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Synaptotagmin activates membrane fusion through a Ca^{2+}-dependent *trans* interaction with phospholipids

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Synaptotagmin-1 is the calcium sensor for neuronal exocytosis, but the mechanism by which it triggers membrane fusion is not fully understood. Here we show that synaptotagmin accelerates SNARE-dependent fusion of liposomes by interacting with neuronal Q-SNARES in a Ca^{2+}-independent manner. Ca^{2+}-dependent binding of synaptotagmin to its own membrane impedes the activation. Preventing this *cis* interaction allows Ca^{2+} to trigger synaptotagmin binding *in trans*, accelerating fusion. However, when an activated SNARE acceptor complex is used, synaptotagmin has no effect on fusion kinetics, suggesting that synaptotagmin operates upstream of SNARE assembly in this system. Our results resolve major discrepancies concerning the effects of full-length synaptotagmin and its C2AB fragment on liposome fusion and shed new light on the interactions of synaptotagmin with SNAREs and membranes. However, our findings also show that the action of synaptotagmin on the fusion-arrested state of docked vesicles *in vivo* is not fully reproduced *in vitro*.

Neurotransmitters are stored in synaptic vesicles that undergo Ca^{2+}-dependent exocytosis upon stimulation. Fusion of synaptic vesicles with the presynaptic plasma membrane is mediated by the neuronal soluble *N*-ethylmaleimide–sensitive factor attachment protein (SNAP) receptor (SNARE) proteins, including synaptobrevin-2 (also referred to as VAMP2) in the membrane of synaptic vesicles and syntaxin-1 and SNAP-25 in the plasma membrane. SNAREs are characterized by conserved stretches of 60–70 amino acid residues, referred to as SNARE motifs. Syntaxin-1 and synaptobrevin-2 each possess a single SNARE motif adjacent to the C-terminal transmembrane domain, whereas SNAP-25 contains two SNARE motifs that are separated by a palmitoylated linker[1,2]. The SNARE motifs are unstructured as monomers[3] but assemble into a tight bundle of four α-helices[4]. SNARE motifs are divided into four conserved subfamilies, referred to as Qa-, Qb-, Qc- and R-SNARE motifs. Each SNARE complex contains one member of each subfamily[5]. The assembly of SNARE complexes is currently believed to be the essential reaction in driving membrane fusion. According to this model, the formation of the SNARE complex is initiated in a *trans* configuration at the N-terminal ends of the SNARE motifs, forming a bridge between the membranes. Assembly then proceeds toward the C-terminal membrane anchor domains, clamping the membranes together and thus overcoming the energy barrier for fusion[6–8].

In contrast to several other SNARE-dependent fusion reactions, neuronal exocytosis is strongly upregulated by calcium[9]. The fast component of Ca^{2+}-dependent release, which is essential for synchronous, action potential–coupled release, is mediated by the proteins synaptotagmin-1, synaptotagmin-2 and probably synaptotagmin-9, which reside in the membrane of synaptic vesicles[2]. Synaptotagmins constitute a family of type I membrane proteins with widespread tissue distribution[10]. The cytoplasmic part of the synaptotagmins contains two C2 domains (referred to as C2A and C2B) that are separated by a short linker[11]. Ca^{2+} binding regulates association of the C2 domains with membranes containing acidic phospholipids[12]. The C2A and C2B domains possess partial coordination sites for Ca^{2+} ions (binding three and two Ca^{2+} ions, respectively)[13,14]. Upon calcium binding, the C2 domains of synaptotagmin-1 are believed to interact with acidic phospholipids and penetrate into the hydrophobic core of the membrane[15–17]. Phosphatidylinositol-4,5-bisphosphate (PIP_2) also seems to influence the calcium affinity of synaptotagmin-1 binding to the membrane[18]. However, there are discrepancies in PIP_2-induced membrane binding between native and recombinant synaptotagmin[19,20], so the role of PIP_2 is still undetermined. Apart from binding to the lipid membrane, synaptotagmin-1 has also been shown to interact with the neuronal SNAREs. It binds to isolated syntaxin-1 (ref. 21) and SNAP-25 (ref. 22) as well as to the binary (containing both SNAP-25 and syntaxin-1)[23] and ternary complexes[24]. SNARE binding occurs in either the presence or absence of calcium, and the relative contributions of these two binding modes are controversial[19].

Disruption of Ca^{2+} binding to either of the C2 domains severely inhibits the function of synaptotagmin-1 in mediating fast synchronous transmitter release, with the disruption of the C2B domain being more severe than that of the C2A domain[25,26]. Furthermore, when mutant synaptotagmins with either reduced or increased Ca^{2+} affinity are expressed in mice lacking synaptotagmin-1, a correlation is observed between their Ca^{2+} affinities and the Ca^{2+} dependence of neurotransmitter release[27,28].

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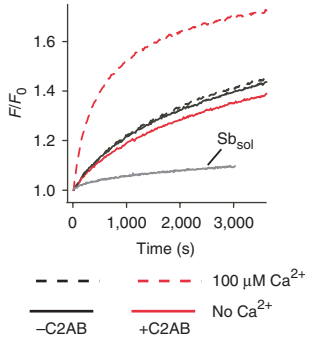

Figure 1 The C2AB fragment of synaptotagmin accelerates liposome fusion mediated by neuronal SNAREs in the presence of Ca^{2+}. A complex of the Q-SNAREs syntaxin-1A (lacking its N-terminal domain, amino acid residues 183–288) and SNAP-25A was reconstituted in liposomes and combined with synaptobrevin-2–containing liposomes (referred to as standard fusion reaction). Fusion was measured by a standard lipid dequenching assay. Both membranes contained 10% (n/n) phosphatidylserine. The C2AB fragment accelerated the reaction in a strictly Ca^{2+}-dependent manner. Ca^{2+} alone did not influence the reaction. In the presence of a ten-fold molar excess of the soluble fragment of synaptobrevin-2 (Sb_{sol}; residues 1–96), fusion was inhibited (gray curve). Fluorescence values were normalized to the initial fluorescence measured in each reaction (denoted as F/F_0). All figures show one representative experiment. Experiments were repeated several times independently, yielding virtually identical results.

In recent years, fusion of liposomes reconstituted with SNARE proteins has been widely used to investigate the mechanisms of SNARE-mediated fusion and its regulation by other proteins[29–31]. In general, fusion is associated with the ability of the SNAREs to form complexes, and it is inhibited when complex formation is impaired, for example by competition with soluble SNARE fragments or by digestion of SNAREs with clostridial neurotoxins. When a fragment of synaptotagmin-1 including both C2 domains (the C2AB fragment) is added to liposomes containing neuronal SNAREs, Ca^{2+}-dependent acceleration of fusion occurs[32]. This effect depends on the presence of acidic phospholipids[33], and it seems to be specific for neuronal SNAREs, as no effect was observed on fusion mediated by SNAREs involved in yeast exocytosis[34]. Furthermore, addition of Ca^{2+} triggers binding of the synaptotagmin-1 fragment both to liposomes containing acidic phospholipids and to liposomes containing syntaxin-1A and SNAP-25A (in the absence of acidic phospholipids), in agreement with previous work[32]. These findings led to the conclusion that, in the presence of Ca^{2+}, synaptotagmin-1 forms a complex with acidic phospholipids that acts on the SNAREs, resulting in enhanced formation of binary and ternary complexes and leading to fusion[34].

Certain aspects of the acceleration by the C2AB fragment, however, are difficult to reconcile with the well-established features of Ca^{2+}- and synaptotagmin-dependent transmitter release in synapses. First, the effect of Ca^{2+} and synaptotagmin-1 on the rate of liposome fusion is moderate. Indeed, the effect on the extent of fusion seems to be stronger than the effect on the rate of fusion, and the maximal rates are still orders of magnitude slower than synaptic exocytosis. Second, in one study, no accelerating effect of calcium was observed when full-length synaptotagmin-1 was reconstituted with synaptobrevin. Rather, the presence of synaptotagmin in the vesicles enhanced fusion in a calcium-independent manner[35], raising questions concerning results obtained with the C2AB fragment.

In the present study, we have thoroughly characterized the effects of the C2AB fragment and membrane-embedded synaptotagmin-1 on liposome fusion mediated by neuronal SNARE proteins. Our data integrate the previously contradictory results into a coherent picture by dissecting the interaction of the C2 domains with membranes containing acidic phospholipids in *cis* and *trans* configurations and with the SNAREs. Our results clarify the molecular interactions between synaptotagmin-1, SNAREs and membranes. However, they also show that the rate-limiting step on which synaptotagmin-1 exerts its action has hitherto not been reproduced *in vitro*.

RESULTS

The C2AB fragment does not influence SNARE assembly

The C2AB fragment of synaptotagmin-1 has been shown to accelerate liposome fusion mediated by neuronal SNAREs[32]. To confirm this result, we reconstituted a complex of the neuronal Q-SNAREs syntaxin-1A (lacking its N-terminal Habc domain) and SNAP-25A into liposomes and combined these with liposomes containing the neuronal R-SNARE synaptobrevin-2. Fusion was measured by a standard assay involving dequenching of fluorescently labeled phospholipids[36]. A robust fusion signal was observed that was inhibited by addition of a soluble fragment of synaptobrevin. The C2AB fragment of synaptotagmin-1, encompassing both C2 domains, accelerated liposome fusion kinetics in the presence of 100 μM Ca^{2+} (**Fig. 1**),

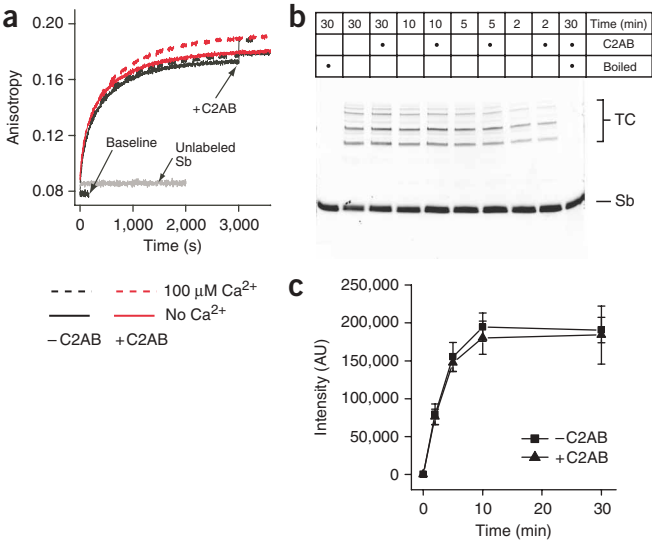

Figure 2 The assembly rate of SNARE complexes on liposomes reconstituted with neuronal Q-SNAREs is not influenced by the C2AB fragment of synaptotagmin. (**a**) Binding of the cytoplasmic fragment of synaptobrevin-2 (Sb) to binary complexes of syntaxin-1A and SNAP-25A reconstituted in liposomes was monitored by fluorescence anisotropy. Preincubating Q-SNARE liposomes with unlabeled synaptobrevin (gray curve) inhibited the reaction. Addition of C2AB fragment at a late time point resulted in a small jump in anisotropy caused by binding to assembled SNARE complexes, confirming that the small differences in anisotropy are not caused by differences in the SNARE assembly rate. (**b**,**c**) As an alternative readout for SNARE assembly, the formation of SDS-resistant complexes was monitored by SDS-PAGE (**b**) and the fluorescent SDS-resistant bands were quantified (**c**). AU, arbitrary units Aliquots from incubation with 100 μM Ca^{2+} in the presence or absence of C2AB fragment were loaded onto an SDS-PAGE gel without prior heating. Aliquots taken after 30 min were also loaded after boiling. No effect of the C2AB fragment on SNARE assembly was observed ($n = 5$; error bars in **c** indicate s.e.m.).

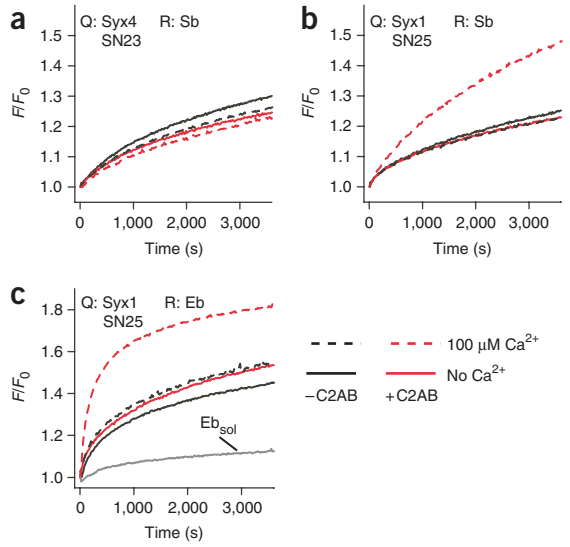

Figure 3 Ca^{2+}-dependent enhancement of fusion by the C2AB fragment of synaptotagmin is specific for neuronal Q-SNAREs syntaxin-1A and SNAP-25A, but not for the neuronal R-SNARE synaptobrevin-2. (**a**) Fusion between liposomes containing the Q-SNAREs syntaxin-4 (Syx4) and SNAP-23 (SN23) and liposomes containing the R-SNARE synaptobrevin-2 (Sb). No Ca^{2+}-dependent enhancement was observed in the presence of the C2AB fragment. (**b**) Fusion between liposomes containing the Q-SNAREs syntaxin-1A (Syx1) and SNAP-25A (SN25) and liposomes containing the R-SNARE synaptobrevin-2 (positive control). (**c**) Fusion between liposomes containing the Q-SNAREs syntaxin-1A and SNAP-25A and liposomes containing the R-SNARE endobrevin (also called VAMP8; abbreviated Eb), showing Ca^{2+}-dependent enhancement by the C2AB fragment of synaptotagmin. Addition of excess amounts of soluble fragment (residues 1–74) of endobrevin (Eb_{sol}) blocked fusion (negative control). See **Figure 1** legend for assay details. In **a** and **b**, full-length syntaxins were used instead of N-terminally truncated versions.

whereas 10 μM Ca^{2+} or single C2 domains of synaptotagmin-1 were ineffective (**Supplementary Fig. 1** online).

It was previously suggested that the C2AB fragment promotes the formation of complexes between syntaxin-1 and SNAP-25, which in turn facilitates fusion[34]. In our system, we use preformed complexes between syntaxin-1A and SNAP-25A, rendering it unlikely that promotion of syntaxin–SNAP-25 assembly causes acceleration of fusion. To test whether formation of the ternary SNARE complex (which is the reaction relevant for fusion) is influenced by synaptotagmin-1, we developed an assay for real-time measurement of SNARE complex formation at concentrations corresponding to those in the liposome fusion experiments. For this purpose, a variant of the cytoplasmic domain of synaptobrevin was generated with a single cysteine at position 79, which was labeled with the fluorescent dye Alexa Fluor 488. Formation of SNARE complexes was then measured as an increase in fluorescence anisotropy. Addition of Q-SNARE liposomes to labeled soluble synaptobrevin in the presence of Ca^{2+} resulted in a rise in fluorescence anisotropy that reached a plateau after approximately 20 min (**Fig. 2a**). In the presence of the C2AB fragment and Ca^{2+}, the kinetics of the anisotropy change were very similar. Similar results were also obtained in the absence of Ca^{2+}. To exclude the possibility that the result was affected by the labeling position on synaptobrevin, we also used a synaptobrevin variant labeled at position 28 and obtained essentially identical results (data not shown). To confirm the results by an independent readout, we monitored the formation of SNARE complexes by SDS-PAGE, allowing for the separation of monomers from the SDS-resistant complexes[37]. The formation of SDS-resistant complexes was not affected by the presence of the C2AB fragment and Ca^{2+} (**Fig. 2b,c**). We concluded that C2AB does not accelerate fusion by influencing SNARE assembly.

The C2AB fragment specifically acts on neuronal Q-SNAREs

Because no effect of the C2AB fragment on SNARE assembly was observed, we examined whether Ca^{2+}-dependent acceleration of liposome fusion requires any direct interaction with the SNAREs. To that end, we replaced syntaxin-1A and SNAP-25A with syntaxin-4 and SNAP-23, respectively, the counterpart SNAREs in constitutive exocytosis. Although the basal fusion rates were comparable between the two sets of SNAREs, no Ca^{2+}-dependent acceleration of fusion by the C2AB fragment was observed when the syntaxin-4–SNAP-23 combination was used (**Fig. 3a,b**). In contrast, replacement of synaptobrevin with another R-SNARE, endobrevin (which functions in the fusion of late endosomes[38]), resulted in a Ca^{2+}-dependent acceleration by the C2AB fragment that was comparable to the acceleration observed with synaptobrevin (**Fig. 3c**). We concluded that a specific interaction between the C2AB fragment and the neuronal Q-SNAREs is required for enhancing fusion.

Given that the R-SNARE itself is evidently not involved in a specific interaction with synaptotagmin, we asked whether the C2AB fragment must interact with the membrane lipids of the R-SNARE vesicle to exert its effect on fusion. Because Ca^{2+}-dependent binding of the C2 domains to membranes requires the presence of acidic phospholipids, we prepared liposomes containing neuronal SNAREs that were devoid of phosphatidylserine in either the Q-SNARE or the R-SNARE membrane. Removal of phosphatidylserine from the Q-SNARE membrane had no effect on the ability of the C2AB fragment to accelerate fusion in the presence of Ca^{2+} (**Fig. 4a**). In contrast, a decrease in the fusion rate was observed when the R-SNARE membrane was devoid of phosphatidylserine (**Fig. 4b**). We also tested the influence of single domains and found that neither the C2A nor the C2B domain alone affected fusion (**Supplementary Fig. 2** online).

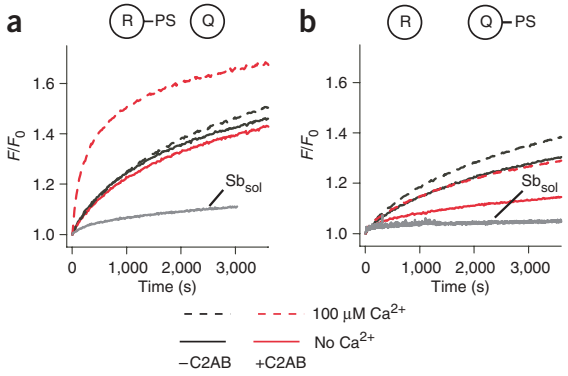

Figure 4 Ca^{2+}-dependent enhancement of fusion by the C2AB fragment of synaptotagmin depends on acidic phospholipids in the R-SNARE membrane. (**a**) Standard fusion reaction (see **Fig. 1**) but with phosphatidylserine present only in the R-SNARE liposomes. Normal Ca^{2+}-dependent enhancement of fusion by the C2AB fragment was observed. (**b**) Standard fusion reaction with phosphatidylserine present only in the Q-SNARE liposomes. Enhancement was largely abolished. Sb_{sol} was used as the negative control (see **Fig. 1**).

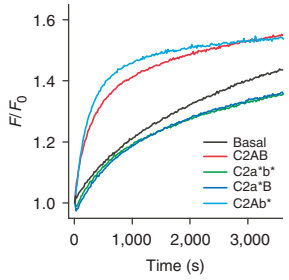

Figure 5 Ca^{2+}-dependent enhancement of fusion by the C2AB fragment of synaptotagmin requires an intact Ca^{2+}-binding site in the C2A but not the C2B domain. C2AB fragments bearing mutations that selectively abolish Ca^{2+} binding in the C2A domain (C2a*B), the C2B domain (C2Ab*) or both domains (C2a*b*) were added to a standard fusion reaction in the presence of 100 μM Ca^{2+}. The basal fusion reaction and the reaction in the presence of wild-type C2AB fragment are shown for comparison. Whereas the mutations of the C2A domain abolished the enhancing effect of the C2AB fragment on fusion, mutations of the C2B domain had no effect.

The Ca^{2+}–C2B domain is not required to accelerate fusion

The experiments presented so far show that acceleration of fusion mediated by the C2AB fragment is specific for neuronal Q-SNAREs and requires an interaction with acidic phospholipids in the R-SNARE membrane. Acceleration depends strictly on Ca^{2+}, although it is not clear whether this is the case for both SNARE and membrane binding, as synaptotagmin is known to interact with the neuronal Q-SNAREs in both the presence and absence of Ca^{2+} (ref. 23). To further investigate the requirement for Ca^{2+} binding to the C2AB fragment, we generated mutant C2AB fragments in which aspartate residues crucial for chelating Ca^{2+} were replaced by alanines, thereby inactivating Ca^{2+} binding to the C2A domain, C2B domain or both. Inactivation of Ca^{2+} binding to both C2 domains abolished the ability of the C2AB fragment to accelerate fusion (**Fig. 5**), in agreement with a previous study[32]. Notably, elimination of Ca^{2+} binding to either the C2A or the C2B domain had divergent effects: whereas Ca^{2+} binding to the C2B domain was dispensable, functional Ca^{2+}-binding sites on the C2A domain were needed for acceleration of liposome fusion.

These findings led us to the following conclusions. First, the interaction of the C2AB fragment with acidic phospholipids is mediated by the C2A domain. Second, as the C2A domain alone is ineffective, it is likely that the C2B domain mediates the interaction with neuronal Q-SNAREs. Third, interaction with the Q-SNAREs does not require Ca^{2+} binding to the C2B domain. Thus, the only Ca^{2+}-dependent interaction that is needed to accelerate liposome fusion seems to be the recruitment of the C2AB fragment to the R-SNARE membrane by the C2A domain. In the following experiments, we therefore repeated our liposome fusion experiments using full-length synaptotagmin reconstituted with SNAREs, thus allowing for discrimination of effects of the C2 domains caused by membrane recruitment from those of other interactions that may be relevant for fusion.

Full-length synaptotagmin accelerates Ca^{2+}-independent fusion

Reconstitution of full-length synaptotagmin with synaptobrevin has been reported to lead to a Ca^{2+}-independent acceleration of liposome fusion[35]. In the next set of experiments, we looked at this reaction in more detail. When we reconstituted full-length recombinant synaptotagmin with the R-SNARE synaptobrevin, these liposomes fused, in the absence of Ca^{2+}, substantially faster with liposomes containing neuronal Q-SNAREs than did liposomes lacking synaptotagmin (**Fig. 6a**), confirming previous findings[35]. When synaptotagmin was inserted into the Q-SNARE liposomes instead, the rate of fusion was reduced compared to the basal reaction (**Fig. 6b**).

Notably, addition of Ca^{2+} reduced the fusion rate to levels comparable to those observed with R-SNARE liposomes lacking synaptotagmin (**Fig. 7a**). To further understand this result, we removed phosphatidylserine from the Q- or the R-SNARE membrane or both. When phosphatidylserine was removed from both membranes, we observed no effect of Ca^{2+} on the reaction (**Fig. 7b**), showing that impairment of fusion after addition of Ca^{2+} probably results from an interaction of synaptotagmin with acidic phospholipids. When only the R-SNARE membrane contained phosphatidylserine, Ca^{2+} led to an impairment of fusion comparable to that observed when both membranes contained phosphatidylserine (**Fig. 7c**). Under this condition, synaptotagmin can interact only with its own membrane (*in cis*). In contrast, acceleration by Ca^{2+} was observed when only the Q-SNARE membrane contained phosphatidylserine (**Fig. 7d**)—that is, under conditions where synaptotagmin can interact in a Ca^{2+}-dependent manner only with the opposing membrane (*in trans*).

These findings led us to two conclusions. First, synaptotagmin must be in the R-SNARE membrane to exert its effect, in agreement with its localization to synaptic vesicles. Second, interactions with both the *cis* and *trans* membranes occur in the presence of Ca^{2+} and a normal complement of acidic phospholipids, with the *cis* interaction being inhibitory and the *trans* interaction being activating and only uncovered when *cis* interactions are prevented.

ΔN-SNARE complex bypasses Syt's effect on liposome fusion

All experiments described so far were carried out using Q-SNARE liposomes in which syntaxin-1A and SNAP-25A were allowed to interact freely with each other. Under these conditions, these SNAREs form a binary complex with 2:1 stoichiometry in which the binding site of synaptobrevin is occupied by a second syntaxin molecule[3]. Assembly kinetics are thus dictated by the displacement of the second syntaxin. There is no evidence that such a 2:1 complex exists *in vivo*, but this artificial interaction explains why the kinetics of liposome fusion mediated by neuronal SNAREs are slow[39]. Consequently, the effects of regulatory proteins on late steps of fusion, downstream of the rate-limiting assembly, cannot be detected in this system. However, formation of the unproductive 2:1 complex can be prevented if a

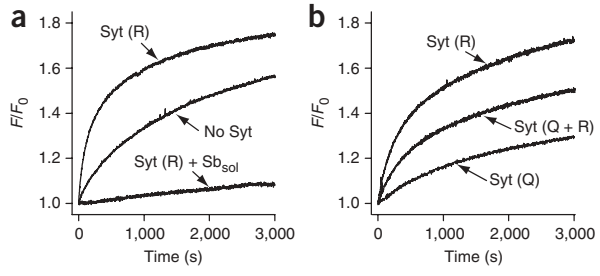

Figure 6 Effects of full-length synaptotagmin inserted in the R- or Q-SNARE membrane on the fusion rate mediated by neuronal SNAREs (standard fusion reaction). (**a**) Reconstitution of full-length synaptotagmin (Syt) with synaptobrevin-2 (R) accelerated fusion in the absence of Ca^{2+}. The reaction was inhibited by excess amounts of soluble synaptobrevin fragment (Sb_{sol}, negative control). (**b**) The accelerating effect of full-length synaptotagmin is restricted to the R-SNARE membrane. Reconstitution with the neuronal Q-SNAREs decreased the fusion rate, with an intermediate rate being observed when synaptotagmin was present in both membranes (Q + R).

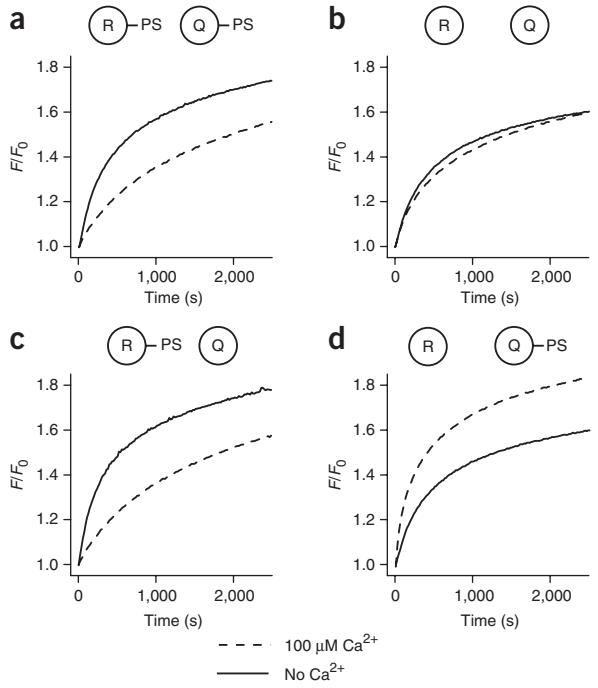

Figure 7 Effects of Ca^{2+} on the fusion of synaptobrevin liposomes containing full-length synaptotagmin with neuronal Q-SNARE liposomes in the presence or absence of acidic phospholipids. (**a**) When phosphatidylserine was present in both Q- and R-SNARE membranes, Ca^{2+} decreased the fusion rate to the level observed in the absence of synaptotagmin. (**b**) When both membranes were devoid of phosphatidylserine, Ca^{2+} had no effect. (**c**) When phosphatidylserine was present only in the R-SNARE membrane, fusion was decreased by Ca^{2+}. (**d**) When phosphatidylserine was present only in the Q-SNARE membrane, fusion was accelerated by Ca^{2+}.

complex is formed between syntaxin-1A, SNAP-25A and a C-terminal fragment (amino acid residues 49–96) of synaptobrevin-2 (denoted as ΔN complex), leaving the N-terminal part of the acceptor free for full-length synaptobrevin to initiate SNARE bundle 'zippering'. Full-length synaptobrevin binds this complex with fast kinetics ($k_{on} = \sim 5 \times 10^5$ M^{-1} s^{-1}; ref. 39). This implies that nucleation of *trans* complex formation—a crucial step that, to our knowledge, was hitherto elusive in *in vitro* experiments—is no longer rate limiting. Accordingly, fusion is accelerated by more than an order of magnitude, a much greater effect than that observed with any of the other proteins we tested so far, including synaptotagmin. We therefore assessed the influence of the C2AB fragment and of full-length synaptotagmin on a fast fusion reaction mediated by the ΔN complex. No marked effects on fusion kinetics were exerted by the C2AB fragment or the full-length protein in the absence or presence of Ca^{2+} (**Fig. 8**). This result clearly shows that both the Ca^{2+}-dependent effects of the C2AB fragment and the Ca^{2+}-independent acceleration by the full-length protein are bypassed if the nucleation of the *trans*-SNARE complex is not rate limiting—that is, if later steps in zippering or fusion become rate limiting.

DISCUSSION

In this study, we used SNARE-mediated fusion of liposomes as a reduced and biochemically accessible model system to learn more about the molecular mechanisms of synaptotagmin in exocytosis. We systematically explored how membrane fusion is influenced by the interactions of the C2AB fragment and a reconstituted full-length variant of synaptotagmin-1 with acidic phospholipids in the R- and Q-SNARE membranes and with SNAREs.

Figure 9 summarizes our main findings. First, Ca^{2+}-dependent binding of the C2AB fragment to the R-SNARE membrane is essential for recruiting synaptotagmin and thus for accelerating fusion (**Fig. 9a**), fully explaining the Ca^{2+}-dependent enhancement of liposome fusion originally observed by Tucker *et al.*[32]. This binding requires an active C2A domain. In contrast, binding of Ca^{2+} to the C2B domain is dispensable in the liposome assay, so it is unclear why Ca^{2+} binding to the C2B domain is essential for the function of synaptotagmin in synaptic transmission. When full-length synaptotagmin is used, the need for the nonphysiological, Ca^{2+}-dependent recruitment step is bypassed. This suggests that the acceleration of fusion by synaptotagmin depends on a direct and Ca^{2+}-independent interaction with the Q-SNAREs in a *trans* configuration (**Fig. 9b**). Acceleration is specific for the neuronal Q-SNAREs, as we observed no acceleration when Q-SNAREs involved in constitutive exocytosis were used. SNARE binding of synaptotagmin probably involves basic residues in the C2B domain. These residues have been shown to be required for the binding of the C2AB domain to the SNARE complex[40], although the significance of Ca^{2+}-independent binding between synaptotagmin and SNAREs has been questioned[19]. The same motif was recently found to be responsible for the accelerating effect of full-length synaptotagmin on liposome fusion[41].

Second, the use of full-length synaptotagmin revealed that synaptotagmin interacts, by virtue of its C2 domains, with both the membrane of its resident R-SNARE liposome (*cis* interaction) and the membrane

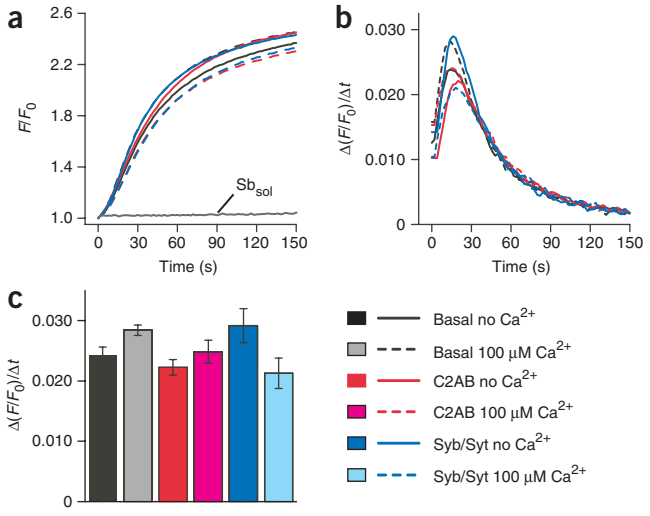

Figure 8 Fusion of synaptobrevin-containing liposomes with liposomes containing a stabilized Q-SNARE acceptor complex is not accelerated by synaptotagmin. (**a**) Comparison of fusion kinetics under basal conditions, in the presence of the C2AB domain, or with R-SNARE liposomes (Syb; synaptobrevin) containing co-reconstituted full-length synaptotagmin (Syt) in the absence or presence of calcium. The rate of fusion was accelerated by at least an order of magnitude compared to the standard fusion reaction, in agreement with our previous report[39]. (**b**) Comparison of the reaction rates derived from the data in **a** by determining the first derivative after filtering to reduce noise. (**c**) Comparison of the maximum reaction rates ($n = 5$; error bars indicate s.e.m.).

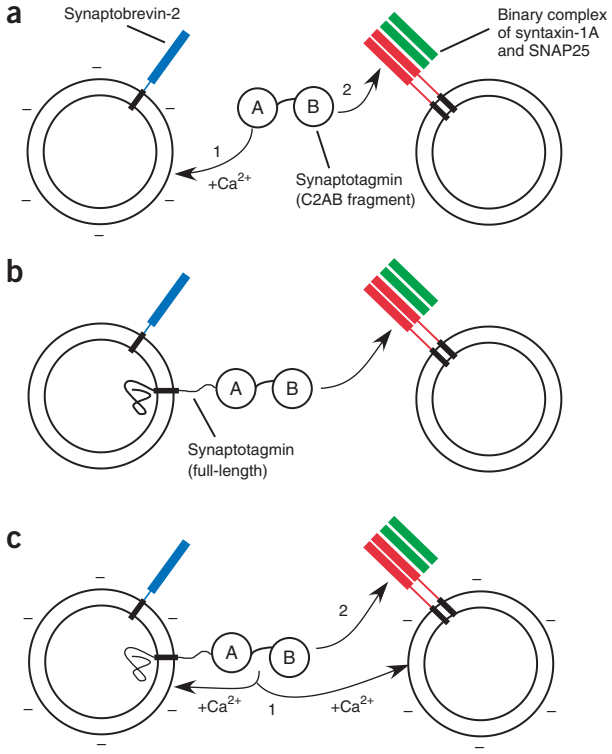

Figure 9 Schematic summary of the interactions between synaptotagmin, SNAREs and membranes containing acidic phospholipids in SNARE-mediated liposome fusion. (**a**) Molecular interactions of the C2AB fragment in SNARE-mediated liposome fusion. First, the C2AB fragment binds through the C2A domain to the synaptobrevin membrane in a Ca^{2+}-dependent manner. Second, the C2B domain interacts with the Q-SNAREs in a Ca^{2+}-independent manner. Both binding reactions are required for acceleration of fusion. (**b**) Molecular interactions of full-length synaptotagmin in the absence of Ca^{2+}. If synaptotagmin is anchored in the R-SNARE membrane through its transmembrane region, the Ca^{2+}-independent interaction with the Q-SNAREs (see **a**) suffices to accelerate fusion. No effect is seen when synaptotagmin is incorporated into the Q-SNARE membrane (not shown). (**c**) Molecular interactions of full-length synaptotagmin in the presence of Ca^{2+}. Synaptotagmin, when inserted into the R-SNARE membrane, interacts with acidic phospholipids in either a *cis* or *trans* configuration. Interaction *in cis* retards fusion, probably by inducing conformational constraints for the Ca^{2+}-independent *trans* interaction with the Q-SNAREs. Interaction *in trans* facilitates fusion. In summary, whenever synaptotagmin can form a bridge between the two liposomes, SNARE assembly and fusion are facilitated.

of the Q-SNARE liposome (*trans* interaction) in a Ca^{2+}-dependent manner. To our surprise, the *cis* interaction inhibits fusion and is dominant; the enhancing effect of the *trans* interaction is revealed only when the *cis* interaction is prevented by the omission of acidic phospholipids from the R-SNARE membrane. This is in agreement with a previous observation that synaptotagmin binds only weakly *in trans* to membranes containing phosphatidylserine when the synaptotagmin vesicles also contain acidic phospholipids[20]. We assume that calcium-induced binding to the R-SNARE membrane causes conformational constraints resulting from the presence of the transmembrane domain as an additional membrane attachment, impairing the *trans* interactions with the Q-SNAREs that are needed for acceleration. Ca^{2+}-induced binding to acidic phospholipids on the opposing membrane, conversely, might facilitate interaction with Q-SNAREs (**Fig. 9c**).

These results help elucidate the molecular mechanism by which synaptotagmin enhances exocytosis in synapses on a millisecond timescale. Although the various interactions of synaptotagmin with SNAREs and membranes *in cis* and *in trans* undoubtedly represent partial reactions involved in mediating Ca^{2+}-dependent exocytosis, thus far it has not been possible to reproduce the physiological stimulation mechanism of synaptotagmin in liposome fusion experiments. In the standard assay used by all laboratories, fusion is largely inhibited and thus proceeds only at a very slow rate because the binding site for synaptobrevin is blocked by a second syntaxin molecule. This configuration certainly does not represent the arrested state in the synapse. Rather, it is controlled by a rate-limiting step that is defined by the displacement kinetics of the second syntaxin, a nonphysiological 'off' pathway of the SNAREs. It is conceivable that the accelerating effects caused by regulatory proteins observed in this and other studies are caused by reversible clustering of R- and Q-SNARE vesicles (see **Fig. 9**). Clustering increases the contact time of the SNAREs *in trans*, which may facilitate syntaxin displacement by synaptobrevin and, consequently, enhance the fusion rate. That contact time may be crucial in *in vitro* reactions was noted in a recent report examining fusion on the single-particle level[42]. In that study, docking of liposomes took much longer than did subsequent steps that led to fusion. Consequently, kinetic effects on this reaction cannot be used directly to draw conclusions about the molecular mechanisms controlling exocytosis. However, synaptotagmin, by virtue of its Ca^{2+}-dependent phospholipid binding *in trans*, may induce very close proximity of membranes that are already docked, which may accelerate fusion[43].

When we used a SNARE acceptor complex in which the *trans* nucleation of SNARE assembly was not rate limiting, synaptotagmin had no effect on the fusion rate. This unexpected result is difficult to reconcile with current views of the mechanism of action of synaptotagmins. We know that this acceptor complex, owing to its artificial nature, is likely to proceed through the late steps of zippering through a nonphysiological pathway, as displacement of the synaptobrevin fragment needed to stabilize the complex will certainly influence the kinetics of fusion. Thus, crucial effects of synaptotagmin on the late stages of zippering may escape detection. However, considering the fast kinetics of synaptobrevin binding and liposome fusion in this system, an alternative mode of action might be conceivable in which synaptotagmin operates upstream of SNARE nucleation, in contrast to the widely favored model in which synaptotagmin acts, together with complexins, upon a partially assembled SNARE complex. For instance, synaptotagmin may bind (reversibly) to the syntaxin–SNAP-25 acceptor complex after vesicle docking but before synaptobrevin nucleation. Such binding *in trans* would position the molecule to interact with both the vesicle and the plasma membrane at a site directly adjacent to the SNARE acceptor complex. Upon Ca^{2+} entry, synaptotagmin would bind both membranes, an event that might be associated with a conformational change and a change in membrane curvature[44], thus repositioning the docked vesicle (as already suggested[43]) and allowing synaptobrevin to nucleate assembly, resulting in immediate fusion. Such a mechanism would explain why calcium-dependent *cis* interaction of synaptotagmin with the vesicle membrane inactivates the molecule: without prior positioning of the C2B domain at the site of fusion by its SNARE interactions, *cis* binding would constrain the molecule, rendering it difficult (if not impossible) for *trans* interactions with the SNAREs to occur.

In summary, our findings show that it is crucial to understand the molecular structure of the late-arrested state of a docked and primed vesicle, particularly with respect to the state of SNARE assembly.

Without such knowledge, it will not be possible to unravel the mechanisms by which late regulators such as synaptotagmin and complexin control the final step of SNARE-mediated fusion. Only if this state can be reproduced experimentally, and the rate-limiting step understood at the molecular level, can the effects of regulatory proteins be integrated into a coherent picture of exocytosis. Thus far, this process has mainly been studied in a reaction that is artificially retarded by a 'wrongly' bound syntaxin.

METHODS

Protein constructs. The SNARE proteins syntaxin-1A, syntaxin-4, SNAP-25A, SNAP-23, synaptobrevin-2 and endobrevin from *Rattus norvegicus* were used. All expression constructs were cloned in the pET28a vector. The expression constructs for the neuronal SNAREs were the full-length syntaxin-1A (amino acid residues 1–288), its SNARE motif including the transmembrane region (183–288), a cysteine-free variant of SNAP-25A (1–206) and synaptobrevin-2 (full-length protein, 1–116; soluble portion, 1–96; C-terminal fragment of the soluble portion, 49–96)[30,39,45,46]. The expression constructs for endobrevin were the full-length protein (1–100) and its soluble portion (1–74)[46,47]. Full-length syntaxin-4 (1–298)[48] and a variant of SNAP-23 in which cysteine residues were replaced with serines were similarly cloned into the pET28a vector using the NdeI and XhoI restriction sites. Single-cysteine variants of synaptobrevin-2 (1–96, S61C and T79C) were also used. For expression and purification of the ΔN complex, syntaxin-1A (183–288) and synaptobrevin-2 (49–96) were cloned into the pETDuet-1 vector using the NcoI/HindIII and NdeI/XhoI restriction sites, respectively. DNA encoding full-length wild-type synaptotagmin 1 (1–421) and the Ca^{2+}-binding mutants of the full-length protein were gifts (see Acknowledgments). See **Supplementary Methods** online for a detailed description of the synaptotagmin constructs used.

Protein purification. Protein purification was carried out essentially as described[30,39]. All proteins were expressed in *Escherichia coli* strain BL21(DE3) and purified by nickel–nitrilotriacetic acid (Ni^{2+}-NTA) affinity chromatography followed by ion-exchange chromatography on the Äkta system (GE Healthcare). The transmembrane region–containing proteins syntaxin-1A (1–288 and 183–288), syntaxin-4 (1–298), synaptobrevin-2 (1–116) and endobrevin (1–100) were purified by ion-exchange chromatography in the presence of 15 mM CHAPS. The binary complexes containing either syntaxin-1A (183–288 or 1–288) and SNAP-25A or syntaxin-4 (1–298) and SNAP-23 were assembled from purified monomers and subsequently purified by ion-exchange chromatography in the presence of 15 mM CHAPS. The ΔN complex (containing syntaxin-1A (183–288), SNAP-25A and synaptobrevin-2 (49–96)) was purified from BL21(DE3) expressing all three proteins, using the pET28a vector for SNAP-25A and the pETDuet-1 vector for syntaxin-1A and synaptobrevin-2. The complex was purified by Ni^{2+}-NTA affinity chromatography followed by ion-exchange chromatography in the presence of 15 mM CHAPS. Full-length synaptotagmin-1 was expressed in the *E. coli* strain BL21(DE3) CodonPlus-RIL (Stratagene). In addition to purification by Ni^{2+}-NTA affinity chromatography and ion-exchange chromatography, size-exclusion chromatography using a HighLoad 26/60 Superdex200 column (GE Healthcare; all in the presence of 0.03% (w/v) *n*-dodecyl-β-D-maltoside) was also done, followed by a second ion exchange step in the presence of 15 mM CHAPS.

Preparation of proteoliposomes and liposome fusion. Liposomes were prepared as described previously[39]; see **Supplementary Methods** for a detailed description of the procedure. When liposomes were devoid of phosphatidylserine, the percentage of phosphatidylcholine was increased accordingly. In experiments where full-length synaptotagmin was reconstituted with SNAREs, the molar ratio of SNAREs to synaptotagmin was 4.5:1, reflecting the ratio of synaptobrevin to synaptotagmin found on synaptic vesicles[49]. Analysis of the liposomes by electron microscopy revealed a relatively homogeneous size distribution, regardless of the presence of synaptotagmin (**Supplementary Fig. 3** online). As a further test for the quality of liposome preparations, all liposomes were routinely analyzed by SDS-PAGE and Coomassie blue staining (**Supplementary Fig. 4** online).

Liposome fusion reactions were performed at 30 °C. For each reaction, 10 μl of labeled liposomes and 15 μl of unlabeled liposomes were mixed in 1.2 ml of buffer containing 20 mM HEPES-KOH (pH 7.4), 120 mM potassium glutamate, 20 mM potassium acetate, 10 mM 1,3-diamino-2-propanol-*N*,*N*,*N*′,*N*′-tetraacetic acid (DPTA) and the appropriate amounts of calcium chloride. This results in final protein concentrations of ~100 nM and 150 nM protein for each liposome population, respectively. Soluble fragments of synaptotagmin were used at concentrations of 500 nM. In inhibition experiments, the soluble fragment of synaptobrevin-2 was used at concentrations of 2 μM. Fluorescence dequenching was measured using wavelengths of 460 nm for excitation and 538 nm for emission in a Fluorolog 3 (Model FL322) or a Fluormax 2 spectrometer equipped with a four-position holder with magnetic stirrer (both from Jobin Yvon). Each panel shows representative examples of experiments that were repeated multiple times. Fluorescence values were normalized to the fluorescence measured at the starting point of each reaction, denoted as F/F_0. In each figure, all curves were obtained on the same day with the same set of liposomes.

Formation of SNARE complexes. To monitor the binding of synaptobrevin to reconstituted binary complexes of syntaxin-1A (1–288) and SNAP-25A, synaptobrevin-2 (1–96) labeled at Cys79 with Alexa Fluor 488 was used. For each reaction, 100 nM Alexa Fluor 488–labeled synaptobrevin-2 was incubated with 500 nM syntaxin-1A–SNAP-25A complex reconstituted in liposomes. For inhibition, 5 μM synaptobrevin-2 (1–96) was used. Fluorescence anisotropy measurements were carried out in a Fluorolog 3 spectrometer in T-configuration equipped for polarization (Model FL322, Jobin Yvon). All experiments were performed in 20 mM HEPES-KOH (pH 7.4), 120 mM potassium glutamate, 20 mM potassium acetate, 10 mM DPTA and the appropriate amounts of calcium chloride. The labeled protein was excited at a wavelength of 490 nm, and emission was measured at 521 nm. The grating factor (G) was calculated as $G = I_{HV}/I_{HH}$ and the anisotropy (r) was calculated as $r = (I_{VV} - G \times I_{VH})/(I_{VV} + 2 \times G \times I_{VH})$, where I is the fluorescence intensity and the first and second subscript letters after I indicate the polarizations of the exciting and emitted light, respectively.

When testing for the formation of SDS-resistant ternary complexes, we used synaptobrevin-2 (1–96) labeled at Cys61 with Alexa Fluor 594. For each reaction, 800 nM labeled protein was mixed with a five-fold molar excess of binary complexes of syntaxin-1A and SNAP-25A reconstituted in liposomes. Aliquots were taken after 2, 5, 10 and 30 min and mixed with sample buffer (final concentrations 50 mM Tris (pH 6.8), 2% (w/v) lithium dodecyl sulfate and 12% (v/v) glycerol). The samples were separated on a 10% polyacrylamide gel[50] without prior heating of the samples and visualized by fluorescence imaging (LAS-1000; Fujifilm) using a filter set consisting of a HQ545/30 excitation filter and a HQ610/75 emission filter. The amount of SDS-resistant ternary complex was quantified by two-dimensional densitometry using the AIDA V4.04 software (Raytest).

Other methods. Protein concentrations were determined using either the Bradford assay[51] or UV absorption. Labeling of single-cysteine variants of synaptobrevin was done according to the manufacturer's instructions (Invitrogen). The fluorescent dyes Alexa Fluor 488 C_5 maleimide and Alexa Fluor 594 C_5 maleimide were purchased from Invitrogen. Experiments with precise Ca^{2+} concentrations were conducted using a DPTA-based buffer system as described[52]. Final Ca^{2+} concentrations in the reaction mixtures were determined using the fluorescent dyes Mag-Fura2 and Fluo5N (Molecular Probes), using a standard curve constructed with a Molecular Probes Ca^{2+} calibration kit.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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