Reconstituted synaptotagmin I mediates vesicle docking, priming, and fusion

Zhao Wang, Huisheng Liu, Yiwen Gu, and Edwin R. Chapman

Department of Neuroscience, Howard Hughes Medical Institute, University of Wisconsin, Madison, WI 53706

The synaptic vesicle protein synaptotagmin I (syt) promotes exocytosis via its ability to penetrate membranes in response to binding Ca^{2+} and through direct interactions with SNARE proteins. However, studies using full-length (FL) membrane-embedded syt in reconstituted fusion assays have yielded conflicting results, including a lack of effect, or even inhibition of fusion, by Ca^{2+}. In this paper, we show that reconstituted FL syt promoted rapid docking of vesicles (<1 min) followed by a priming step (3–9 min) that was required for subsequent Ca^{2+}-triggered fusion between v- and t-SNARE liposomes. Moreover, fusion occurred only when phosphatidylinositol 4,5-bisphosphate was included in the target membrane. This system also recapitulates some of the effects of syt mutations that alter synaptic transmission in neurons. Finally, we demonstrate that the cytoplasmic domain of syt exhibited mixed agonist/antagonist activity during regulated membrane fusion in vitro and in cells. Together, these findings reveal further convergence of reconstituted and cell-based systems.

Introduction

Elucidation of the molecular mechanisms that underlie Ca^{2+}-triggered membrane fusion and neurotransmitter release at synapses can be directly addressed through in vitro fusion assays using reconstituted SNARE proteins. SNAREs form the core of a conserved membrane fusion complex in neurons, with v-SNAREs (synaptobrevin [syb]) binding to t-SNAREs (syntaxin and SNAP-25), thereby pulling the membranes together to catalyze fusion (Weber et al., 1998). This system has been used to study accessory proteins that regulate fusion, including the Ca^{2+} sensor for exocytosis synaptotagmin I (syt). Syt is anchored to synaptic vesicles (SVs) via a single membrane-spanning domain. To simplify the study of syt, most studies make use of the cytoplasmic domain of protein (which harbors both Ca^{2+}-sensing motifs C2A and C2B and is therefore designated C2AB; Tucker et al., 2004; Schaub et al., 2006; Stein et al., 2007; Chicka et al., 2008; Gaffaney et al., 2008; Xue et al., 2008).

Recent studies have attempted to address the impact of full-length (FL) membrane-embedded syt on fusion in vitro. In one study, Ca^{2+} was without effect (Mahal et al., 2002), whereas in another study, Ca^{2+}–syt inhibited fusion. In this latter study, Ca^{2+}–syt was able to stimulate fusion only when phosphatidylserine (PS) was removed from the v-SNARE vesicle (Vr) membrane (Stein et al., 2007); the physiological relevance of this finding is unclear, as PS is present on both the SV and target membrane in vivo (Takamori et al., 2006). A third study reported Ca^{2+}-triggered fusion using reconstituted FL syt, but in this case, fusion was triggered by only a narrow range of [Ca^{2+}], centered around 10 µM (Lee et al., 2010). At [Ca^{2+}] ≥ 25 µM, stimulation of fusion was not observed even though higher concentrations of Ca^{2+} are achieved at release sites (Llinás et al., 1992, 1995), and robust neurotransmitter release occurs at tens to hundreds of micrometer [Ca^{2+}] (Thomas et al., 1993; Heidelberger et al., 1994; Heinemann et al., 1994; Bollmann et al., 2000; Voets, 2000). Finally, in the most recent study, Ca^{2+}-triggered fusion occurred but only at Ca^{2+} concentrations ≥2 mM (Kyoung et al., 2011), a value far above the physiological range.

To date, reconstituted membrane fusion systems incorporating FL syt, which mimic the native state, have yet to be described. Here, we define an FL syt-regulated membrane fusion assay that more accurately recapitulates several fundamental aspects of syt-regulated exocytosis at synapses.

Correspondence to Edwin R. Chapman: chapman@wisc.edu

Abbreviations used in this paper: FL, full length; KO, knockout; LDCV, large dense core vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Rh, rhodamine; RRP, readily releasable pool; SRP, slowly releasable pool; SV, synaptic vesicle; syb, synaptobrevin; TMD, transmembrane domain; WT, wild type.

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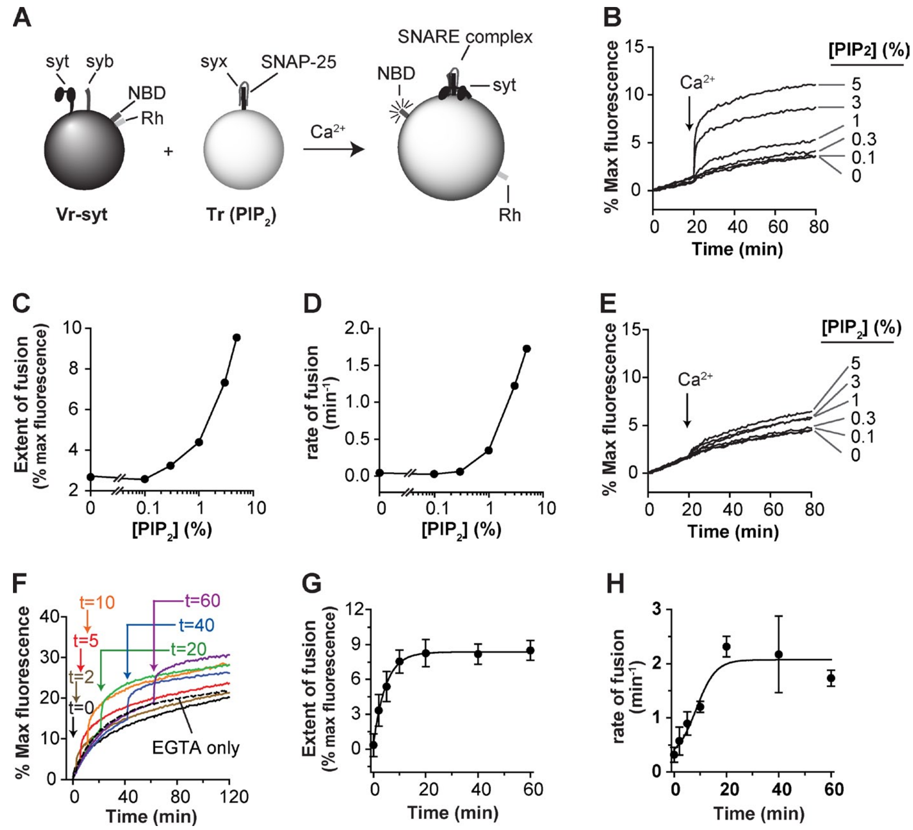


Figure 1. **Reconstitution of active FL syt.** (A) A schematic diagram of the in vitro FL syt-regulated fusion assay. (B) Titration of PIP_2 in Tr. Vesicles bearing FL syt and syb (Vr-syt) were preincubated with Tr, which harbored the indicated amount of PIP_2, for 20 min in the presence of 0.2 mM EGTA before the addition of 1 mM Ca^{2+}. Fusion was monitored for another 60 min after Ca^{2+} injection. (C and D) Increasing [PIP_2] resulted in increases in the extent (C) and initial rate (D) of regulated fusion. (E) In reactions lacking FL syt, membrane fusion was only slightly promoted by Ca^{2+}, and this small effect occurred when the PIP_2 levels were ≥1%. (B–E) Experiments were repeated twice, and similar results were obtained. (F) Optimization of the preincubation time. Fusion was monitored as in B. The duration of the preincubation step was varied as indicated. (G and H) The extent (G) and initial rate (H) of regulated fusion were plotted versus the preincubation time (n = 4). The data shown are represented as mean ± SEM.

Results

Effect of PIP_2 on Ca^{2+}–syt-regulated fusion

In some of the earlier studies of FL syt, a critical lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), was not included in the reconstituted vesicles (Mahal et al., 2002; Stein et al., 2007). PIP_2 plays an essential role in the Ca^{2+}-triggered exocytosis of large dense core vesicles (LDCVs) in neuroendocrine cells (Eberhard et al., 1990; Hay et al., 1995) and might also play a key role in SV exocytosis (Zheng et al., 2004), although this latter issue remains to be fully explored. In neurons and neuroendocrine cells, PIP_2 is concentrated on the inner leaflet of the plasma membrane and is absent from secretory vesicles (Holz et al., 2000; Micheva et al., 2001). Ca^{2+}-independent interactions with PIP_2 have been shown to steer the membrane penetration activity of syt toward the PIP_2-harboring membrane (i.e., the plasma membrane), rather than the vesicle membrane, in response to Ca^{2+} (Bai et al., 2004). As syt stimulates fusion by selectively acting on the target membrane (Chicka et al., 2008) and as interactions with the vesicle membrane are favored kinetically (Bai et al., 2000), we hypothesized that PIP_2-mediated steering of syt would be essential for productive fusion (Bai et al., 2004). To test this, we titrated [PIP_2] in t-SNARE vesicles (Tr) and reconstituted syt in Vr (Vr-syt; Fig. 1, A–D). Fusion between Vr-syt and Tr was monitored by loss of FRET between a lipidic donor–acceptor pair, as shown in Fig. 1 A; in brief, vesicles were mixed and monitored for 20 min, Ca^{2+} was injected, and fusion was monitored for an additional 60 min (Fig. 1 B). When PIP_2 was <1% (molar ratio relative to total lipid), Ca^{2+}-stimulated fusion was not observed; at ≥1%, Ca^{2+}-triggered membrane fusion became apparent, and both extent and rate of the fusion were further enhanced by increasing the PIP_2 on Tr up to 5%, the highest concentration tested (we note that PIP_2 has been estimated to reach 6% of the total lipid within rafts in cells; Fig. 1, B–D; James et al., 2008). Ca^{2+}-triggered fusion was not observed when syt was not present on Vr (Fig. 1 E). These data indicate that PIP_2 is a critical effector for the action of FL syt during regulated fusion.

The Ca^{2+}-free preincubation step was critical; omission of this step resulted in fusion reactions that were not triggered by Ca^{2+} to any appreciable degree (Fig. 1 F). The half-maximal rate and extent of fusion occurred with preincubations of 9 and 3 min, respectively (Fig. 1, G and H), so a 20-min incubation time was selected for all subsequent reactions.

To determine whether the membrane fusion signal observed in these experiments reflected hemi- or full fusion, we used dithionite to selectively quench fluorophores on the outer leaflet, thereby revealing the fusion signal from only the inner leaflet. The fluorescence signal was reduced by ∼60% at all stages of the fusion reaction (Fig. S1), demonstrating equal lipid mixing in both the inner and outer leaflets. These data indicate that full fusion occurs during all phases of these reconstituted vesicle fusion reactions.

Specificity of the phosphatidylinositol bisphosphate requirement for regulated fusion

To further probe the role of PIP_2 during fusion, we used the drug neomycin, which is an antibiotic that specifically binds to PIP_2 (Griffin et al., 1980) and has been shown to inhibit synaptic transmission (Zheng et al., 2004). We titrated neomycin into FL syt-regulated fusion reactions and found that 30 µM neomycin abolished Ca^{2+}-triggered fusion (IC_{50}= 6.9 µM; Fig. 2, A and B), a concentration that was without effect on C2AB-regulated fusion reactions. Complete inhibition of Ca^{2+} C2AB-regulated fusion required millimolar concentrations of neomycin (IC_{50} = 357 µM; Fig. 2, C and D) and is likely to be a nonspecific effect.

Cell membranes contain several phosphatidylinositol bisphosphates, but only Ptdlins(4,5)P_2 is required for exocytosis (Eberhard et al., 1990; Hay et al., 1995; Tucker et al., 2003). To determine whether syt binds to PIP_2 specifically to regulate fusion, three different phosphatidylinositol bisphosphates were reconstituted into Tr (Fig. 2 E). Only Ptdlins(4,5)P_2 was able to stimulate fast and robust Ca^{2+}-dependent fusion; Ptdlins(3,5)P_2 or Ptdlins(3,4)P_2 was significantly less effective (extent and rate of fusion reduced >35% compared with PIP_2; Fig. 2, F and G).

Optimal syt density and the Ca^{2+} sensitivity of fusion

To determine the number of copies of syt per vesicle required for optimal fusion activity, we titrated the amount of reconstituted syt molecules incorporated into Vr (Fig. 3, A and B); 3% PIP_2 was included in all Tr. Vr lacking syt exhibited only small responses to Ca^{2+}; however, inclusion of syt in these vesicles, even at relatively low copy numbers (six per vesicle), resulted in fusion that was strongly stimulated, in terms of both rate and extent, by Ca^{2+}. Increasing the amount of syt further enhanced the Ca^{2+}-dependent response until saturation was reached at 30 copies of syt per vesicle; the maximal response occurred between 12 and 30 copies. Interestingly, this range coincides with the finding that SVs harbor 15 copies of syt (Takamori et al., 2006).

The Ca^{2+} sensitivity of syt-promoted fusion was determined using Vr that harbored 30 copies of syt and Tr with 3% PIP_2. Corrections for these measurements are detailed in Fig. S2 (A–C), and the dose–response curve is shown in Fig. 3 C. The $[Ca^{2+}]_{1/2}$ was 250 µM, and the Hill coefficient was 1.5, indicating some degree of cooperativity. This Ca^{2+} sensitivity is similar to the half-maximal [Ca^{2+}] for exocytosis from goldfish retinal bipolar neurons (194 µM; Heidelberger et al., 1994) but is less than estimates from autaptic cultures of hippocampal neurons (Burgalossi et al., 2010) or the calyx of Held (Bollmann et al., 2000; Schneggenburger and Neher, 2000). However, it should be noted that the Ca^{2+} sensitivity is directly related to the concentration of anionic phospholipids in the in vitro system (Tucker et al., 2004), and lower $[Ca^{2+}]_{1/2}$ values can be obtained using a higher mole fraction of PS (Tucker et al., 2004).

In stark contrast to a previous study in which a lack of response was observed at high [Ca^{2+}] (Lee et al., 2010), we found robust fusion activity at all Ca^{2+} concentrations tested, a result that more accurately reflects the in vivo behavior of the regulated fusion machinery (Heidelberger et al., 1994; Heinemann et al., 1994; Bollmann et al., 2000; Voets, 2000). We also confirmed that fusion was mediated by trans-SNARE pairing, as the cytoplasmic domains of the t-SNARE heterodimer (cd t-SNAREs; Fig. 3, D and F) and syb (cd syb; Fig. 3, E and F) completely blocked lipid mixing.

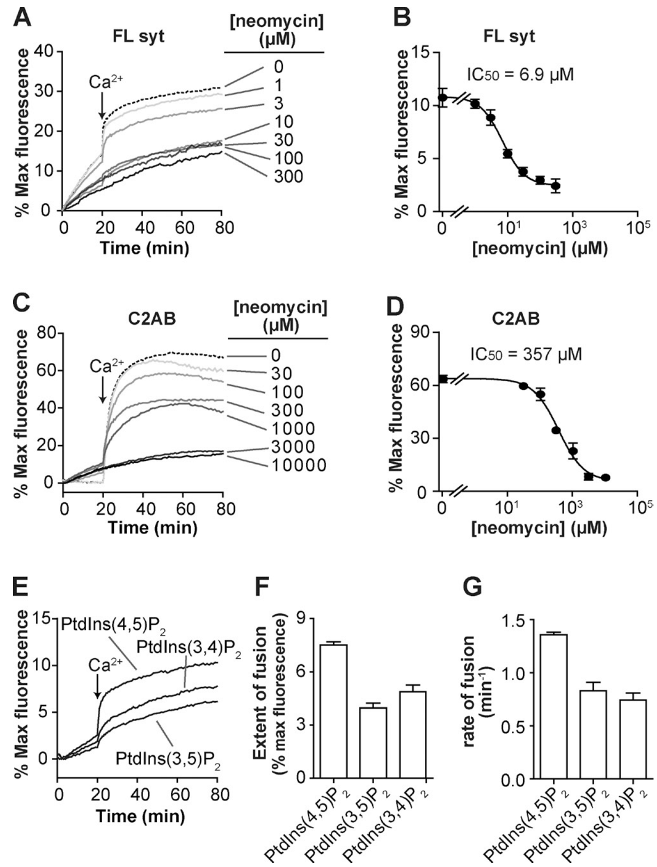


Figure 2. **Phosphatidylinositol bisphosphate specificity for syt-regulated fusion.** (A) Fusion assays were performed as described in Fig. 1 B but in the presence of the indicated [neomycin]. (B) Quantification of the data from A. (C and D) The same experiments were performed as in A and B but using fusion assays in which FL syt was replaced by 3 µM C2AB. (E) Tr containing three different phosphatidylinositol bisphosphates was mixed with Vr-syt vesicles, and fusion was monitored as in Fig. 1 B. (F and G) The extent (F) and initial rate (G) of regulated fusion were calculated and plotted. Data were obtained from three or more independent trials. All data shown are represented as mean ± SEM.

Topological requirements for PS, PIP_2, and syt

PS is the major acidic phospholipid in neurons and is crucial for syt to penetrate and bend membranes to promote fusion (Bhalla et al., 2005; Hui et al., 2009). Interestingly, when PS was absent from Tr, regulated fusion was abolished (Fig. 4 A). In contrast, fusion was largely unaffected by omission of PS from Vr (Fig. 4 A). These results are consistent with a model in which the C2 domains of FL syt act, in trans, on the target membrane to stimulate fusion. Moreover, these findings contrast the mechanism of fusion regulated by the cytoplasmic domain of syt, designated C2AB, which requires the presence of PS on both Vr and Tr membranes (Bhalla et al., 2005).

Analogous to PS, PIP_2 must also be present on the target membrane in order for Ca^{2+}–syt to stimulate fusion (Fig. 4 B). This finding further indicates that syt executes its function by acting on the t-SNARE membrane, as predicted from earlier biochemical studies (Chicka et al., 2008; Hui et al., 2009).

We also addressed the topological requirements for syt and found that fusion was stimulated by Ca^{2+} only when syt was

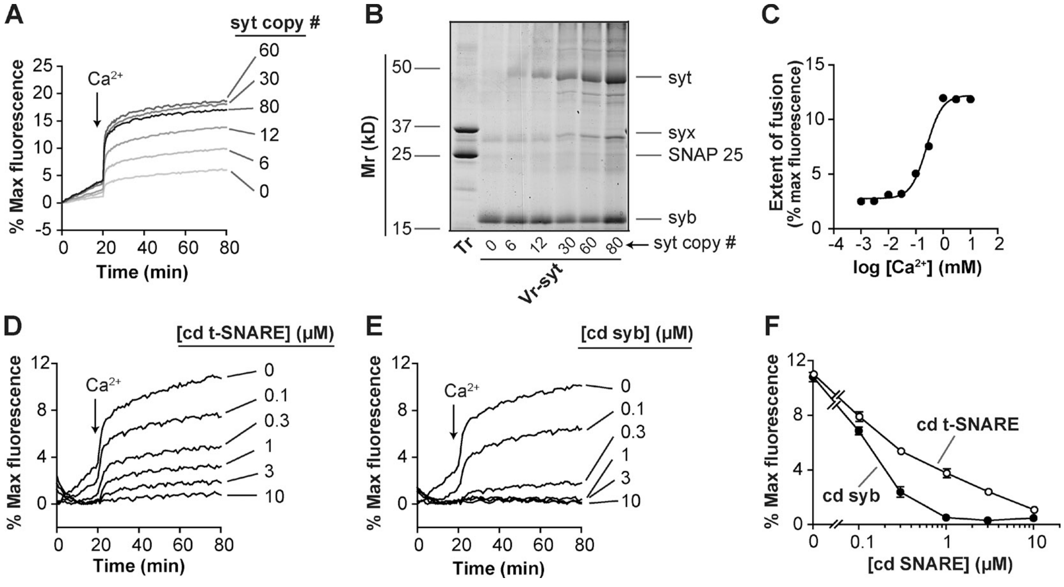


Figure 3. **Physiological densities of FL syt regulate fusion via a SNARE-dependent mechanism.** (A) Titration of reconstituted FL syt in Vr alters the rate and extent of Ca^{2+}-promoted membrane fusion. The experiments were performed as described in Fig. 1 B; the average copy number of syt per vesicle is indicated. (B) Samples from A were subjected to SDS-PAGE and stained with Coomassie blue. Mr, molecular mass. (C) Ca^{2+} dose response from fusion assays containing 30 copies of syt in Vr and 3% PIP_2 in Tr. The corrected data were fitted with a sigmoidal curve; the $[Ca^{2+}]_{1/2}$ was 250 μM, and the Hill coefficient was 1.5. Experiments were repeated twice, and similar results were obtained. (D–F) The cytoplasmic domains of SNAREs block syt-promoted fusion. (D and E) Increasing concentrations of cd t-SNAREs (D) or cd syb (E) were preincubated with Tr and Vr-syt. Membrane fusion was monitored using the same protocol as shown in Fig. 1 B. (F) The final extent of fusion at 80 min was plotted as a function of [cd SNARE] (n = 3). All data shown are represented as mean ± SEM.

present on Vr (Fig. 4 C). The finding that syt must be localized to the vesicle membrane is consistent the targeting of this protein to vesicles in vivo and with the notion that syt acts as a docking factor via interactions with t-SNAREs and PIP_2 (Reist et al., 1998; Bai et al., 2004; de Wit et al., 2009).

Mutational analysis of syt during regulated fusion

To further address the mechanism by which reconstituted syt regulates fusion, we examined mutant forms of the protein. A positively charged patch on the side of C2B plays a critical role in PIP_2-mediated steering of syt to insure Ca^{2+}-triggered penetration into the target membrane (Bai et al., 2004). Steering activity in vitro (Bai et al., 2004) and SV exocytosis in vivo (Mackler and Reist, 2001; Loewen et al., 2006; Takamori et al., 2006) were both impaired by mutations (K326 and 327A) that neutralize these positive charges (Fig. 5 A). More specifically, these mutations resulted in a 40% reduction in neurotransmitter release at the *Drosophila melanogaster* neuromuscular junction (Loewen et al., 2006) and a 50% reduction in autaptic cultures of hippocampal neurons (Takamori et al., 2006). These findings are in reasonable agreement with the ~70% reduction observed in our simplified, reduced in vitro fusion assay (Fig. 5 B).

Several additional syt mutations have been characterized. One such mutant, designated *AD1*, which has been studied in detail in *Drosophila* (Broadie et al., 1994; DiAntonio and Schwarz, 1994; Yoshihara and Littleton, 2002), lacks the C2B domain, resulting in a strong loss-of-function phenotype (Fig. 5 A). Consistent with the fly physiology, the AD1 mutant was unable to promote membrane fusion in response to Ca^{2+} (Fig. 5 C), thus confirming that the C2B domain of syt is indispensable for regulated fusion (Broadie et al., 1994; Yoshihara and Littleton, 2002; Gaffaney et al., 2008).

We also mutated Ca^{2+} ligands in either the C2A or C2B domain of FL syt (Fig. 5 A). In earlier in vitro fusion assays using C2AB, Ca^{2+} ligand mutations in the C2A domain resulted in a more severe loss of activity than analogous mutations in the C2B domain (Bhalla et al., 2005; Stein et al., 2007). However, in neurons, mutation of Ca^{2+} ligands in C2A are tolerated, but Ca^{2+} ligand mutations in C2B completely disrupt the ability of syt to drive synchronous SV exocytosis (Mackler et al., 2002; Nishiki and Augustine, 2004). We found that the disparity between cell-based and in vitro fusion assays was partially resolved via the use of FL reconstituted syt; mutations in the C2B domain disrupted most of the ability of membrane-embedded syt to stimulate fusion in response to Ca^{2+}, whereas mutations in C2A were less deleterious (35% reduction in the extent of fusion; Fig. 5 D). Despite this convergence regarding the C2B domain, the reconstituted fusion assay still fails to recapitulate the lack of effect or gain of function reported for Ca^{2+} ligand mutations in the C2A domain (Robinson et al., 2002; Stevens and Sullivan, 2003).

The transmembrane domain (TMD) of syt is not necessary for syt to regulate neuronal exocytosis (Hui et al., 2009). For example, when C2AB was targeted to SVs by fusing it with the SV protein synaptophysin, synchronous neurotransmitter was fully restored in syt knockout (KO) neurons (Hui et al., 2009). To extend this observation to the reconstituted system, we linked C2AB to Vr via conjugation with maleimide-phosphatidylethanolamine (PE).

Interestingly, conjugated C2AB stimulated fusion in response to Ca^{2+} in a manner analogous to FL syt (Fig. S3, A–C). These data further validate the observation that the TMD of syt is dispensable for fusion.

Context-dependent mixed antagonist/agonist activity of C2AB

C2AB stimulates fusion in vitro (Tucker et al., 2004) but has been shown to inhibit fusion in PC12 (Desai et al., 2000) and chromaffin cells (Rickman et al., 2004). Although these findings might appear to be contradictory, we note that C2AB was only able to inhibit fusion in cells to a limited degree, suggesting that this protein fragment might have mixed agonist/antagonist activity. In addition, it is possible that C2AB is less efficacious in terms of regulating fusion than the FL protein. In this case and in the presence of intact syt, addition of C2AB would be predicted to diminish fusion to some degree. The new system reported here, based on the reconstitution of active FL syt, makes it possible to test these ideas.

We titrated C2AB into fusion assays that contained FL syt; at relatively low concentrations, C2AB slightly, but reproducibly, inhibited Ca^{2+}-promoted membrane (Fig. 6, A–C). In contrast, in the absence of FL syt, C2AB only stimulated fusion, and this effect required relatively high concentrations of the protein (≥3 µM; Fig. S4, A and B). Interestingly, the rate of fusion was reduced by increasing [C2AB] in the presence of FL syt. These results agree with our general finding that in response to Ca^{2+}, FL syt-regulated fusion occurs with faster kinetics than C2AB-regulated fusion reactions (Fig. 7).

To extend these experiments to a native system, we expressed C2AB in cultured chromaffin cells (Fig. S4 C). In wild-type (WT) cells, overexpression of C2AB reduced the rate of exocytosis from the readily releasable pool (RRP) of vesicles (Fig. 6, D–F). Interestingly, overexpression of C2AB in syt KO chromaffin cells did not inhibit fusion but rather restored the size the RRP and partially restored the fast rate of release (Fig. 6, D–F). Overexpression of C2AB in either WT or syt KO cells had no significant effect on the size or release rate of the slowly releasable pool (SRP) of vesicles or the sustained phase of release (Fig. S4, D–G). Together, these results are consistent with a previous study demonstrating that endogenous syt functions to regulate release from the RRP (Voets et al., 2001). Thus, in reconstituted systems and in cells, C2AB can partially inhibit fusion in the presence of FL syt but acts only to stimulate fusion in the absence of the FL protein. These findings are interpreted in the Discussion section.

Systematic comparison of fusion reactions regulated by FL or the cytoplasmic domain of syt

In the course of analyzing the impact of FL syt on fusion, we incorporated three important modifications in the fusion assay: incorporation of PIP_2 in Tr, addition of PE to both Tr and Vr, and the inclusion of a Ca^{2+}-free preincubation step. To clarify potential differences between the FL protein versus C2AB, each of these conditions was systematically explored using both proteins (Figs. 7 [A–C] and S5 [A–C]).

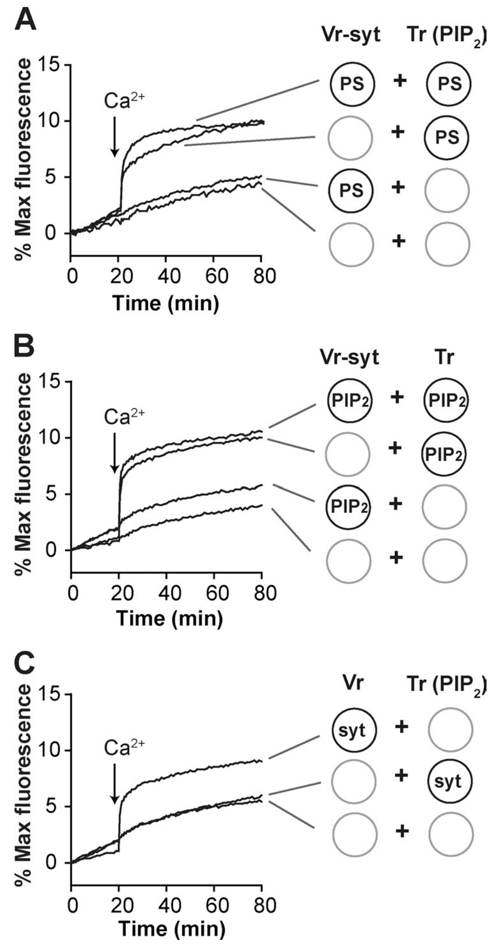


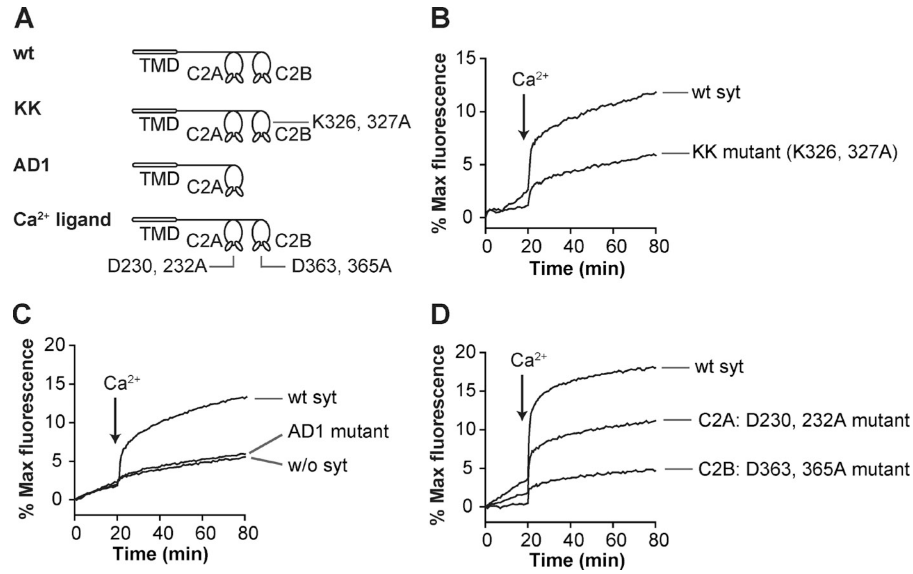
Figure 4. **Topological requirements for PS, PIP_2, and syt during reconstituted membrane fusion.** (A and B) PS (A) or PIP_2 (3%; B) was reconstituted into either Vr with 30 copies of syt or Tr. In A, all Tr contained 3% PIP_2; in B, all Tr contained 25% PS, and all Vr contained 15% PS. Fusion assays were performed as described in Fig 1 B. Both PS and PIP_2 are required in Tr, but not Vr. (C) 30 copies of syt were reconstituted into either Vr or Tr; syt must be present on Vr to stimulate fusion. Representative examples from three or more independent trials are shown.

PIP_2 and the preincubation step were both essential for FL syt to stimulate fusion in response to Ca^{2+} but were not required for C2AB to regulate fusion. These findings suggest that the FL protein might act via a somewhat distinct mechanism than C2AB, as detailed in the next section. Inclusion of PE enhanced fusion reactions regulated by both FL syt and C2AB. Finally, in response to Ca^{2+}, FL syt-regulated fusion was less efficient than C2AB-regulated fusion but occurred with faster kinetics.

A distinct mechanism of FL syt and C2AB-mediated fusion

C2AB promotes fusion, in part, by aggregating vesicles in response to Ca^{2+} and thereby enhancing v- and t-SNARE pairing (Hui et al., 2011). Our finding that FL syt stimulates fusion in response to Ca^{2+} only after Vr and Tr have been preincubated together in EGTA, in conjunction with a study indicating that membrane-embedded syt mediates secretory vesicle docking in cells (via interactions with t-SNAREs; de Wit et al., 2009),

Figure 5. **Syt loss-of-function mutations that impair synaptic transmission also disrupt syt function in vitro.** (A) A schematic diagram of syt mutants; each mutant has been previously characterized in intact synapses. (B–D) Fusion assays, using Vr harboring each mutant shown in A, were performed as described in Fig. 1 B. Representative examples from three or more independent trials are shown.

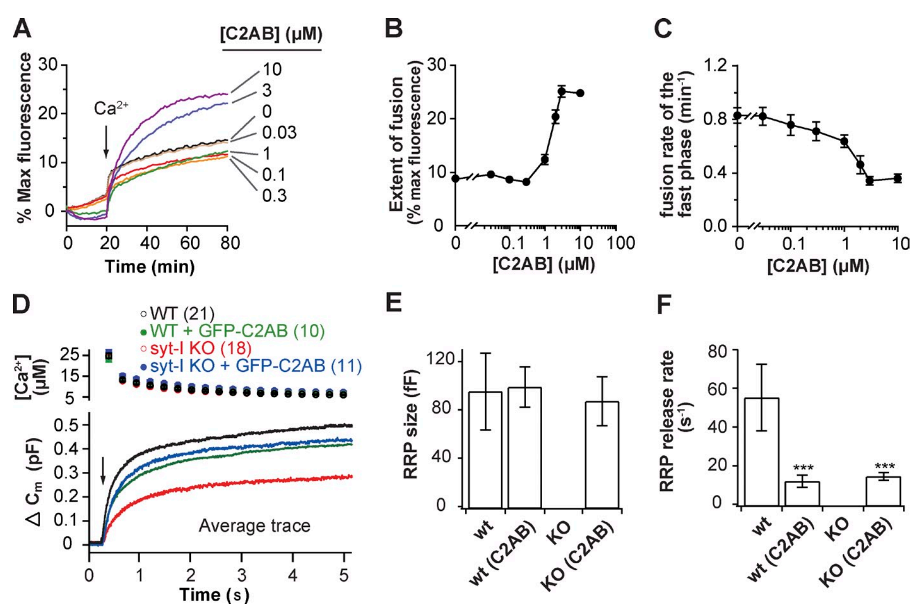


prompted experiments to determine whether FL syt promotes docking in our in vitro assay. To test this, we measured vesicle aggregation in fusion reactions that contained FL syt or C2AB and found that the FL membrane-embedded protein drove rapid aggregation (<1 min) in EGTA; addition of Ca^{2+} did not result in further vesicle aggregation but did stimulate fusion, presumably by acting on predocked vesicle complexes (Fig. 8, A and B). When FL syt was omitted from the system, we did not observe appreciable aggregation (Fig. 8 A). In sharp contrast to experiments using FL syt, aggregation was not observed in C2AB-regulated reactions until Ca^{2+} was added (Fig. 8 B).

To confirm that vesicle aggregation involved docking between Tr and Vr, a docking assay was used (Fig. 8 C). Vr, harboring either syt, syb, or both proteins, were mixed with Tr that were immobilized on beads using avidin and biotin. Vesicles that harbored either syt or syb were pulled down to some extent by Tr, but much more robust docking was observed when both proteins were present on the Vr. Inclusion of PIP_2 further enhanced docking mediated by both vesicular proteins ($P < 0.05$; Fig. 8 D). These findings agree with studies reporting that native syt mediates LDCV docking in chromaffin cells (de Wit et al., 2009) and SV docking in neurons (Reist et al., 1998) and with recent findings that syb is critical for docking of LDCVs in PC12 cells (unpublished data). Interestingly, PIP_2 failed to promote docking when Vr harbored only FL syt. This might be a result of the relatively weak interaction between syt and PIP_2 under Ca^{2+}-free conditions, such that putative docking interactions were disrupted during the washing steps.

Our comparisons of FL syt and C2AB are summarized in Fig. 8 E, illustrating that they act via somewhat distinct mechanisms. Finally, we note that aggregation occurs more rapidly (complete in <1 min) than the priming step characterized in Fig. 1, G and H ($t_{1/2}$ = 3–9 min), suggesting the existence of a post-docking step that has yet to be defined in molecular terms but might involve the assembly of trans-SNARE complexes.

Figure 6. **C2AB exhibits context-dependent mixed antagonist/agonist activity.** (A) C2AB was titrated into fusion assays that contained FL syt in Vr. C2AB inhibited FL syt and Ca^{2+}-promoted fusion at relatively low concentrations (<1 µM) but promoted fusion at relatively high concentrations (≥1 µM). (B and C) The extent (B) and initial rate (C) of fusion (only the Ca^{2+}-dependent component was analyzed) were plotted versus [C2AB] (n = 3). (D) Average C_m traces of WT and syt KO chromaffin cells that did and did not overexpress GFP-C2AB in response to a steplike elevation of $[Ca^{2+}]_i$ generated by flash photolysis of caged Ca^{2+} (arrow indicates the flash). (E and F) The amplitudes (the size of the RRP) and rates of the fast release component were plotted for each condition (n ≥ 10). C2AB inhibited release in the presence of endogenous syt but partially rescued fast release in the absence of native syt. fF, femtofarad. All data shown are represented as mean ± SEM.



Discussion

In the current study, we draw six major conclusions. First, PIP_2 is absolutely required for membrane-embedded FL syt to regulate SNARE-mediated membrane fusion; moreover, Vr and Tr must be preincubated together to prime fusion before the Ca^{2+} trigger. Second, PIP_2 and PS are required only in the target membrane, consistent with models in which syt acts on the plasma membrane. Third, the FL syt system described here recapitulates three steps in the secretory pathway that occur in vivo: docking, priming, and subsequent Ca^{2+}-triggered fusion. In contrast, C2AB promotes all three steps only in response to Ca^{2+} (Hui et al., 2011). Fourth, the effects of several syt mutations in the FL syt fusion system described here more closely mirror the effects of these mutations on synaptic transmission in vivo, as compared with previous work focused on C2AB (Bhalla et al., 2005; Stein et al., 2007). For example, Ca^{2+} ligand mutations in C2B completely disrupt Ca^{2+}-triggered fusion in our FL syt-regulated fusion assay and in vivo but have little effect on C2AB-regulated fusion reactions. Fifth, the number of syt molecules needed per vesicle to drive efficient fusion closely mirrors the syt density on SVs in vivo (~15 copies/vesicle); in contrast, higher concentrations of C2AB are needed to drive fusion (e.g., 1 µM C2AB vs. 10 nM FL syt). Finally, we also addressed the ability of C2AB to both inhibit as well as stimulate fusion in vitro and in cells; in the presence of membrane-embedded or native syt, this protein fragment exhibits mixed agonist/antagonist activity, and in the absence of FL syt, this fragment acts only to stimulate fusion.

One of the most striking findings in the current study was the absolute requirement for a preincubation step before the Ca^{2+} trigger; if syt/Vr and PIP_2/Tr were not allowed to interact before the Ca^{2+} signal, regulated fusion was not observed. Hence, there appears to be a novel priming step that involves both syt and PIP_2. Interestingly, the $t_{1/2}$ for priming is longer (3–9 min) than the time requirements for docking (<1 min). We speculate that this lag might involve the relatively slow partial assembly of trans-SNARE pairs, and this idea will be tested in future studies using fluorescence probes to monitor SNARE structure. Further analysis of this step will be of interest, as it is known that in cells, vesicles must undergo priming reactions after docking to become fusion competent (Zenisek et al., 2000). Priming in vivo involves several additional factors that are not included in our fusion system (e.g., Munc13; Brose et al., 2000; Martin, 2002), so the priming step reported here does not reflect all the priming reactions that have been identified in cells.

We note that when PIP_2 was included in both Tr and Vr, Ca^{2+}-triggered fusion was not compromised, as compared with the condition in which PIP_2 was only present on Tr (Fig. 4 B). If weak Ca^{2+}-independent interactions with PIP_2 serve to steer the C2 domains of syt toward the target membrane (i.e., the plasma membrane; Bai et al., 2004), inclusion of PIP_2 in the Vr membrane might have been expected to inhibit fusion, but this did not occur. This is probably because PIP_2 and t-SNAREs on the target membrane act in a synergistic manner to steer the Ca^{2+}-triggered membrane penetration activity of syt to the target membrane even when PIP_2 is present on both membranes.

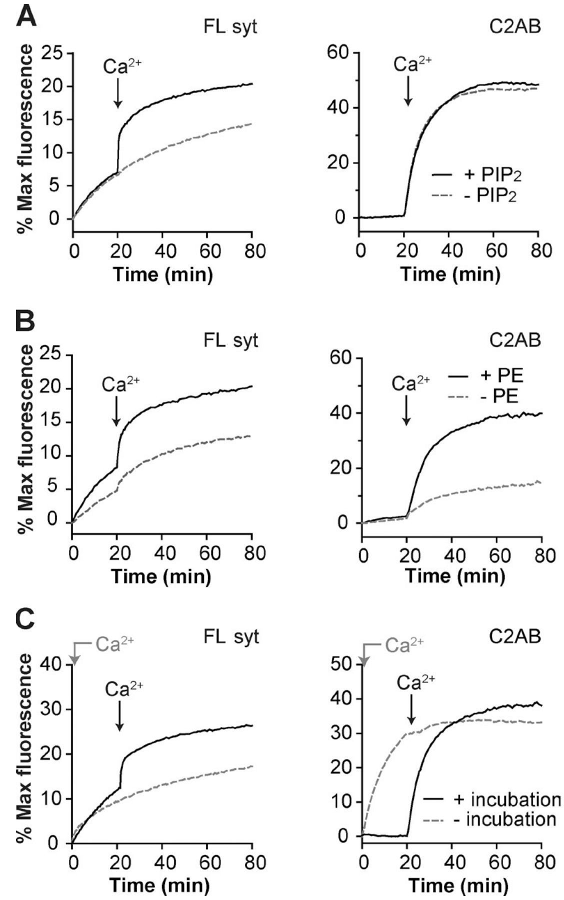


Figure 7. **PIP_2 and a preincubation step are required for FL syt-regulated fusion but are not needed for C2AB-regulated fusion activity.** (A–C) Fusion assays were performed as described in Fig. 1 B, with modifications. FL syt was used in all experiments shown in the left panels; C2AB was used in all experiments shown in the right panels. Assays were performed with or without PIP_2 (A), with or without 30% PE (B), and with or without a 20-min preincubation step in EGTA before the Ca^{2+} trigger (C). Other than these specific omissions, all other components remained constant. Injection of Ca^{2+} is indicated by arrows (gray arrows show Ca^{2+} injected at 0 min, and black arrows show Ca^{2+} injected at 20 min).

Indeed, recent studies indicate that PIP_2 interacts with t-SNAREs (Murray and Tamm, 2009), and the relevant target for syt might correspond to a complex composed of these components (Tucker et al., 2003) that binds the C2 domains of FL syt avidly enough to mediate efficient steering.

Another surprising finding was that FL syt, when reconstituted into only Tr, failed to promote fusion in response to Ca^{2+} (Fig. 4 C). This result contrasts the ability of C2AB, when fused to a plasma membrane–targeting motif, to rescue the syt KO phenotype in neurons (Hui et al., 2009). Although some degree of targeting to SVs cannot be ruled out in the rescue experiments, an alternative interpretation is that the N-terminal region of syt, which contains the sole TMD of the protein, prevents syt from stimulating fusion when reconstituted into the target membrane. This possibility is consistent with the finding that C2AB can partially rescue exocytosis in syt KO chromaffin cells; clearly, this soluble protein fragment is active in reconstituted systems and in living cells. So, it is plausible that C2AB, when in the plasma membrane, might be

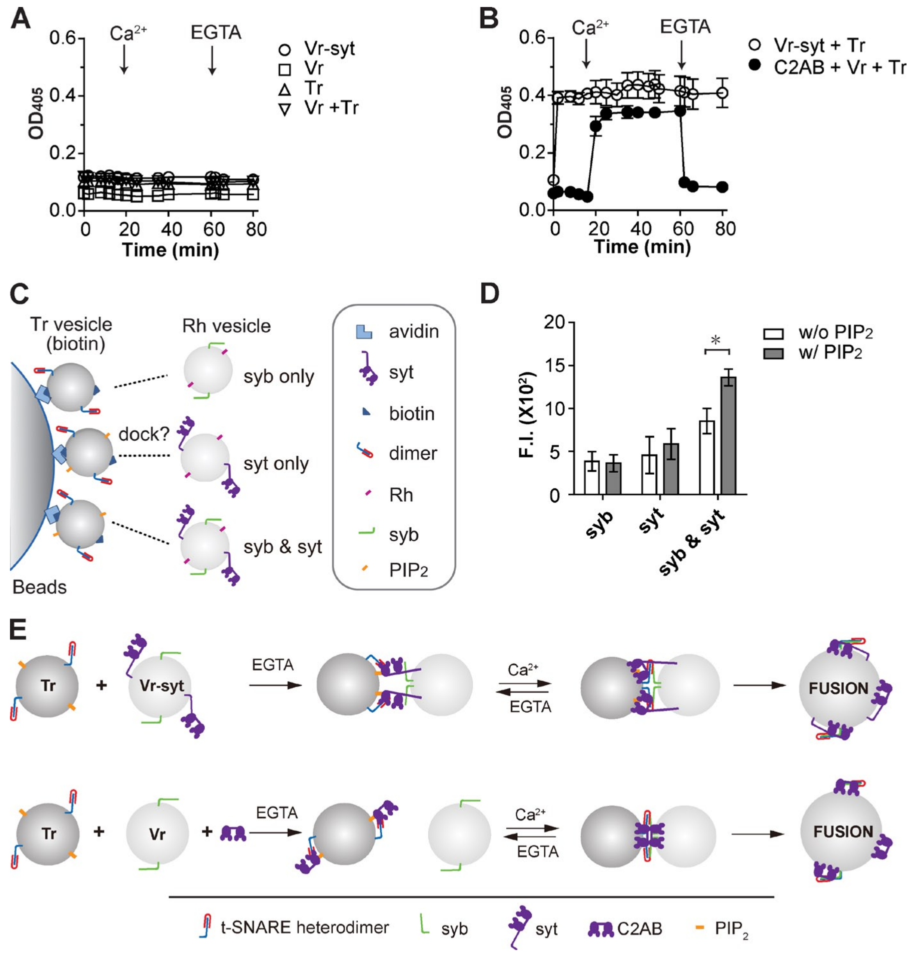


Figure 8. **FL syt and C2AB promote fusion via different mechanisms.** (A and B) FL syt, but not C2AB, mediates rapid Ca^{2+}-independent docking/aggregation of SNARE-bearing vesicles. Vesicle aggregation (OD_{405}) was monitored as a function of time; injection of Ca^{2+} (1 mM final) or EGTA (0.2 mM final) is indicated by arrows. (C) Illustration of the bead pull-down docking assay. (D) Direct measure of Vr and Tr docking. Tr was tethered to beads and used to pull down Rh-labeled Vr that harbored either FL syt, syb, or both proteins together. Protein-free Rh-labeled vesicles were used as a control; signals obtained under this condition were used to correct for background binding, so the plotted data report only specific binding (n = 3). F.I., fluorescence intensity. *, $P < 0.05$. (B and D) The data shown are represented as mean ± SEM. (E) A model summarizing the mechanism of action of FL syt versus C2AB.

rendered inactive by inclusion of the N-terminal domain. These findings raise the issue of whether the fraction of syt isoforms that is localized to the plasma membrane at steady-state (presumably after fusion) has any function in membrane fusion reactions in vivo.

A weakness of earlier in vitro fusion studies concerned the disparity between the effects of syt mutations on fusion in vitro versus the effects of these mutations on exocytosis from cells, as determined using genetic and electrophysiological approaches. Namely, in neurons, Ca^{2+} ligand mutations in C2A are tolerated or even lead to a slight gain of function (Robinson et al., 2002; Stevens and Sullivan, 2003), whereas analogous mutations in the C2B domain completely disrupt function (Mackler et al., 2002; Nishiki and Augustine, 2004). In contrast, similar mutations in C2AB, analyzed in reconstituted fusion reactions, led to markedly different results; Ca^{2+} ligand mutations in C2A resulted in greater losses in activity than did mutations in C2B (Bhalla et al., 2005; Stein et al., 2007). Here, we show that in the FL syt/PIP_2 fusion assay, mutations in the C2B domain completely disrupt function and thus mimic observations based on intact synapses. Although mutations in C2A do not yet recapitulate the synaptic physiology phenotype, they are clearly less deleterious than mutations in C2B. However, it should also be noted that expression of FL syt that harbors a mutation in a Ca^{2+} ligand in the C2A domain does result in reductions in secretion in PC12 cells (Wang et al., 2006).

A key concern regarding earlier in vitro studies based on the cytoplasmic domain of syt was the fact that overexpression of C2AB in WT PC12 cells (Desai et al., 2000; Tucker et al., 2003) or chromaffin cells (Rickman et al., 2004) inhibits exocytosis to some extent, suggesting that C2AB might not provide a valid means to study the positive role played by syt during fusion. Here, we resolved this controversy by documenting the mixed agonist/antagonist activity of C2AB in both in vitro and cell-based experiments. Namely, we used two systems in which FL syt was functional: the reconstituted fusion assay described here and WT chromaffin cells. We also had variants of each system that lacked FL syt (i.e., omission of FL syt in Vr and use of syt KO chromaffin cells). We found that in the absence of FL syt, C2AB stimulated fusion in both systems and did not exhibit any inhibitory activity. In contrast, when FL syt was present, low concentrations of C2AB partially inhibited fusion in both reconstituted fusion reactions and in chromaffin cells. The implication from this latter experiment is that, in some ways, FL syt works better than C2AB and that C2AB can interfere, to some degree, with the action of the intact protein. Indeed, FL syt and C2AB appear to regulate fusion via somewhat distinct mechanisms (Fig. 8). Together, these results indicate that C2AB has mixed agonist/antagonist activity in the presence of FL syt but acts only as an agonist in the absence of the FL protein. We note that another tandem C2 domain protein Doc2 (Orita et al., 1996; Groffen et al., 2010;

Yao et al., 2011), thought to regulate SV and LDCV exocytosis in a manner analogous to syt, lacks a membrane anchor and is soluble. Also, several syt isoforms have potential splice variants lacking a TMD. Hence, it will be interesting to determine whether these soluble proteins regulate fusion in a manner analogous to the C2AB domain of syt.

Unlike our previous work on C2AB, FL syt appeared to be unable to clamp fusion in the reconstituted system. In fact, in the absence of Ca^{2+}, membrane fusion was enhanced by increasing the syt copy number on Vr. This lack of clamping activity is probably a result of the strong spontaneous fusion of small unilamellar vesicles during the Ca^{2+}-free docking step. Indeed, we have recently shown that aggregation of v- and t-SNARE small unilamellar vesicles is sufficient to stimulate fusion to some extent, and this Ca^{2+}-independent component of fusion would obscure the potential clamping activity of FL syt (Loewen et al., 2006; Stein et al., 2007; Hui et al., 2011). Future studies using giant unilamellar vesicle target membranes or using lower temperatures might reduce the Ca^{2+}-independent fusion rate, making it possible to probe for clamping activity by comparing the fusion of Vr that do and do not harbor FL syt. It should be noted that vesicles prepared using different batches of lipids exhibited different degrees of Ca^{2+}-independent fusion, but all of the data in each individual panel are generated from the same stock of lipids.

In a previous study (Chicka et al., 2008), under Ca^{2+}-free conditions, C2AB was proposed to clamp SNARE assembly at a step after vesicle docking. Our vesicle aggregation data (Fig. 8 B) indicate that in EGTA, vesicles were largely nonaggregated/undocked (Fig. 8 B). Thus, a more plausible explanation for C2AB-mediated clamping activity might be that this protein fragment down-regulates fusion upstream of the docking/aggregation step.

In summary, we have reconstituted active FL syt and found that this protein is required for docking, priming, and fusion in an in vitro system. The next avenue of study will be to determine how each of these steps is related to changes in the structure of SNARE proteins and the assembly of SNARE complexes.

Materials and methods

DNA constructs

cDNA encoding rat syt was provided by T.C. Südhof (Stanford University, Menlo Park, CA). The D374 mutation was corrected by substituting this residue with glycine. A plasmid for the expression of recombinant mouse syb 2 was provided by J.E. Rothman (Yale University, New Haven, CT; Weber et al., 1998). FL t-SNARE heterodimers were generated, as previously described, by subcloning cDNA encoding FL rat SNAP-25B and rat syntaxin 1A into the pRSFDuet-1 vector (EMD; Chicka et al., 2008). Point mutations were generated by QuikChange mutagenesis (Agilent Technologies).

Protein expression and purification

Recombinant proteins were purified as previously described (Gaffaney et al., 2008). In brief, *Escherichia coli* was grown at 37°C to an A_{600} of 0.8, and protein expression was induced with 0.4 mm isopropyl 1-thio-β-D-galactopyranoside. After 4 h, bacteria were collected by centrifugation, lysed via sonication, and then extracted with 3% Triton X-100 for 3 h at 4°C. Insoluble material was removed by centrifugation (at 17K *g* for 25 min), and the supernatant was applied to an Ni^{2+} column using an ÄKTAFPLC system (GE Healthcare). Bound protein was washed extensively with resuspension buffer (25 mM Hepes-KOH, 400 mM KCl, 50 mM imidazole, 10% glycerol, and 5 mM 2-mercaptoethanol) containing 1% Triton X-100 followed by a wash buffer (25 mM Hepes-KOH, 400 mM KCl, 50 mM imidazole, 10% glycerol, 5 mM 2-mercaptoethanol, and 1% *n*-octyl glucoside); his-tagged proteins were eluted in the wash buffer with 500 mM imidazole.

Vesicle preparation

Lipids were purchased from Avanti Polar Lipids, Inc. Proteoliposomes were prepared as previously described (Tucker et al., 2004). In brief, lipids (Vr: 15% PS, 30% PE, and 55% phosphatidylcholine [PC]; Tr: 25% PS, 30% PE, 42% PC, and 3% PIP_2) were dried under a stream of nitrogen and resuspended in elution buffer (25 mM Hepes, 400 mM KCl, 10% glycerol, 1 mM dithiothreitol, and 1% *n*-octyl glucoside) plus the indicated proteins. Mixtures were diluted in dialysis buffer (25 mM Hepes, 100 mM KCl, 10% glycerol, and 1 mM dithiothreitol) and centrifuged for 5 h at 290K *g* in an Accudenz gradient (Accurate Chemical & Scientific Corporation). Vesicles were collected (1.2 ml) from the 0 and 30% Accudenz interface.

In vitro fusion assay

Fusion reactions (total volume of 100 µl) were composed of 8 µl of Tr (8 nM final concentration), 1 µl of NBD-rhodamine (Rh)–labeled Vr (1 nM final concentration) that bear syt, and buffer (25 mM Hepes, 100 mM KCl, and 1 mM dithiothreitol). Mixtures were preincubated at 37°C for 20 min in the presence of 0.2 mM EGTA followed by injection of 1 mM Ca^{2+}; fusion was monitored for an additional hour. At the end of each run, 20 µl of the detergent *n*-dodecyl-β-D-maltoside was added to each reaction to yield the maximum fluorescence signals at infinite dilution of the FRET donor–acceptor pair. NBD dequenching was monitored using a plate reader (HT Synergy; BioTek). Statistical significance was evaluated by using the two-tailed unpaired Student's *t* test (***, $P < 0.001$). All data shown are represented as mean ± SEM.

Conjugation of C2AB to Vr

The lone endogenous cysteine in C2AB (C277) was mutated to an alanine, and glycine 96 at the N terminus was mutated to cysteine using the mutagenesis method described in the DNA constructs section. For labeling, lipid mixtures that contained maleimide-PE (Avanti Polar Lipids, Inc.) were incubated with the mutated form of C2AB (G96C and C277A) for 30 min. DTT was then added to block the remaining maleimide functional groups. Then, syb was incubated with the mixtures for 20 min, and samples were diluted with fusion assay buffer and dialyzed against this buffer overnight. The dialyzed vesicles were purified on an Accudenz gradient.

Preparation of mouse chromaffin cells

Adrenal glands were removed from newborn WT and syt KO mice and digested with 1 ml Dispase II (Roche) for 20 min at 37°C to obtain isolated chromaffin cells. Cells were incubated in DME supplemented with penicillin/streptomycin (40,000 U/L and 40 mg/L; Invitrogen) and 10% FBS at 37°C with 5% CO_2. Tails from syt KO pups were kept for genotyping. All procedures involving animals were performed in accordance with the guidelines of the National Institutes of Health, as approved by the University of Wisconsin-Madison Animal Care and Use Committee.

Lentivirus constructs and infection of chromaffin cells

GFP was fused to the N terminus of the cytoplasmic domain of syt C2AB (residues 96–421) and subcloned into the lentiviral vector pLox Syn-DsRed-Syn-GFP (provided by F. Gomez-Scholl, University of Seville, Seville, Spain). Because cultured chromaffin cells are viable for relatively short periods of time (5 d) and protein expression using the synapsin promoter in pLox is slow, we replaced the synapsin promoter, via XbaI and EcoRI restriction sites, with a cytomegalovirus promoter, which results in faster protein expression (2 d). The GFP tag was used to identify infected chromaffin cells for recordings.

Lentiviral particles were generated by transfecting HEK293T cells with the modified lentiviral construct plus two other packaging vectors encoding VSV-G and Δ8.9. The supernatant was collected after 48–72 h, purified by filtration through a 0.45-µm filter, and centrifuged at 70K *g* for 2 h to concentrate the virus. Viral particles were resuspended in PBS, and the titer was determined. For overexpression of GFP-C2AB, cells were plated on polylysine-coated coverslips and infected with virus for 3 d. Electrophysiological recordings were performed between 3 and 4 d in vitro.

Ca^{2+} uncaging and $[Ca^{2+}]_i$ measurement

Homogenous global elevation of $[Ca^{2+}]_i$ was achieved by photolysis of the caged Ca^{2+} compound nitrophenyl-EGTA (NP-EGTA; Invitrogen) with a UV light source, as previously described (Xu et al., 1997). In brief, steplike elevations of $[Ca^{2+}]_i$ were elicited via a UV flash generated from a flash lamp (Rapp OptoElectronic GmbH). The flash was followed by excitation, via a monochromator (Polychrome V; TILL Photonics), that alternated between 350 and 380 nm, allowing for ratiometric determination of the Ca^{2+} concentration according to the equation (Grynkiewicz et al., 1985) $[Ca^{2+}]_i = K_{eff} \times (R - R_{min})/(R_{max} - R)$, in which K_{eff}, R_{min}, and R_{max} are constants obtained from in vivo calibration. R was calculated as F_{350}/F_{380} after background subtraction. Fluorescence signals were monitored using a photodiode detector (TILL Photonics). The NP-EGTA pipette solution contained 110 mM CsCl, 5 mM NP-EGTA, 2 mM NaCl, 4 mM $CaCl_2$, 2 mM MgATP, 0.3 mM GTP, 0.2 mM Fura-6F, and 35 mM Hepes, adjusted to pH 7.2 using CsOH or HCl (osmolarity of 300 mOsm). The free Ca^{2+} concentration in the pipette solution was determined to be ~200 nM.

Membrane capacitance (C_m) measurements

The C_m of chromaffin cells was monitored in real time using an amplifier (EPC 10 Double; HEKA) with a conventional whole-cell patch clamp configuration. A sine + dc protocol was applied using the Lockin extension of the Pulse program (HEKA). Chromaffin cells were voltage clamped at a holding potential of −70 mV, and a sine wave voltage command (20 mV at 977 Hz) was applied. Currents were filtered at 2.9 kHz and sampled at 15.6 kHz. The bath solution contained 140 mM NaCl, 2.5 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, and 10 mM glucose (adjusted to pH 7.4 with NaOH at 308 mOsm).

Vesicle aggregation assay

To monitor vesicle aggregation, the optical density of samples was measured at 405 nm using a BioPhotometer Plus (Eppendorf). 5 µl of Tr was incubated with either 5 µl of Vr-syt or Vr vesicles plus 1 µM C2AB in the presence of 0.2 mM EGTA for 20 min. Then, 1 mM Ca^{2+} was injected, and aggregation was monitored for 40 min. EGTA was added at the end of each run to assay for reversibility.

Data analysis for C_m

Data analysis was performed using IGOR Pro software (version 5.05; WaveMetrics). Statistical significance was evaluated using the Kruskal–Wallis test for multiple comparisons of groups with nonnormal distributions. Offline analysis of $[Ca^{2+}]_i$ data was performed by measuring the fluorescence intensity from individual chromaffin cells; data were analyzed using IGOR Pro software (version 5.05). $[Ca^{2+}]_i$ was calculated from the equation derived by Grynkiewicz et al. (1985), as detailed in the Ca^{2+} uncaging and $[Ca^{2+}]_i$ measurement section. For C_m responses in flash photolysis experiments, the size and release rate of three distinct release components—the RRP, the SRP, and the sustained release of vesicles—were determined as described in previous studies (Voets, 2000; Sørensen et al., 2003). In brief, a triple exponential function was used to fit the C_m responses as follows:

$$f(t) = A_0 + \sum_{i=1}^{3} A_i \times \left(1 - \exp\left(-(t - t_0)/\tau_i\right)\right) \text{ for } t > t_0,$$

in which A_0 is the capacitance of the cell before flash, and t_0 is the time of flash. The amplitudes (A_i) and time constants (τ_i) of the two faster exponentials define the size and release kinetics of the fast and the slow exocytotic burst, respectively. The third exponential represents the sustained component.

Bead pull-down docking assay

Avidin beads (Thermo Fisher Scientific) were first blocked with protein-free liposome (PS, PC, and PE at 15, 55, and 30%, respectively) at 4°C overnight. Beads were then washed with fusion assay buffer three times, and 40 µl of bead slurry was incubated with 10 µl of Tr bearing biotin-PE at room temperature for 15 min. The samples were then washed with the same buffer and incubated with Vr that did and did not harbor reconstituted proteins (i.e., FL syt and syb, as indicated in Fig. 8 C) labeled with 1.5% Rh-PE at 4°C for 30 min. After washing three times with fusion buffer, 50 µl of 2.5% *n*-dodecyl-β-D-maltoside (Sigma-Aldrich) was added to solubilize the Rh-PE. Beads were removed by centrifugation (4,600 rpm in a microfuge for 1 min), and the Rh signal in the supernatant was measured using a plate reader.

Immunostaining

Chromaffin cells were costained with a mouse monoclonal antibody directed against the C2AB domain of syt (41.1; provided by Reinhard Jahn, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany) and, to visualize LDCVs, a rabbit polyclonal antibody directed against chromogranin B. The cells were fixed in PBS with 4% PFA, permeabilized, and blocked with 0.1% Triton X-100 plus 10% goat serum stained with primary antibodies for 2 h, washed with PBS three times, and then incubated with either Cy3-tagged anti–mouse or Alexa Fluor 647–tagged anti–rabbit (Jackson ImmunoResearch Laboratories, Inc.) secondary antibodies for 1 h at room temperature. Coverslips were then mounted in Fluoromount (SouthernBiotech), and images were acquired on an upright confocal microscope (FluoView 1000; Olympus) with a 60× 1.10 NA water immersion lens.

Online supplemental material

We included five supplemental figures to address the following issues: (1) whether Ca^{2+}–syt promoted full fusion or hemifusion (Fig. S1); (2) the Ca^{2+} sensitivity of syt-promoted fusion (Fig. S2); (3) whether C2AB, covalently linked to phospholipids, mimics FL syt during regulated membrane fusion (Fig. S3); and (4) control experiments for Figs. 7 and 8 (Figs. S4 and S5, respectively). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201104079/DC1.

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