/mL for each type of colored liposomes [based on liposome sizes (3) and space required for lipids (4)]. In Table S1, the composition of the liposomes for all data shown in Figs. 1–4 and Figs. S2 and S3 are shown.

**Fluorescence Cross-Correlation Spectroscopy (FCCS) Setup.** For simultaneous two-photon excitation of differently labelled liposomes we used a titanium-sapphire laser (800 nm, 87 MHz, Fig. S1A). The laser beam was expanded using a lens system and coupled into a dichroic mirror (715 DSCPXR; AHF) into a UPlanSapo 60×/1.2-W water immersion objective (Olympus). The emitted photons passed through the objective and the dichroic mirror. Scattered light from excitation beam was blocked by a short pass filter (E700SP2; AHF). The emission was collected using a second lens system, separated by a second dichroic mirror (590 DCXR; AHF), filtered in each direction with a band pass filter (HQ 645/75 and HQ 535/50; AHF) and collected by separate avalanche photodiodes (APD) (SPC-AOMR-13; Perkin-Elmer). The transistor–transistor logic (TTL) signals from the APD were analyzed using a four-channel router (PRT 400; PicooQuant) and a time-correlated single photon counting (TCSPC) card (TimeHarpoon; PicooQuant) and saved in time-tagged time-resolved (TTTR) format. The correlation was processed using a homemade program.

For the measurements without or with 100 μM Ca2+ either the buffer containing 20 mM Hepes, 150 mM potassium chloride, 1 mM EGTA or the buffer containing 20 mM Hepes, 150 mM potassium chloride, 1 mM EGTA, 1 mM calcium chloride was used. The reaction volume was 100 μL. The measurement was started by diluting the red and green lipid stock solutions into the corresponding reaction buffer and loading a droplet (20 μL) onto the coverslip after short vortexing. The final concentration of the liposomes was ~10 nM for each color (corresponding to ~0.09 mg/mL lipids). The signal traces for the TP-FCCS analysis were recorded six times for 12 seconds for each droplet resulting in a total measuring time of 72 seconds per droplet. This procedure was repeated several times with different droplets from the same solution. Each experiment using different liposome protein and lipid compositions as well as Ca2+ concentrations was repeated at least one time with fresh liposome and buffer preparations.

**Tethering Assay and Binding of Labeled, Soluble C2AB-Fragments.** The tethering assay has been described in detail (3). In general, the average number of particles in the focal detection volume that carry Oregon Green-labeled lipids, Nc, can be calculated from the inverse of the autocorrelation amplitude for the Oregon Green fluorescence Nc = Gd(0)−1 at small lag times (green line in Fig. S1B). Here, a particle can be either a single liposome or a particle consisting of two or more tethered liposomes for which at least one liposome also contains Oregon Green-labeled lipids. Under our experimental conditions, the influence of different liposome/particle compositions on Nc can be neglected (3). In the same manner the average particle number for Texas Red-labeled particles, Nc, can be calculated from the inverse of the autocorrelation amplitude for the Texas Red fluorescence.
Red fluorescence $N_{\text{rg}} = G_{\text{rg}}(0)^{-1}$ at small lag times (red line in Fig. S1B). The average particle number in the focal detection volume that carries both types of labeled lipids, $N_{\text{rg}}$, was calculated from the particle numbers $N_g$ and $N_r$ and the cross-correlation amplitude for the Texas Red and Oregon Green fluorescence (blue line in Fig. S1B) at small lag times: $N_{\text{rg}} = G_{\text{rg}}(0)N_gN_r$. By comparing this number of double-labeled particles, $N_{\text{rg}}$, with the total number of particles carrying green labels, $N_g$, the tethering percentage can be calculated: Tethering (%) = $N_{\text{rg}}/N_g$ · 100. Only in the case of Fig. 3A this percentage was calculated by $N_{\text{rg}}/N_r$ · 100 because here the number of green-labeled C2AB fragment was present in large excess in most cases in comparison with the number of red liposomes. Therefore, the tethering percentage $N_{\text{rg}}/N_r$ · 100 represents the percentage of liposomes carrying significant amounts of C2AB fragments in comparison with the total amount of liposomes. On the contrary, the $N_{\text{rg}}/N_g$ · 100 used for Fig. 3B represents the amount of C2AB fragment attached to liposomes. In this case only relative extents in C2AB fragments binding can be given at higher percentages of bound C2AB, because a liposome carrying many green C2AB fragments is a lot brighter than a single-labeled C2AB fragment. However, even though only relative bound fractions can be exactly concluded from the analysis shown in Fig. 3B it provides clear evidence that at 215 nM C2AB a significantly smaller fraction of C2AB is bound to the membranes than at 50 nM C2AB. This can only be explained by a saturation of the membranes. Because full tethering is still not observed at 860 nM soluble C2AB (Fig. 2), this provides evidence that clustering occurs at saturating concentrations.

Fig. S1. TP-FCCS tethering assay. (A) In the TP-FCCS set-up fluorescence fluctuations caused by diffusion of labeled liposomes through the microscopic two-photon excitation volume (dimensions ∼200–500 nm) are recorded and analyzed by correlation functions. (B) Schematic presentation of the correlation functions. Amplitudes of the red or green auto-correlation curves (correspondingly colored curves) are inversely proportional to the average number of red- or green-labeled liposomes in the detection volume, respectively. For example, the amplitudes of 0.5 of the red and green curves reflect approximately two red- or green-labeled particles that are on average in the excitation volume. Cross-correlation amplitude (blue) relative to the autocorrelation amplitudes is a direct measure for the proportion of tethered red-green liposomes in the total liposome population. (C) Exemplary measured autocorrelation curve of Texas-Red labeled, synaptotagmin-containing vesicles (red) and of the cross-correlation (blue) with Oregon-Green–labeled liposomes. Left, no or little tethering with an inactive mutant of synaptotagmin-1 (D178A D230A D232A D309A K325A K326A D363A D365A). Right, 100% tethering by wild-type synaptotagmin-1. For more details see ref. 1.

Fig. S2. Tethering of liposomes mediated by membrane-bound synaptotagmin-1. Dependence on the Ca\(^{2+}\) concentration and on the presence of PiP\(_2\) in the target membrane. Tethering was measured as in Fig. 1. (A) Ca\(^{2+}\)-concentration dependence of membrane tethering by wild-type synaptotagmin in the absence of PiP\(_2\) in the target membrane. (B) Ca\(^{2+}\)-titration curves of synaptotagmin mutants. At Ca\(^{2+}\) concentrations of about \(\sim 8.5\) μM the tethering for both, C2A*b* and C2a*B*, is lower than at \(\sim 100\) μM (red curves) but in the presence of 1 mol% PiP\(_2\) in the target membrane full tethering is observed (green curves). In contrast to wild-type synaptotagmin, no significant increase of tethering with increasing Ca\(^{2+}\) concentrations can be observed for the double mutant a*b* (black curve). See Fig. 1 legend for an explanation of the synaptotagmin variants.

Fig. S3. Membrane tethering by synaptotagmin in the presence of 5 and 12% PS in the synaptotagmin-containing membrane. (A) Presence of 12% PS prevented membrane tethering in a very similar fashion as 20% PS (Fig. 1), regardless of whether the target membrane contains PS only or PS plus PiP\(_2\). (B) A total of 5% PS is not sufficient anymore to inhibit the activity of synaptotagmin-1 by cis-binding if either 100 μM Ca\(^{2+}\) is present in the solution or 1% PiP\(_2\) in the target membrane. Only the absence of both allows an inactivation of synaptotagmin tethering by the presence of 5% PS in the synaptotagmin-1–containing membranes.

Table S1. Percentages of lipid composition

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Chol, cholesterol; OGPE, Oregon green phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PiP\(_2\), phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; TRPE, Texas red phosphatidylethanolamine.