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 Ca\(^{2+}\) triggering. However, recent studies suggested that upon membrane contact synaptotagmin may not only bind in trans to the target membrane but also in cis to its own membrane. Using a sensitive membrane tethery assay we have now dissected the structural requirements and concentration ranges for Ca\(^{2+}\)-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 Ca\(^{2+}\)-binding sites in the C2 domains and a basic cluster involved in membrane binding were disrupted we show that Ca\(^{2+}\)-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 cis- and 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.

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phosphatidylserine) (PS) (for more details see Table S1). Osmes contained acidic phospholipids (20% of all lipids were
labeled with Oregon green-DHPE. Unless indicated otherwise, target liposomes were labeled by using 1.5 mol% of all lipids
bearing liposomes were free of acidic phospholipids, whereas acceptor liposomes contained 1 mol% Texas red-DHPE, whereas the protein-free liposomes were labeled with 1 mol% of all lipids
that were labeled without synaptotagmin-1 (control in Fig. 1) or with an inactive mutant in which Ca2+ binding in both C2 domains as
well as the polybasic stretch was inactivated (K326A, K327A; KAKA mutant). Synaptotagmin-1 was incorporated in
a 1:1000 molar protein-to-lipid ratio into liposomes that were labeled with 1 mol% Texas red-DHPE, whereas the protein-free
liposomes contained acidic phospholipids (20% of all lipids were phosphatidylserine) (PS) (for more details see Table S1).
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 in 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.

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) (3) and mutations in which the polybasic stretch of the C2B domain was inactivated (K326A, K327A; KAKA mutant). Synaptotagmin-1 was incorporated in a 1:1,000 molar protein-to-lipid ratio into liposomes that were labeled with 1 mol% Texas red-DHPE, whereas the protein-free target liposomes were labeled by using 1.5 mol% of all lipids Oregon green-DHPE. Unless indicated otherwise, target liposomes contained acidic phospholipids (20% of all lipids were phosphatidylserine) (PS) (for more details see Table S1).

In the first set of experiments (Fig. 1A), the synaptotagmin-1 bearing liposomes were free of acidic phospholipids to exclude cis-binding. Under these conditions, moderate tethering was observed that was enhanced more than twofold upon addition of 100 μM Ca2+ (red bars in Fig. 1L) and reverted when Ca2+ was chelated with 500 μM EGTA (<5% tethering). A total of 1 mM Mg2+ did not influence membrane tethering. Tethering was dependent on synaptotagmin-1 because no tethering was observed without synaptotagmin-1 (control in Fig. 1) or with an inactive mutant in which Ca2+ binding in both C2 domains as well as the polybasic stretch was inactivated (<5% tethering in all cases). We can safely exclude membrane fusion under any of the conditions tested in this work, because membrane fusion would result in Förster resonance energy transfer and decreased lifetimes of Oregon green-DHPE (23), which was not observed.

Upon disruption of Ca2+-binding of either the C2A or the C2B domain still a maximum tethering as with wild-type synapto-
tagmin-1 was observed in the presence of 100 μM Ca2+ (a*B and A*b in Fig. 1L). However, when the Ca2+ concentration was reduced (8.5 μM Ca2+), the tethering activity of both mutants was lower (about 10–20% above the level of no Ca2+, Fig. S2B, red lines). When both Ca2+-binding domains were disrupted, no Ca2+-dependent enhancement of tethering was observable (C2a*b* in Fig. 1L) even when the Ca2+ concentration was increased to 880 μM in agreement with previous observations (17, 22) (see also Fig. S2B, black line). Calcium-independent tethering is mediated by the polybasic lysine patch on the C2B domain (10, 17), because removal of charges (KAKA mutant) virtually abolished tethering while Ca2+-dependent tethering remained unaffected (KAKA in Fig. 1L).

To investigate whether the presence of PiP2 enhances tethering, the experiments described above were repeated using target liposomes that, in addition to 20% PS, also contained 1 mol% PiP2 (Fig. 1B). No major tethering differences were observed when using no or 100 μM Ca2+. This finding is not surprising because already full tethering is observed even without PiP2 in the presence of 100 μM Ca2+ (Fig. 1L). However, at reduced Ca2+ concentrations (~8.5 μM) more tethering was observed with the mutants C2a*B and C2aB* when PiP2 was present in the target membrane (Fig. S2B, green lines).

To examine whether binding of synaptotagmin-1 to its own membrane affects its tethering activity, the experiments were repeated using synaptotagmin-1-bearing liposomes containing 20% PS (Fig. 1C and D). Most strikingly, the presence of PS almost completely prevented membrane tethering in all conditions, regardless of whether the target membrane contains PS only or PS plus PiP2. Very similar observations were made when 12% PS was used, a concentration corresponding to that of...
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 reversed 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 effected by soluble C2AB domains in the presence of Ca\(^{2+}\) requires saturation of the membrane surface with C2AB domains (Discussion).

In the final set of experiments, we investigated liposome tethering by binding of membrane-anchored synaptotagmin-I to SNAREs (1, 5–7) (Fig. 4). To rule out trans-binding to acidic phospholipids, the SNAREs were reconstituted into liposomes lacking acidic phospholipids. Efficient tethering was observed when target liposomes containing either syntaxin-1A (183–288) alone (Sx1A), a binary Syntaxin 1A-SNAP-25 complex (Sx1A-SN25) or a fully assembled ternary complex consisting of synaptobrevin 2 (1–96), SNAP-25, and syntaxin 1A (Sx1A-SN25-Sb2) were used (Fig. 4A). This tethering was significantly larger than tethering mediated by SNARE proteins in the absence of synaptotagmin (Fig. 4B). Binding was also not due to nonspecific adsorption because it was not prevented by adding 10 mg l\(^{-1}\) BSA. Addition of Ca\(^{2+}\) 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 Ca\(^{2+}\) 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. 4 D and E). Intriguingly, both basal and Ca\(^{2+}\) 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 Ca\(^{2+}\), to synaptotagmin-I–mediated tethering of membranes. 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 numerous previous reports addressing the membrane-binding properties of isolated C2 domain fragments (3, 9, 10). In the absence of Ca\(^{2+}\), moderate trans-tethering by the basic cluster occurs. Full tethering by any C2 domain was observed in the presence of 100 μM Ca\(^{2+}\). At around 8.5 μM Ca\(^{2+}\) full tethering...
was only observed when both C2 domains were intact or when 1% PI(4,5)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 at least two domains or even only the C2B domain are capable of clustering (217, 27) have shown that soluble fragments containing both C2 domains is in trans. Alternatively, it is conceivable that C2AB molecules are capable of trans-interactions that are only sufficiently strong for tethersing 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 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 consistent with previous findings in our study (22). 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(4,5)P₂), and phosphatidylinositol-4-phosphate (PI(4)P)] were purchased as 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% PI(4,5)P₂, 20% PE (including 1% TRPE or 1.5% OGPE), 10% cholesterol, 0 or 1% PI(4)P, 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-SNARE experiments (see below). Finally, the lipids were spread on microplates as described (28) and 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.

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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 acids 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-1 (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) and the synaptotagmin-1 (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 lipid mixtures protein was added to achieve a protein:lipid ratio of 1:1,000, 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 lipo-some 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 with 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 collimated 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 555/50; AHF) and collected by separate avalanche photodiodes (APD) (SPC-AMR-13; Perkins-Elmer). The transistor–transistor logic (TTL) signals from the APD were analyzed using a four-channel router (PRT 400; PicoQuant) and a time-correlated single photon counting (TCSPC) card (TimeHarp200; PicoQuant) 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, 1.1 mM calcium chloride was used. The reaction volume was 100 μL. The measurement was started by diluting the red and green liposome 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, Np, can be calculated from the inverse of the autocorrelation amplitude for the Oregon Green fluorescence Np = G0(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 Np can be neglected (3). In the same manner the average particle number for Texas Red-labeled particles, Np, can be calculated from the inverse of the autocorrelation amplitude for the Texas Red fluorescence.
Red fluorescence $N_r = G_r(0)^{-1}$ 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, $N_{rg}$, 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: $N_{rg} = G_{rg}(0) N_g N_r$. By comparing this number of double-labeled particles, $N_{rg}$, with the total number of particles carrying green labels, $N_g$, the tethering percentage can be calculated: Tethering (%) = $N_{rg}/N_g$ · 100. Only in the case of Fig. 3A this percentage was calculated by $N_{rg}/N_r$ · 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 $N_{rg}/N_r$ · 100 represents the percentage of liposomes carrying significant amounts of C2AB fragments in comparison with the total amount of liposomes. On the contrary, the $N_{rg}/N_g$ · 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.


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**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 \(\sim 8.5\) \(\mu\)M the tethering for both, C2A*b* and C2a*B*, is lower than at \(\sim 100\) \(\mu\)M (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 \(\mu\)M 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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<th>PC</th>
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<th>PS</th>
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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.

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