Solution single-vesicle assay reveals PIP₂-mediated sequential actions of synaptotagmin-1 on SNAREs

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Synaptotagmin-1 (Syt1) is a major Ca²⁺ sensor for synchronous neurotransmitter release, which requires vesicle fusion mediated by SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). Syt1 utilizes its diverse interactions with target membrane (t-) SNARE, SNAREpin, and phospholipids, to regulate vesicle fusion. To dissect the functions of Syt1, we apply a single-molecule technique, alternating-laser excitation (ALEX), which is capable of sorting out subpopulations of fusion intermediates and measuring their kinetics in solution. The results show that Syt1 undergoes at least three distinct steps prior to lipid mixing. First, without Ca²⁺, Syt1 mediates vesicle docking by directly binding to t-SNARE/phosphatidylinositol 4,5-bisphosphate (PIP₂) complex and increases the docking rate by 10³ times. Second, synaptobrevin-2 binding to t-SNARE displaces Syt1 from SNAREpin. Third, with Ca²⁺, Syt1 rebinds to SNAREpin, which again requires PIP₂. Thus without Ca²⁺, Syt1 may bring vesicles to the plasma membrane in proximity via binding to t-SNARE/PIP₂ to help SNAREpin formation and then, upon Ca²⁺ influx, it may rebound to SNAREpin, which may trigger synchronous fusion. The results show that ALEX is a powerful method to dissect multiple kinetic steps in the vesicle fusion pathway.

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Introduction

Neurons use Ca²⁺-triggered exocytosis of synaptic vesicles to release neurotransmitters for interneuronal communication. To release neurotransmitters, synaptic vesicles undergo several steps; tethering and docking onto the presynaptic plasma membrane, priming that prepares vesicles to be fusion ready, and membrane fusion triggered by Ca²⁺ influx (Sudhof, 2004). Vesicle fusion is mediated by the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins: t-SNAREs syntaxin-1A and SNAP-25 on the plasma membrane and v-SNARE VAMP-2 (or synaptobrevin-2) on the synaptic vesicles (Weber *et al*, 1998; Jahn and Scheller, 2006; Sudhof and Rothman, 2009). SNAREs from two membranes engage and form a parallel four-helix bundle (Poirier *et al*, 1998; Sutton *et al*, 1998), which brings membranes into close proximity, facilitating fusion (Sudhof and Rothman, 2009).

Synaptotagmin-1 (Syt1), a vesicle protein that has two Ca²⁺-binding tandem C2 domains (C2A and C2B), is a major Ca²⁺ sensor for neuroexocytosis (Brose *et al*, 1992; Fernandez-Chacon *et al*, 2001; Chapman, 2008). The disruption of *Syt1* gene typically abolishes the rapid Ca²⁺-dependent synchronous exocytosis (DiAntonio *et al*, 1993; Littleton *et al*, 1993; Nonet *et al*, 1993; Geppert *et al*, 1994). Syt1 is known to bind the SNARE complex (or SNAREpin) as well as phospholipids in the presence of Ca²⁺ (Schiavo *et al*, 1996; Zhang *et al*, 2002; Rickman *et al*, 2004, 2006; Bai *et al*, 2004b; Bhalla *et al*, 2006; Tang *et al*, 2006; Lynch *et al*, 2007; Connell *et al*, 2008; Hui *et al*, 2011). The SNARE complex binding may play a role in declamping the complexin clamp that holds vesicles at the primed state until the Ca²⁺ influx or it may just assist SNARE complex formation (Chapman, 2008; Rizo and Rosenmund, 2008; Sudhof and Rothman, 2009). Meanwhile, the phospholipid binding may function to induce a membrane curvature that is favourable for fusion (Martens *et al*, 2007; Hui *et al*, 2009) or it simply serves to enhance the Ca²⁺ sensitivity for C2 domain (Brose *et al*, 1992; Schiavo *et al*, 1996).

Besides its Ca²⁺ sensing activity, a new emerging role of Syt1 is its functions prior to fusion (Bai *et al*, 2004a; Chapman, 2008; de Wit *et al*, 2009; van den Bogaart *et al*, 2011b). It has been proposed that this Syt1 function involves again the binding of Syt1 to SNAREs (Loewen *et al*, 2006; Rickman *et al*, 2006), although the specifics of this interaction is not clear. Several morphological studies using EM showed that the knockout of Syt1 decreased the docked vesicles on the plasma membrane (Reist *et al*, 1998; Liu *et al*, 2009; de Wit *et al*, 2009), which suggested that Syt1 plays a role in synaptic vesicle docking (or tethering) (Reist *et al*, 1998; Loewen *et al*, 2006; Rickman *et al*, 2006; de Wit *et al*, 2009). A recent study in adrenal chromaffin cells has revealed that the binding partner of Syt1 is binary t-SNARE and this specific interaction plays an important role in vesicle docking (de Wit *et al*, 2009). In contrast, an *in-vitro* study using

proteoliposomes showed that Syt1 has strong affinity to phosphatidylinositol 4,5-bisphosphate (PIP₂) without Ca²⁺, which led to a proposition that Syt1 acts as a steering factor to bring vesicles closely to the plasma membrane prior to the Ca²⁺ influx (Bai *et al*, 2004a). Recent work also showed that Syt1 tethers vesicles without Ca²⁺, but does not require its specific interaction with t-SNARE (van den Bogaart *et al*, 2011b). Thus, there is ample evidence that Syt1 is involved in a process upstream of the Ca²⁺ action, but it is still controversial whether it interacts with t-SNARE or PIP₂, or both, to induce vesicle tethering. It is also unknown how Syt1 helps vesicle tethering kinetically and what happens to Syt1 after SNAREpin is formed (Chapman, 2008; Rizo and Rosenmund, 2008).

The bulk *in-vitro* fluorescence assay using proteoliposomes appears to be an ideal tool to dissect interactions of Syt1 with t-SNARE and PIP₂ (Weber *et al*, 1998). The anticipated transition of Syt1's interactions, first with t-SNARE (and/or PIP₂) and subsequently with the SNARE complex (and/or PIP₂) can be examined effectively through such a well-defined *in-vitro* setting. However, the fundamental weakness of this technique is its inability to discriminate the earlier steps, happening before lipid mixing from the fusion step. For example, vesicle docking is undetected in this fluorescence-based method, because the docking of a vesicle pair does not give rise to the change in the fluorescence signal. Therefore, this method is not adequate to study Syt1's interactions with t-SNARE or PIP₂, and subsequently with the SNARE complex, which will be followed by lipid mixing.

Here, we introduce a new single-molecule technique, alternating-laser excitation (ALEX), which is capable of sorting out the subpopulations of fusion products, such as unreacted vesicles, fused vesicles, and fusion intermediate of docked-but-unfused species and measuring the kinetics of each subpopulation in solution (Figure 1A; Supplementary Figure S1; Kapanidis *et al*, 2004; Lee *et al*, 2005). The docked and fused vesicles have been discriminated by a single-vesicle assay by immobilizing vesicles on a surface or lipid bilayer (Bowen *et al*, 2004; Fix *et al*, 2004; Liu *et al*, 2005; Yoon *et al*, 2006; Kyoung *et al*, 2011). But this method often suffers from non-specific binding of vesicles on surface. Recently, as a method bypassing surface immobilization, fluorescence cross-correlation spectroscopy (FCCS) has been used to monitor docking and fusion kinetics (Cypionka *et al*, 2009). However, FCCS observes several vesicles at the same time and thereby does not provide direct measurement of full subpopulations of fusion products. In comparison with these methods, ALEX detects vesicles at the single-vesicle level without the need of surface immobilization and directly provides full subpopulations and the kinetics of fusion products.

We used ALEX to dissect the interactions of membrane-anchored Syt1 with t-SNARE, PIP₂, and the SNARE complex prior to lipid mixing and studied the effect of Syt1 on docking and fusion kinetics. We found that Syt1 interacts with both t-SNARE and PIP₂, which gives rise to vesicle tethering without Ca²⁺. When VAMP-2 binds to t-SNARE to form the SNARE complex, Syt1 dissociates from the complex. However, when Ca²⁺ is introduced, Syt1 rebinds to the SNARE complex. We find that PIP₂ is required in this final step too. Thus, in the absence of Ca²⁺ Syt1 may function to bring vesicles to the plasma membrane in proximity through

t-SNARE/PIP₂ interaction, which helps SNAREpin formation, and finally binds to SNAREpin and membrane again in the presence of Ca²⁺, which may trigger membrane fusion. Kinetically, the docking rate of vesicles containing Syt1 is as much as 10³ times higher than that of SNAREs-only vesicles. Moreover, the fusion step is found to be the rate-limiting step for Syt1-containing vesicles in the absence of Ca²⁺. The results demonstrate that ALEX is a powerful tool for discriminating the subpopulations and measuring the kinetics of docking and fusion of vesicles in solution, which will be useful in studying the functions of SNAREs and regulatory proteins in vesicle fusion.

Results

ALEX sorts out subpopulations and measures their kinetics in an *in-vitro* fusion assay

The bulk *in-vitro* fusion assay using fluorescently labelled proteoliposomes has been an important tool for investigating the functions of SNAREs and their regulatory proteins (Weber *et al*, 1998). In this method, liposomes reconstituted with t-SNAREs (t-vesicles) are mixed with another population of liposomes reconstituted with v-SNAREs (v-vesicles). As fusion progresses, the reaction builds up a mixture of fusion intermediates and products as well as unreacted v- and t-vesicles. However, this bulk method is not effective in dissecting subpopulations corresponding to these individual species, and the results are often ambiguous (Smith and Weisshaar, 2011).

In ALEX, we detect and analyse single-vesicle species diffusing in and out of a confocal volume (Figure 1A) and sort them into subpopulations according to their fluorescence characteristics (Figures 1B–F). We dope t-vesicles with fluorescence donor (DiI) and v-vesicles with fluorescence acceptor (DiD). We focus both donor- and acceptor-excitation lasers into a small confocal volume (Figure 1A). The reaction mixture is sufficiently diluted (~100 pM vesicle concentration) that only a single particle diffuses into the detection volume at a given moment. The donor- and acceptor-excitation lasers alternate sufficiently fast to excite and detect both the donor and acceptor fluorescence before the vesicle diffuses out of the volume. Figures 1B–E show typical time traces, where each fluorescent burst represents a single vesicle, a pair/aggregate of docked vesicle, or a fused vesicle, diffusing in and out of the confocal volume. Each single burst contains three types of fluorescence intensities, I_D^D , I_D^A , and I_A^A , where I_D^D and I_D^A are the donor and acceptor fluorescence intensity, respectively, when the vesicle is irradiated with the donor-excitation laser, and I_A^A is the acceptor fluorescence intensity when irradiated with the acceptor-excitation laser. These three intensities are used to calculate two parameters, **S**, a sorting number, and **E**, the fluorescence resonance energy transfer (FRET) efficiency of each vesicle (for detail, see Materials and methods) (Kapanidis *et al*, 2004). Briefly, **S** becomes 1 for a vesicle responding only to the donor-excitation laser but not to the acceptor-excitation laser, which represents a single t-vesicle, while it is 0 for that responding to the acceptor-excitation laser but not to the donor-excitation laser, which represents a single v-vesicle. For a vesicle pair that contains both t- and v-vesicles, **S** becomes 0.5 by responding to both lasers. The **E** value is calculated based on the equation $E = I_D^A / (I_D^D + I_D^A)$, which reports the degree of

Figure 1 Sorting subpopulations of vesicle mixture by single vesicle assay, alternating-laser excitation (ALEX). (A) Schematic description of single-molecule alternating-laser excitation set-up (Supplementary Figure S1). Donor- and acceptor-excitation lasers are alternated faster (400 μ s) than the transit time ($\sim$ 5 ms) of a vesicle through the confocal excitation volume ($\sim$ 1 femto-liter). Dilution to $\sim$ 100 pM vesicle concentration ensures that one vesicle passes through the excitation volume at a given time. The fluorescent emissions of donor and acceptor are detected separately, which results in fluorescence time traces. (B–E) Typical fluorescence time traces of vesicles. A random event of diffusing in and out of a vesicle generates a fluorescent burst (or spike) in time traces. Each time trace contains three different photon streams: I_D^D , fluorescent emissions of donor dyes (DiI) excited by donor-excitation laser (green line), I_D^A , fluorescent emissions of acceptor dyes (DiD) excited by donor-excitation laser, which are FRET signals (orange line), and I_A^A , fluorescent emissions of acceptor dyes excited by acceptor-excitation laser (red line). (B) Time trace of t-vesicle, reconstituted with t-SNARE and doped with DiI. (C) Time trace of v-vesicle, reconstituted with VAMP-2 and doped with DiD. (D) Time trace of docked-but-unfused vesicle. Both I_D^D and I_A^A present significant intensity. (E) Time trace of fused vesicle. Due to FRET, the intensity of I_D^D is reduced, while that of I_D^A is increased. (F) Two-dimensional E (FRET efficiency)-S (sorting number) graph. Three fluorescent intensities of a burst are used to calculate S and E (see ‘Definition and calculation of E and S of a single vesicle’). The unreacted, docked, and fused vesicles have characteristic values of S and E, and, as a result, occupy different areas in E-S graph: t-vesicle only (green box), v-vesicle only (red box), docked vesicle (purple box), and fused vesicle (orange oblique). (G) Comparison of bulk FRET assay with ALEX. For bulk FRET assay, T (reconstituted with t-SNARE) and V (reconstituted with VAMP-2) were mixed to be 50 μ M lipid concentration at 35°C (black line). From single-exponential fitting, fusion rates were obtained; $k_{\text{fusion, bulk}} = 1.03 \pm 0.02 \times 10^{-3} \text{ s}^{-1}$ and $k_{\text{fusion, ALEX}} = 1.19 \pm 0.08 \times 10^{-3} \text{ s}^{-1}$, respectively. As a control, SNAP-25 was not added (grey line). For ALEX, the mixture was incubated at 35°C and diluted to be 3 μ M lipid concentration at the selected time points and then measured for 5 min. The fractions of docked and fused vesicles (purple box and orange oblique, respectively) were obtained from three independent measurements. Dotted line denotes the level of random coincidence. (H–J) 2D E-S graphs obtained by ALEX. Each dot denotes a vesicle. (H) T-V mixture after 60 s incubation (blue arrow in G), and (I) after 2400 s incubation (red arrow in G). (J) No SNAP-25 control after 2400 s incubation (green arrow in G). Colour scale bar indicates the number of vesicles.

lipid mixing. In the two-dimensional (2D) display of S and E parameters, subpopulations corresponding to unreacted v- and t-vesicles, docked vesicles, and fused vesicles are resolved into distinct areas (Figure 1F).

We prepared v-vesicle with VAMP-2 (V, the lipid-to-protein ratio L/P = 300) and t-vesicle with t-SNARE (T, L/P = 300). Figure 1G depicts the typical results of the bulk measurement of the T-V mixture at 35°C, which shows fusion between T and V through the increase of FRET (black line), while the control without SNAP-25 shows no FRET increase (grey line). The 2D E-S graph (Figure 1H), taken 1 min after incubating the T-V mixture at 35°C (Figure 1G, blue arrow), shows considerable subpopulation of unreacted vesicles (green and red boxes), while docked and fused vesicles are rare (purple box and orange oblique, respectively). However, after 40 min incubation (Figure 1G, red arrow), the population of fused vesicles was considerably increased, while the populations of unreacted vesicles were decreased and docked vesicles were still scarce (Figure 1I). As a control, when SNAP-25 is not included, no fused vesicles were observed even after 40 min incubation (Figure 1G, green arrow), but unreacted

v- and t-vesicles are clearly seen (Figure 1J). The E values of donor-only (T) and acceptor-only (V) vesicles in the 2D graph are determined by fluorescent backgrounds. For example, E of donor-only vesicles (0.13) was generated by the leakage (donor emission detected by acceptor emission detector), and the high E of acceptor-only vesicles was caused by the direct excitation of acceptor dyes by donor-excitation laser (Lee *et al*, 2005).

We then quantified the subpopulations of docked and fused vesicles from the 2D E-S graph. A simple quantitative analysis (Supplementary data) using this E-S graph was used to measure the fractions of fused and docked vesicles. In this analysis, we select bursts using ‘acceptor photon search’ (Supplementary Figure S2; Kapanidis *et al*, 2006). To select fluorescent bursts from the time traces (Figures 1B–E), a threshold photon count above the background is used (Kapanidis *et al*, 2004). Typically, all photon counts, that is, the sum of donor and acceptor intensities ($I_D^D + I_D^A + I_A^A$), are used for burst search to present all fluorescent species in 2D E-S graphs (Figure 1H–J). However, this searching method selects docked and fused vesicles, which contain both donor

and acceptor dyes, more efficiently than unreacted vesicles. For this reason, when quantitative analysis of subpopulations is required, I_A^A is used for burst search (acceptor photon search) (Kapanidis *et al*, 2006). Because unreacted v-vesicle, docked, and fused vesicles have similar intensities of I_A^A , this 'acceptor photon search' provides unbiased quantitative measurement of the subpopulations (see Supplementary Figure S2 for comparison between two burst searching methods). We found that the fusion kinetics measured from the fractions of fused vesicles agrees well with the bulk measurement (Figure 1G). We estimate that ~5% of docked vesicles is the background, which stems from two vesicles entering into the detection volume simultaneously without docking. The subpopulation of docked vesicles was not seen, indicative of a significantly slower rate of docking than the rate of fusion for the T-V reaction (Smith and Weisshaar, 2011).

Both binary t-SNARE and PIP₂ are required for Syt1-mediated docking

Previously, using a surface single-vesicle assay, it has been demonstrated that membrane-anchored Syt1 is capable of promoting vesicle docking and fusion in a Ca²⁺-independent manner (Lee *et al*, 2010). However, because both Syt1 and VAMP-2 were present in v-vesicle, it was unclear whether such an effect was due to the direct interaction between Syt1 and the target membrane components (t-SNARE and PIP₂) or due to its catalytic role in helping VAMP-2 interaction with t-SNARE. To investigate the possible direct interaction between Syt1 and t-SNARE *in trans*, we prepared v-vesicle without VAMP-2 but containing Syt1 (**S** in Figure 2A, L/P = 600). We included 1% PIP₂ in t-vesicle (**T**) (L/P = 500 for t-SNARE). We mixed **S** with **T** to induce docking at room temperature for 5 min at 10 μ M lipid concentration and analysed the mixture using ALEX at 3 μ M lipid concentration

Figure 2 Syt1 is able to mediate vesicle docking by interacting with both t-SNARE and PIP₂. (A) Schematic description of vesicle docking measurement. **S**, v-vesicle without VAMP-2 but containing Syt1. **T**, t-vesicle with t-SNARE and 1% PIP₂. After mixing two vesicles, the mixture was analysed by ALEX. (B–E) 2D *E-S* graphs for mixture of t- and v-vesicles. (B) Mixture of **S** and **T**. Significant amounts of docked vesicles were observed (purple box). (C) Mixture of **S** and **S**_{Y311N} (v-vesicles incorporated with Syt1 Y311N mutant) and **T**. (D) Mixture of **S** and **T**_{no PIP₂} (t-vesicle incorporated with t-SNARE but without PIP₂). (E) Mixture of **S** and **T**_{syx-only} (t-vesicle containing only syntaxin-1A but containing 1% PIP₂). (F) Positions of SNAP-25 mutations in SNARE complex. (G–I) Three SNAP-25 mutants of SNAP-25 D51K/E52K, D179K/D186K, and BoNT/A were used to reconstitute **T** (**T**_{D51K/E52K}, **T**_{D179K/E186K}, and **T**_{BoNT/A}, respectively), and each one of them was mixed with **S**. (G) **T**_{D51K/E52K}-**S**; (H) **T**_{D179K/E186K}-**S**; (I) **T**_{BoNT/A}-**S**. (J) Time-dependent T-S docking. For detailed method, see 'ALEX measurements for the docking and fusion kinetics' in Supplementary data. The error bars within 5 min were obtained from 10 independent measurements, while those after 5 min from three measurements. (K) Bar graph of the fraction of the docking subpopulations. Error bars (standard deviation) were obtained from more than three independent experiments, and >1000 vesicles were analysed in each measurement (**P* < 0.005). (L) PIP₂ only control. We mixed protein-free liposome, containing 1% PIP₂, and **S**. (M) The t-SNARE/PIP₂ molar ratio determines docking efficiency of **T**_{D51K/E52K}-**S**. We kept 1% PIP₂, but varied the amounts of t-SNARE constructed with SNAP-25 D51K/E52K mutant, and then measured the docking efficiency of **T**_{D51K/E52K}-**S**. We used initial input molar ratio of t-SNARE/PIP₂ and the incorporation efficiency of t-SNARE into vesicle (60 ± 11%; Supplementary Figure S5) to determine the t-SNARE/PIP₂ ratio. Error bars were obtained from more than three independent experiments.

(~100 pM vesicle concentration). The 2D *E-S* graph of the **T-S** mixture (Figure 2B) shows a considerable docking population (purple box). A simple quantitative analysis (Supplementary data; Supplementary Figure S2) using this *E-S* graph shows that 40% of **S** are paired (or docked) with **T** (Figure 2K). To confirm that vesicle docking occurred through Syt1, we prepared an Syt1 mutant (Syt1 Y311N) that has a single point mutation in the C2B domain, which is known to reduce Syt1's binding to t-SNARE (Yoshihara and Littleton, 2002; Rickman *et al*, 2006). Indeed, with Syt1 Y311N vesicle docking was significantly impaired nearly to the background level of 7% (Figures 2C and K). This result shows that Syt1 is responsible for vesicle docking observed for **T-S** (Figure 2B).

In **T**, there are two potential binding partners for Syt1, t-SNARE and PIP₂ (Tucker *et al*, 2003; Bai *et al*, 2004a). To find which interaction is dominant for vesicle docking, we prepared two types of **T**; one without PIP₂ (**T_{no PIP2}**) and the other without SNAP-25 (**T_{syx-only}**) while keeping other components the same. The absence of PIP₂ significantly reduced the subpopulation of docked vesicles (Figure 2D), indicating that PIP₂ is essential for Syt1-induced vesicle docking. Likewise, when SNAP-25 is absent docking population was reduced to the similar level in the case without PIP₂ (Figure 2E). This shows that the docking by Syt1 required both PIP₂ and binary t-SNARE.

To further confirm the need of binary t-SNARE for Syt1 docking, we reconstituted t-vesicle with the SNAP-25 D51K/E52K mutant (Figure 2F), which is known to disrupt the interaction between Syt1 and t-SNARE without interfering with formation of SNARE complexes (Rickman *et al*, 2006; Supplementary Figure S3). As expected, the subpopulation of docked vesicles was considerably reduced for this mutant (Figure 2G). Thus, this result shows that Syt1 directly interacts with t-SNARE for docking. On the other hand, when two other SNAP-25 mutants D179K/D186K in which two aspartic acids in the middle were changed to lysines and BoNT/A in which the C-terminal 9 residues are cleaved were used (Figure 2F), the subpopulation of docked vesicles were similar to that for wild type (Figures 2H and I). Thus, the results suggest that Syt1 interacts with the central region of the SNAP-25 SNARE motif, where positions 51 and 52 are located rather than the C-terminal region or the face where 179 and 186 residues are located (Figure 2F), consistent with the previous single FRET result (Choi *et al*, 2010).

Then, we tested if PIP₂ offers a specific binding for Syt1 or it is simply to add the surface negative charge in the target membrane. We increased the PS concentration from 15 to 25% in **T**. This increased the docked population marginally from 7 to 10% (Figure 2K; Supplementary Figure S4). In sharp contrast, adding just 1% PIP₂ increased the docking population to 40%. Therefore, the results show that the stimulation of docking by Syt1 is highly specific to PIP₂.

Overall, the ALEX results support that Syt1 is capable of inducing vesicle docking in the absence of VAMP-2 and Syt1 uses its interaction with both t-SNARE and PIP₂, consistent with the *in-vivo* observation in chromaffin cells (de Wit *et al*, 2009).

The kinetics of Syt1-mediated vesicle docking

Next, we investigated how fast the docking between **T** and **S** occurs (Figure 2J). To measure the docking kinetics, we collected the ALEX data for 5 min soon after mixing **T** and **S** with 3 μ M final lipid concentration. Then, the data were divided into five time bins and the fractions of docked

vesicles were obtained for each time bin. For later times than 5 min, the mixture was incubated at room temperature for a desired duration and then measured for 5 min as described above. Because of the delay between mixing and measurement, the earliest time point we obtained was 35 s. We found that the docking became saturated to 40% even at 35 s, the earliest time point that we obtained. Thus, the low limit of the docking rate ($k_{\text{dock, T-S}}$) between **T** and **S** was $1.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (Supplementary data). This is close to the diffusion-limit rate constant (k_{limit}) of $3.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ for vesicles (Supplementary data). The average collision number ($k_{\text{limit}}/k_{\text{dock, T-S}}$) before docking is only 30. This collision number is much smaller than 10^4 – 10^5 collisions of **T-V** for docking, a number previously reported (Cypionka *et al*, 2009; Smith and Weisshaar, 2011) and also confirmed in this work (see below). Overall, we found that Syt1 enhances the rate of vesicle docking by $\sim 10^3$ times when compared with that of SNARE-only vesicles.

The t-SNARE/PIP₂ ratio determines Syt1 binding to either PIP₂ or t-SNARE/PIP₂ complex

How does PIP₂ cooperate with t-SNARE to promote Syt1-mediated docking? PIP₂ itself is known to interact with Syt1 in the absence of Ca²⁺ (Bai *et al*, 2004a,b). Indeed, PIP₂ alone, in the absence of t-SNARE in **T**, was able to induce a similar (or higher) level of vesicle docking in the case of the presence of both t-SNARE and PIP₂ (Figure 2L). Why is it then those t-vesicles containing PIP₂ together with syntaxin or t-SNARE with the SNAP-25 D51K/E52K mutant cannot mediate docking? One scenario might be that syntaxin and t-SNARE interferes with the direct PIP₂-Syt1 interaction. It has been shown that PIP₂ is localized with syntaxin at the site where exocytosis occurs in PC12 cells (Aoyagi *et al*, 2005). The basic juxtamembrane region of syntaxin could interact with acidic PIP₂ electrostatically, which might induce the clustering of PIP₂ in the vicinity of syntaxin (Wagner and Tamm, 2001; Kweon *et al*, 2002; Vicogne *et al*, 2006; James *et al*, 2008). Recently, it has been shown that the electrostatic interactions between PIP₂ and syntaxin are responsible for the syntaxin clustering (van den Bogaart *et al*, 2011a). This putative PIP₂ syntaxin clustering may lessen the direct PIP₂ Syt1 interaction.

To test this possibility, we determined the threshold t-SNARE/PIP₂ ratio in which PIP₂ can interact directly with Syt1. We prepared t-vesicles, containing different t-SNARE/PIP₂ ratios (Figure 2M), by keeping 1% PIP₂ but varying the amount of t-SNARE (Supplementary Figure S6), constructed with SNAP-25 D51K/E52K that does not bind Syt1 but is expected to have the ability to sequester PIP₂. When the t-SNARE concentration is high (above 1:24 ratio of t-SNARE/PIP₂) the subpopulation of docked vesicles was close to the background level (Figure 2M), which implies that the direct interaction between PIP₂ and Syt1 was blocked. However, when the t-SNARE concentration is sufficiently low (below t-SNARE/PIP₂ ratio of 1:32) the subpopulation of docked vesicles appeared and at ratio of 1:64 the subpopulation was close to that of PIP₂-only vesicle.

These results suggest there exist two binding modes of Syt1 without Ca²⁺. In the absence or low copy number of t-SNARE, Syt1 may interact with PIP₂ directly (Bai *et al*, 2004a; van den Bogaart *et al*, 2011b). In the presence of sufficiently large number of t-SNARE, however, the direct

interaction between Syt1 and PIP₂ is suppressed, and Syt1 interacts with t-SNARE/PIP₂ complex, instead. Thus, the results suggest that the direct interaction between Syt1 and PIP₂ is only possible when the t-SNARE/PIP₂ ratio is non-physiologically low, consistent with the EM study that showed tethering by Syt1 required t-SNARE on the plasma membrane (de Wit *et al*, 2009).

Tethering by Syt1 increases vesicle fusion: fusion is the rate-limiting step

In the previous *in-vitro* studies, it has been reported that incorporation of membrane-anchored Syt1 into v-vesicle stimulated fusion without Ca²⁺ (Mahal *et al*, 2002; Stein *et al*, 2007). Although the detailed mechanism of this enhancement was unclear, a recent single-vesicle assay using surface immobilization showed that Syt1 increased both docking and fusion (Lee *et al*, 2010). We tested whether in our solution system the enhancement of vesicle docking by Syt1 also increases the rate of fusion, and further investigated which step is rate limiting between docking and fusion. For

this, we prepared two types of v-vesicles; one containing VAMP-2 (L/P = 500; **V**) and the other containing both VAMP-2 and Syt1 (L/P = 500 and 900, respectively, **SV**), and then incubated the **T-V** or the **T-SV** mixture (10 μ M) at room temperature for 5 min. Here, we took the advantage of ALEX that observes not only docked vesicles but also fused vesicles in the same solution (5 min measurement). The 2D **E-S** graph of **T-V** shows negligible amounts of the docked and fused subpopulations (Figure 3A), which implies that the docking efficiency by VAMP-2 was very low under these conditions. However, when Syt1 was incorporated into v-vesicle (**T-SV**), the subpopulations of both docked and fused vesicles were dramatically increased (Figure 3B); fusion was enhanced as much as by factor of 27 (Supplementary Figure S7). When we used SNAP-25 D51K/E52K mutant, which impairs Syt1-induced tethering to t-SNARE (Figure 2G) but still had fusion activity (43% of the fusion activity of WT SNAP-25; Supplementary Figure S3), both docking and fusion populations were considerably reduced (Figure 3C). These results strongly support that t-SNARE·Syt1 interaction is more

Figure 3 Tethering by Syt1 enhances vesicle fusion. (A) 2D **E-S** graph of the mixture of **T** and **V**, (B) **T** and **SV**, (C) **T_{D51K/E52K}-SV**. **T**, t-vesicle reconstituted with t-SNARE and 1% PIP₂. **T_{D51K/E52K}**, the same as **T** except for using SNAP-25 D51K/E52K mutant. **V**, v-vesicle reconstituted with VAMP-2. **SV**, v-vesicle reconstituted with both VAMP-2 and Syt1. The data were obtained by incubating the mixture for 5 min at room temperature and measured for 5 min by ALEX. Purple and orange boxes denote docked and fused vesicles, respectively. (D) Bar graph for the fractions of the docked and fused subpopulations, analysed from . Error bars were obtained from at least three independent measurements (* $P < 0.005$). (E) Time-dependent measurement of docked and fused vesicles of **T-SV**. We used the same method for Figure 2J but using **T-SV**. The fractions of docked and fused vesicles for each time bin were obtained from 10 measurements. Purple, red, and black squares represent the fractions of docked, fused, and the sum of docked and fused vesicles, respectively. From double-exponential fitting, the average fusion rate was obtained, $k_{T-SV} = 3.7 \pm 0.9 \times 10^{-3} \text{ s}^{-1}$. This value is similar to that of bulk FRET from (G); $k_{T-SV, \text{bulk}} = 2.9 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$. Expanded view is present in inset. (F) Time-dependent measurement of docked and fused vesicles of **T-V**. The same method for **T-SV** was used. Expanded view is present in inset. The dotted line indicates average fraction of random coincidence. (G and H) Bulk FRET measurements for **T-SV** and **T-V**, depending on vesicle concentration, respectively, at room temperature.

effective than t-SNARE-VAMP-2 interaction for vesicle tethering and this efficient tethering increases the rate of membrane fusion (Figure 3D).

We then measured the docking and fusion kinetics of **T-SV**. Figure 3E presents time-dependent subpopulations of docked and fused vesicles. It clearly shows that the docking between **T-SV** occurred within 35 s, similarly to the case with **T-S**, which was followed by a slow fusion with the rate constant $k_{\text{fusion}} = 3.7 \pm 0.9 \times 10^{-3} \text{ s}^{-1}$. This fusion rate is comparable to that of bulk FRET ($k_{\text{fusion}} = 2.9 \pm 0.1 \times 10^{-3} \text{ s}^{-1}$; Figure 3G). Thus, fusion is the rate-limiting step for **T-SV** in this work. At 35 s, the fraction of complex, that is, the sum of docked and fused vesicles, was $\sim 50\%$ and then saturated at 65%, which was 25% more than the saturated fraction for **T-S** (Figure 1J). The rate of the complex build-up after 35 s was $2.1 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$, which is comparable to the fusion rate. This is reasonable considering that fusion decreases the concentration of docked vesicle and the docking increases to maintain the steady state. We note that 35% of **SV** never form docked vesicles, but all the docked vesicles fused in our solution measurement.

However, without Syt1 in v-vesicles (**V**), both docking and fusion were nearly undetectable until 2500 s under the same experimental conditions as those of **T-SV** (room temperature, 3 μM lipid concentration) (Figure 3F). The population of docked vesicles was not distinguishable from the background level of 5% (random coincidence), while fused population was above the background level but it was very slowly accumulated ($k_{\text{fusion}} = 1.16 \pm 0.25 \times 10^{-4} \text{ s}^{-1}$) (Supplementary Figure S8). Because docked vesicles were not accumulated, docking should be here the rate-limiting step for **T-V**. Because the docking population was not observed, the fusion rate of **T-V** could not be determined.

To confirm that fusion and docking are the rate-limiting steps for **T-SV** and **T-V**, respectively, we performed bulk FRET measurement while varying the vesicle concentration (Figures 3G and H). If fusion is the rate-limiting step, then fusion rate would be independent of vesicle concentration. On the other hand, if docking rate is the rate-limiting step, then fusion rate will be increased as vesicle concentration is increased. Indeed, **T-SV** presented no change in fusion rate (Figure 3G), while in the case of **T-V** more efficient fusion was

observed as vesicle concentration was increased (Figure 3H). This supports that fusion is the rate-limiting step for **T-SV**, while docking is the rate-limiting step for **T-V**. By assuming that the observed fusion rate of Figure 3F (and Supplementary Figure S8) corresponds to the docking rate, we determined the docking rate of **T-V**, $k_{\text{dock}, \text{T-V}} = 8.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (Supplementary data), which matches well with the previous measurements (Cypionka *et al*, 2009; Smith and Weisshaar, 2011) and it is 10^3 times slower than that of **T-S** (or **T-SV**).

Previous bulk fusion studies have shown that Syt1 increases fusion (Mahal *et al*, 2002; Stein *et al*, 2007). Our results clearly demonstrated that such increment was originated from the enhanced docking by Syt1. It is to be noted that the docking and fusion rate of **T-SV** *in vitro* is still considerably slower compared with the fusion *in vivo*, occurring within 1 ms (Borst and Sakmann, 1996; Sabatini and Regehr, 1996; Schneggenburger and Neher, 2000). Although effective in resolving docking from fusion in the present experiments, one weakness of ALEX is a slow time resolution (30 s) at this moment, which limits its application to the faster fusion kinetics.

VAMP-2 displaces Syt1 from t-SNARE upon SNARE complex formation

It is likely that Syt1 binding to t-SNARE precedes SNARE complex formation (Figure 3B). An intriguing question is then: does Syt1 stay bound to or is it detached from the SNARE complex upon SNARE assembly? To answer this question, we applied soluble VAMP-2 (aa 1–94, sVAMP-2) to the **T-S** mixture in which the docked vesicle pairs are accumulated. sVAMP-2 forms ternary complex with t-SNARE, but it is unable to induce fusion due to its lack of transmembrane domain. We reasoned that if Syt1 remains to be bound to the complex, sVAMP-2 would not change the docked subpopulation, while if VAMP-2 competes with Syt1 for t-SNARE binding, it would reduce the docked subpopulation (Figure 4A, upper panel). The results show that the number of docked vesicles is significantly dropped to 32% of that of **T-S** (Figure 4A, lower panel). When **T** was incubated with sVAMP-2 prior to mixing with **S** (Figure 4B), the subpopulation of the docked vesicles was reduced to 19% of that of **T-S**

Figure 4 Soluble VAMP-2 reduces the docked subpopulation by Syt1. (A) Top, schematic description of soluble VAMP-2 (sVAMP-2) test. **T** and **S** were incubated together for 5 min at room temperature, and then 1 μM of sVAMP-2 was added to the mixture, followed by 15 min incubation. Bottom, 2D **E-S** graph of the mixture. (B) Top, **T** was pre-incubated with sVAMP-2 for 15 min, and then mixed with **S** for 5 min. Bottom, 2D **E-S** graph of the mixture. (C) Bar graph for the fraction of docked vesicles. **T-S** + sVAMP-2 and (**T** + sVAMP-2)-**S** from the results of Figure 4A and B; respectively. **T-S** + sVAMP-2_{1–70} and **T-S** + sVAMP-2_{1–59} denote the docking fraction after adding sVAMP-2_{1–70} and sVAMP-2_{1–59}. As a control, we added 3 μM BSA instead of sVAMP-2 (**T-S** + BSA). Error bars were obtained from four independent measurements (* $P < 0.005$).

(Figure 4B). This result shows that SNARE complex formation weakens the Syt1 interaction with SNAREs significantly.

There is strong evidence that SNAREs are partially zippered in the priming state, which is followed by the full zippering upon fusion step (Pobbat *et al*, 2006; Sorensen *et al*, 2006; Walter *et al*, 2010). Thus, we tested whether the displacement of Syt1 by VAMP-2 may occur at the partial zippering state, by using C-terminal truncations of sVAMP-2 (aa 1–70 and aa 1–59) (Sorensen *et al*, 2006). We found that both truncations of sVAMP-2 also reduced the population of docked vesicles (Figure 4C), which indicates that the displacement of Syt1 by VAMP-2 may occur before fusion.

Ca^{2+} induces rebinding of Syt1 to ternary SNARE complex

Although it has been known that ternary SNARE-Syt1 interaction becomes stronger by Ca^{2+} and this interaction is believed to play an important role in Ca^{2+} -triggered vesicle fusion (Zhang *et al*, 2002; Bai *et al*, 2004b; Chapman, 2008; Choi *et al*, 2010; Lee *et al*, 2010), the detailed mechanism of ternary SNARE-Syt1 interaction has been poorly understood (Chapman, 2008; Rizo and Rosenmund, 2008). Our result in Figure 4 showed that ternary SNARE-Syt1 interaction without Ca^{2+} is too weak to maintain vesicle docking. However, when we added $10\ \mu\text{M}$ Ca^{2+} in this mixture (Figure 5A), we observed a significant level of vesicle docking similar to the level of T-S (Figure 5B). This result suggests that Ca^{2+} strengthens the ternary SNARE-Syt1 interaction and the enhanced interaction promotes vesicle docking, consistent with the previous results that the interaction between C2AB and SNARE complex is enhanced by Ca^{2+} (Zhang *et al*, 2002; Bai *et al*, 2004b).

However, when PIP_2 is absent from T the subpopulation of docked vesicles was considerably reduced, again indicating that PIP_2 plays an important role in Ca^{2+} -induced Syt1 binding to the ternary SNARE complex (Figure 5C). Also, when SNAP-25 D51K/E52K was used, Ca^{2+} -induced redocking of vesicles did not happen (Figure 5D). Thus, the results show that Ca^{2+} -induced vesicle redocking occurs through Syt1's interaction with the ternary SNARE complex and PIP_2 . It appears that the central region of the SNARE complex is responsible for the interaction with Syt1, consistent with recent single-molecule study (Choi *et al*, 2010). We also tested whether Ca^{2+} -induced redocking can occur at the partial zippering state, using C-terminal truncations of sVAMP-2, and observed redocking (Figure 5E). This indicates that Ca^{2+} -induced redocking occurs before fusion.

Discussion

A number of *in-vitro* studies have shown that Syt1 has the ability to interact with t-SNARE, ternary SNARE complex, and PIP_2 , but the function of each interaction in exocytosis has been poorly understood (Chapman, 2008). In this work, we studied how membrane-anchored Syt1 uses its Ca^{2+} -independent and -dependent interactions with SNAREs and with membrane lipids for vesicle docking and fusion. In order to investigate the functions of Syt1, we used a single-molecule technique, ALEX, which resolves subpopulations of individual components and measures their kinetics in the fusion reaction mixture without surface immobilization (Figures 1F and 3E). We found that Syt1 used two types of binding modes with SNAREs, which was mainly controlled

Figure 5 Ca^{2+} induces rebinding of Syt1 to ternary SNARE complex. (A) Schematic description of Syt1 rebinding experiments. To the mixture of Figure 4B, $10\ \mu\text{M}$ Ca^{2+} was added at room temperature. (B) 2D E-S graph obtained from the mixture of Figure 5A. (C) 2D E-S graph obtained from the mixture of Figure 5A, but without PIP_2 on T. (D) 2D E-S graph obtained from the mixture of Figure 5A, but T was constructed with SNAP-25 D51K/E52K mutant. (E) Bar graph for the fraction of docked vesicles with and without Ca^{2+} . We tested wild type and three mutants of SNAP-25, the case of no PIP_2 on T, and C-terminal truncations of sVAMP-2 (1–70 and 1–59). Error bars were obtained from four independent measurements (* $P < 0.005$).

by Ca²⁺. In the absence of Ca²⁺, Syt1 interacted with t-SNARE, which is strong enough to induce vesicle tethering (Figure 2B) and increases the docking rate by >10³ times than that of SNARE-only vesicles. This tethering mode helps ternary SNARE complex formation, which resulted in significant enhancement in fusion rate (Figure 3B). When sVAMP-2 was added to such docked vesicles, Syt1 lost its binding to t-SNARE, which resulted in reduced subpopulation of docked vesicles (Figure 4A). However, when 10 μM Ca²⁺ was added, Syt1 rebound to the ternary SNARE complex (Figure 5B). For binding to both t-SNARE (Ca²⁺ independent) and ternary SNARE complex (Ca²⁺ dependent), PIP₂ was required on t-vesicles. With Syt1, the fusion step was the rate limiting. However, without Syt1, docking became the rate-limiting step in solution measurement.

These findings lead us to propose a sequential mechanism by which Syt1 interacts with SNARE complexes depending on the level of Ca²⁺ (Figure 6). First, in the absence of Ca²⁺, Syt1 brings vesicles to the plasma membrane in proximity by directly interacting with the plasma membrane components t-SNARE-PIP₂ (Rickman *et al*, 2006; de Wit *et al*, 2009). Then, because two membranes are geometrically close, VAMP-2 can efficiently search and bind t-SNARE to form the ternary SNARE complex. As the ternary complex is formed, Syt1 loses its contact with t-SNARE. Then, upon the Ca²⁺ arrival, Syt1 rebinds to the SNARE complex together with PIP₂. It appears that Syt1 binds to the central region of ternary SNARE complex (Choi *et al*, 2010) and these processes occur before full zippering of the SNARE complex.

Although the function of Syt1 in the absence of Ca²⁺ is controversial, evidence from *in-vivo* studies supports its tethering role (Reist *et al*, 1998; Loewen *et al*, 2006; Rickman *et al*, 2006; Liu *et al*, 2009; de Wit *et al*, 2009). For example, the EM study of an Syt1 mutant AD3 in *Drosophila*, corresponding to Syt1 Y311N mutant, showed the reduced number of docked vesicles (Reist *et al*, 1998). SNAP-25 D51K/E52K/E55K mutant, which disrupted t-SNARE-Syt1 interaction (Figure 2D), also reduced exocytosis significantly in neuroendocrine PC12 cells (Rickman *et al*, 2006). Indeed, recent EM study of adrenal chromaffin cells

provided direct evidence that this SNAP-25 mutant reduced the population of docked vesicles (de Wit *et al*, 2009). Although mouse Syt1 KO neurons have yielded inconsistent results (Geppert *et al*, 1994; Nishiki and Augustine, 2004a, b; Maximov and Sudhof, 2005; Burgalossi *et al*, 2010), recent study has shown that the disparity mainly came from the preparation of hippocampal neurons: Syt1 KO in autaptic cultures, that is, a single cell innervates itself, resulted in negligible change in the size of readily releasable pool (RRP) of synaptic vesicles, but in dissociated mass cultures, connected with multiple cells, resulted in reduction in the size of RRP and the population of docked vesicles (Liu *et al*, 2009). Although it is still not clear why two cultures gave different results, at least the results of dissociated mass cultures, morphologically closer to *in-vivo* neuron than autapses, support the function of Syt1 as a tethering or docking factor.

In contrast to the requirement of both t-SNARE and PIP₂ for Syt1-mediated vesicle tethering, a recent *in-vitro* study showed that Syt1 tethers proteoliposomes without its specific interaction with t-SNARE (Bai *et al*, 2004a; van den Bogaart *et al*, 2011b). This seemingly contradictory result can be explained by the two binding modes for Syt1 in the absence of Ca²⁺ (Figure 2M). Syt1 interacts with PIP₂ with a high affinity (Figure 2J), strong enough to induce vesicle tethering, while its interaction with t-SNARE is not sufficiently strong to sustain such vesicle tethering in the absence of PIP₂ (Figure 2D). However, when syntaxin-1A coexists with PIP₂, its basic juxta-membrane region could attract PIP₂ electrostatically and promotes the clustering of PIP₂ near syntaxin-1A (Kweon *et al*, 2002; Aoyagi *et al*, 2005; James *et al*, 2008; van den Bogaart *et al*, 2011a). Thus, when there is enough copy number of t-SNARE, this PIP₂ clustering near syntaxin-1A may dampen the direct PIP₂ Syt1 interaction while promoting the interaction between t-SNARE-PIP₂ cluster and Syt1 (Figure 2M). In the previous work (van den Bogaart *et al*, 2011b), Jahn and co-workers used a low salt concentration and a low L/P ratio (2000:1). Under such limiting conditions, it is likely that Syt1 interacts with PIP₂ directly because the t-SNARE/PIP₂ is too low. Thus, our results suggest that Syt1 use its interaction with t-SNARE-PIP₂ to induce vesicle tethering.

This study shows that PIP₂ is consistently involved in the functions of Syt1, for both Ca²⁺-independent t-SNARE interaction and Ca²⁺-dependent ternary SNARE interaction. PIP₂ is a key anionic phospholipid on the cytoplasmic leaflet of the plasma membrane and has been known to be required for Ca²⁺-triggered vesicle fusion (Hay *et al*, 1995; Holz *et al*, 2000). Although the detailed role of PIP₂ in the exocytosis is still unclear, electrophysiological studies showed that PIP₂ regulates the size of the releasable vesicle pool of chromaffin cells (Gong *et al*, 2005; Milosevic *et al*, 2005). In addition, as discussed above, PIP₂ is localized with syntaxin and LDCVs are observed to be docked where PIP₂ is colocalized with syntaxin (Aoyagi *et al*, 2005; James *et al*, 2008; Lam *et al*, 2008). We note that our results are in line with and further support these observations, showing that the tethering by Syt1 required both PIP₂ and t-SNARE (Figure 2K), which may corresponds to the prefusion step of vesicle priming.

We showed that the addition of sVAMP-2 reduced the number of docked vesicles significantly, compared with that of T-S (Figure 4C), which implies that SNARE complex formation weakens the Syt1 interaction with SNAREs. In the previous work, the assay using glutathione S-transferase

Figure 6 Model for sequential actions of synaptotagmin-1 on SNAREs. In the absence of Ca²⁺, Syt1 interacts with t-SNARE-PIP₂, which brings vesicle to the plasma membrane in proximity. Because of this close geometry of two membranes, VAMP-2 can efficiently search and bind t-SNARE to form the SNARE complex. During this step, Syt1 loses its contact with SNAREs. When Ca²⁺ arrives, Syt1 rebinds to the SNARE complex together with PIP₂.

(GST)-tagged proteins showed that t-SNARE-VAMP-2 interaction is stronger than that of t-SNARE-Syt1 (Rickman *et al*, 2006). However, in the same work, sVAMP-2 did not disrupt the t-SNARE-Syt1 complex. This discrepancy with our work may come from that, in the assay using GST tagged proteins, t-SNARE and Syt1 were not incorporated into liposomes but simply mixed in solution, so that the effects of phospholipids, such as PIP₂, which play an important role in Syt1-SNARE interactions, were not taken into account. We note that in recent single-vesicle assay the pre-incubation of sVAMP-2 with t-SNARE reduced vesicle docking for v-vesicle containing both Syt1 and VAMP-2 (Kyoung *et al*, 2011), which is in line with our result of Figure 4B and supports our proposition that sVAMP-2 disrupts the t-SNARE-Syt1 complex.

In summary, our work shows the direct evidence that Syt1 uses its diverse interactions with SNAREs and phospholipids, particularly PIP₂, to enhance vesicle fusion, which results in Syt1's sequential actions on SNAREs. We resolved full subpopulations of fusion reaction mixture and their kinetics using a single-molecule technique, ALEX, which should be very useful for future study on the functions of SNAREs, regulatory proteins, and phospholipids, at each step of vesicle docking and fusion.

Materials and methods

Protein and lipid preparation, proteoliposome reconstitution, Dynamic light scattering and co-floatation assay, bulk FRET assays, ALEX set-up, and simple quantitative analysis are described in Supplementary data.

Definition and calculation of *E* and *S* of a single vesicle

From ALEX measurement, the fluorescence time traces of vesicles were obtained as raw data (Figures 1B–E). Because the lasers are focused tightly in femto-liter volume (Figure 1A), only one vesicle passes through the excitation volume randomly in sufficiently diluted solution. This diffusing-in-and-out event of a vesicle appears as a fluorescent burst in the time trace (Figures 1B–E). The key of this technique is to alternate two lasers (400 μs) sufficiently faster than the transit time (5 ms) of a vesicle through the excitation volume (Kapanidis *et al*, 2004). By alternating two lasers, we can obtain three different types of photons for each burst (single vesicle): I_D^D is a fluorescent emission of donor dyes excited by donor-excitation laser (532 nm); I_D^A is a fluorescent emission of acceptor dyes excited by donor-excitation laser (532 nm), which is a FRET signal; and I_A^A is a fluorescent emission of acceptor dyes excited by acceptor-excitation laser (633 nm). These three intensities can be used to distinguish among vesicles, such as unreacted, docked-but-unfused, and fused vesicles. For example, t-vesicle doped with donor dyes emits only I_D^D (Figure 1B, green line), because this vesicle does not contain acceptor dyes, while v-vesicle doped with acceptor dyes emits only I_A^A (Figure 1C, red line). In the case of docked vesicle, because it contains both donor and acceptor dyes, it emits both I_D^D and I_A^A (Figure 1D). In the docked state, the lipids of two vesicles are not mixed and thus the FRET signal is negligible, which results in low intensity of I_D^D (orange line in Figure 1D). In the case of fused vesicle, because it contains both donor and acceptor dyes but the FRET signal is strong, it emits low I_D^D (due to energy transfer to acceptor dyes) but strong I_D^A and I_A^A (Figure 1E). As a result, the pattern of three fluorescent intensities of a

burst can be used to identify the status of a vesicle. To present these subpopulations in a graph, two parameters of *S* (Stoichiometry ratio or Sorting number in this work) and *E* (FRET efficiency) were used (Kapanidis *et al*, 2004). *S* and *E* of each burst are calculated by:

$$S = \frac{I_D^D + I_D^A}{I_D^D + I_D^A + I_A^A},$$

$$E = \frac{I_D^A}{I_D^D + I_D^A}.$$

Here, *S* is used as a sorting number. Theoretically, *S* of t-vesicle is 1, because $I_A^A \approx 0$ (no acceptor dyes), while *S* of v-vesicle is 0, because $I_D^D + I_D^A \approx 0$ (no donor dyes). In the cases of docked and fused vesicles, because they have both donor and acceptor dyes ($I_D^D + I_D^A \approx I_A^A$), *S* becomes 0.5. Then, docked and fused vesicles are discriminated by *E*: docked vesicle has low *E*, but fused one has high *E* by lipid mixing. Therefore, the bursts of unreacted, docked, and fused vesicles occupy different areas in *E*-*S* graph (Figure 1F).

ALEX measurements for the subpopulations of vesicle docking and fusion

For vesicle docking and fusion measurement using ALEX technique (Figures 2 and 3), t- and v-vesicles, incorporated with desired proteins, were mixed and incubated for 5 min at room temperature in vesicle buffer (25 mM HEPES (pH 7.4), 100 mM KCl, and 100 μM EGTA). We used 10 μM final lipid concentration for incubation to minimize possible vesicle aggregation or multiply docked vesicles. For ALEX, we further diluted the mixture by three times to achieve single-vesicle detection. The diluted mixture was put into measurement chamber, which was made by sandwiching two cover glasses together with silicon gasket (S4810, Sigma). All data were analysed by home-built software. For sVAMP-2 displacement, *T* and *S* were incubated for 5 min at room temperature, and then 1 μM sVAMP-2 was added to the mixture, followed by 15 min incubation (Figure 4A). We also tested the effect of sVAMP-2 by pre-incubating *T* with sVAMP-2 for 15 min before adding *S* (Figure 4B). As a control, we did the same experiment using 3 μM BSA. For Ca²⁺-induced rebinding of Syt1 (Figure 5), we added CaCl₂ (10 μM final concentration) to the final mixture of Figure 4B, and after additional 5 min incubation the mixture was analysed by ALEX. For this experiment only, we did not add EGTA in the buffer. For the docking and fusion kinetics measurement by ALEX, see Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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