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One SNARE complex is sufficient for membrane fusion

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In eukaryotes, most intracellular membrane fusion reactions are mediated by the interaction of SNARE proteins that are present in both fusing membranes. However, the minimal number of SNARE complexes needed for membrane fusion is not known. Here we show unambiguously that one SNARE complex is sufficient for membrane fusion. We performed controlled *in vitro* Förster resonance energy transfer (FRET) experiments and found that liposomes bearing only a single SNARE molecule are still capable of fusion with other liposomes or with purified synaptic vesicles. Furthermore, we demonstrated that multiple SNARE complexes do not act cooperatively, showing that synergy between several SNARE complexes is not needed for membrane fusion. Our findings shed new light on the mechanism of SNARE-mediated membrane fusion and call for a revision of current views of fusion events such as the fast release of neurotransmitters.

SNAREs are an evolutionarily conserved superfamily of small transmembrane or membrane-anchored proteins that possess a conserved domain of about 60–70 amino acid residues, termed the SNARE motif. SNARE proteins have a vital role in eukaryotic life, as they mediate all intracellular fusion reactions except mitochondrial fusion. Thus, they are essential for a wide range of cellular processes, including cell growth, cytokinesis and synaptic transmission[1,2]. Isolated SNARE motifs are unfolded and have no secondary structure. When membranes with complementary sets of SNARE proteins are mixed, the SNAREs assemble *in trans*. Assembly proceeds from the N-terminal ends to the C-terminal membrane anchors and results in a tight coiled-coil complex (core complex). This process pulls the membranes together and initializes fusion, resulting in the SNARE proteins being present in a single membrane (*cis* complex)[1,2].

Membrane fusion can be reproduced *in vitro* using purified SNARE proteins reconstituted in liposomes. For instance, liposomes that contain the neuronal SNARE proteins syntaxin-1 and SNAP-25 from the plasma membrane fuse with liposomes containing synaptobrevin-2 from synaptic vesicles[3]. Although this indicates that SNARE proteins are sufficient to mediate membrane fusion, mechanistic details of the fusion reaction are lacking[1–3]. Most importantly, it is still unknown how many SNARE proteins are needed for membrane fusion. Although single-molecule studies have shown that a single SNARE complex is sufficient for membrane docking[4,5], the only estimates available for membrane fusion range from 3 to 15 SNARE complexes[6–9]. These numbers are largely based on the *in vivo* titration of inhibitors while fusion kinetics are being measured. An estimate of at least three SNARE complexes was obtained when researchers inhibited exocytosis in PC12 cells with a soluble fragment of synaptobrevin[6]. In contrast, a much higher number (10–15 SNARE complexes) was inferred when a group inhibited neuronal exocytosis by titrating botulinum neurotoxins, which inactivate SNAREs through specific proteolysis[7]. Lastly, an electrophysiology study in PC12 cells involving syntaxin mutants indicated that between five and eight SNARE complexes are needed for membrane fusion, on the basis of a model of steric hindrance[8]. The wide range of estimates is explained by the fact that all determinations have so far been based on indirect approaches. No direct measurements of the minimal number of SNARE complexes needed for membrane fusion have been carried out.

Here we measure SNARE-mediated fusion directly, using liposomes in which the number of SNAREs is progressively reduced to an average of less than one molecule per liposome. In these experiments, we have taken advantage of the observation that fusion is greatly accelerated when a 1:1 acceptor complex of syntaxin-1 (residues 183–288) and SNAP-25 is stabilized with a synaptobrevin-2 fragment (residues 49–96)[10]. Using fluorescence correlation spectroscopy in combination with FRET measurements, we have recently demonstrated that with the stabilized acceptor complex, docking of the liposomes proceeds even faster (by more than ten-fold) than membrane fusion[11]. The stabilization of the complex prevents the formation of a so-called 2:1 complex, in which the binding site of synaptobrevin is erroneously occupied by a second syntaxin, resulting in a kinetically trapped dead end[12,13]. In addition, the truncated version of syntaxin lacks the N-terminal Habc domain that downregulates syntaxin's ability to enter SNARE complexes[14]. Thus, the stabilized acceptor complex ensures that all SNAP-25 and syntaxin molecules can participate in core-complex formation, allowing us to measure SNARE-mediated fusion at very low protein-to-lipid (p/l) ratios and hence to determine the minimum number of SNAREs needed for fusion.

RESULTS

Characterization of the proteoliposomes

To prepare liposomes with, on average, less than one SNARE molecule per liposome, we purified variants of synaptobrevin-2 and the

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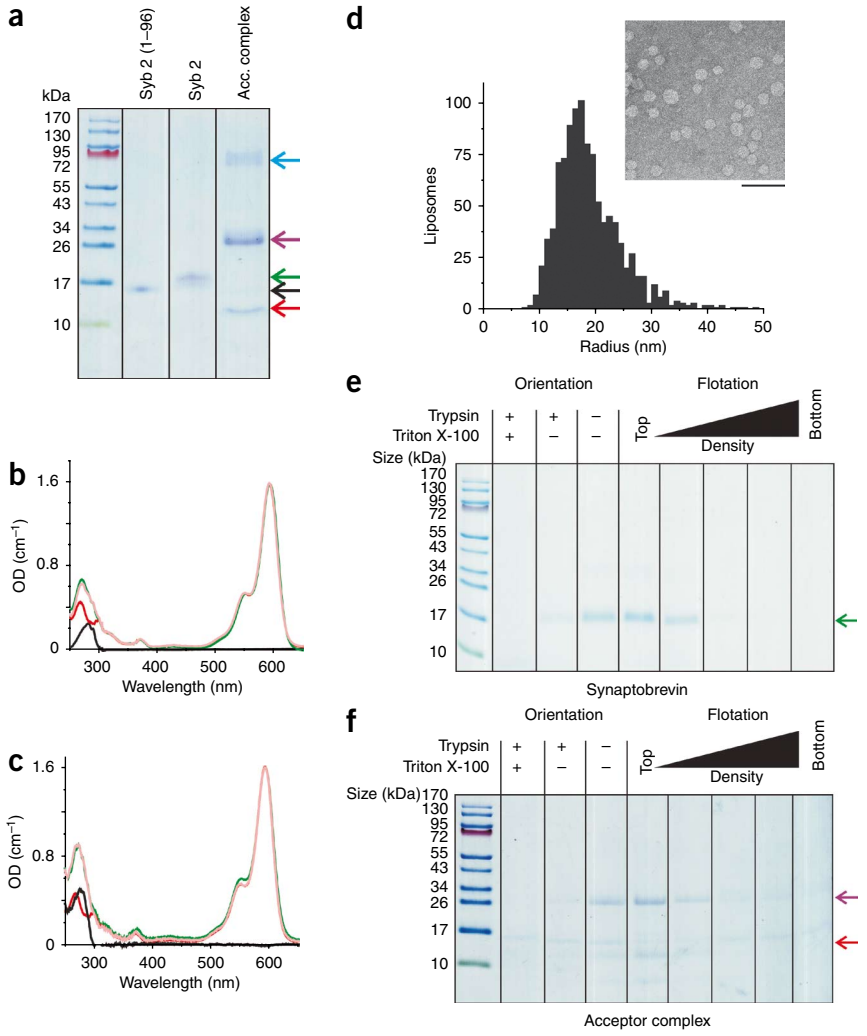

Figure 1 Characterization of the SNARE-containing liposomes. (**a**) Synaptobrevin-$2_{1–96}$ (black arrow), synaptobrevin-2 (green arrow) and the synaptobrevin-$2_{49–96}$ stabilized acceptor complex (blue arrow) consisting of SNAP-25 (purple) and syntaxin-1A (residues 183–288, 289Cys; red) were free of contaminants when analyzed by SDS-PAGE (Coomassie blue staining). Syb, synaptobrevin. (**b**,**c**) The absorption spectra of 18 μM synaptobrevin (green curve in **b**) and the acceptor complex (green curve in **c**), both labeled with Texas red. The absorption of 18 μM unlabeled protein (black) and Texas red (red) and the sum of both (pink) are also shown. The overlap between the pink and the green curves indicates stoichiometric labeling. OD, optical density. (**d**) Negative-staining electron microscopy (inset; scale bar, 100 nm) showed that the proteins were reconstituted in monodisperse liposomes with an average radius of 17.9 ± 5.8 nm (s.d.). (**e**,**f**) All proteins were fully incorporated in the membrane, as verified by density-gradient flotation on a 30–80% gradient of Nycodenz (Nycomed). We used partial proteolysis to determine the orientations of the proteins in the liposomes. In the absence of detergent, only correctly oriented SNAREs (those with their cytoplasmic domains outside the liposome) are cleaved by trypsin, whereas all proteins are proteolyzed in the presence of Triton X-100. About 50% of the synaptobrevin (**e**) and 80% of the acceptor complex (**f**) were correctly oriented in the liposomes. Electron microscopy, flotation gradients and trypsin digestion have been described[37].

stabilized acceptor complex (rat sequence) containing single cysteines and labeled them with Texas red. For our experiments, a high labeling efficiency was essential to rule out the possibility that we might underestimate the number of SNAREs owing to unlabeled protein. Indeed, for Texas red, the labeling was stoichiometric, with efficiencies of 90–110% as assessed by UV-visible spectroscopy (**Fig. 1a–c**). Also important was that the proteins were reconstituted in a monodisperse liposome population; we confirmed this by electron microscopy, finding that the average radius of the liposomes was 17.9 ± 5.8 nm (s.d.; **Fig. 1d**). Density-gradient flotation experiments indicated that all proteins were fully incorporated in the membrane. Furthermore, partial proteolysis experiments showed that 50–80% of the molecules were correctly oriented—that is, with their cytoplasmic domains on the outside (**Fig. 1e**,**f**). We have shown in the past that all correctly oriented synaptobrevin in the proteoliposomes is capable of complex formation[15]. We subsequently determined the distribution of fluorescently labeled SNARE proteins over the liposomes.

We measured the number of labeled SNARE proteins in individual liposomes using sequential photobleaching. This single-molecule technique has been employed to determine the number of fluorescently labeled proteins encapsulated in lipid vesicles[16] and allows up to approximately eight fluorophores[17] to be resolved sequentially through discrete photobleaching steps. For the sequential photobleaching, we first embedded the liposomes containing the Texas red–labeled proteins in an agarose matrix and recorded a time series of two-photon excitation microscopy images (**Fig. 2a**). Control experiments with unlabeled protein confirmed that the fluorescence signal was specific for the Texas red label. Furthermore, experiments with the fluid-phase marker calcein encapsulated in the lumen of the liposomes showed that the liposomes remained intact during the embedding procedure. Subsequently, we selected for the liposomes by employing an offset above the level of the background signal, as described[18] (**Fig. 2b**,**c**). We then determined the number of fluorescently labeled proteins for each liposome by counting the discrete photobleaching steps in the time trace (**Fig. 2d**).

Notably, the distribution of the SNARE proteins was well-described by a Poisson function, suggesting that no protein homo-oligomerization occurred before reconstitution. For a molar p/l ratio of 1:4,000, we measured an average of 1.4 synaptobrevin and 1.2 syntaxin molecules per liposome (**Fig. 2e**,**f**), which is within the range calculated for 100% protein incorporation when assuming an average surface area per lipid headgroup of 0.7 nm^2 (2.8 copies per liposome)[19]. In addition, the number of proteins scaled to the p/l ratio, and an average of 0.4 synaptobrevin and 0.35 syntaxin molecules were present per liposome when the liposomes were prepared at a p/l ratio of 1:16,000, again demonstrating that the SNAREs were completely, yet randomly, distributed over the liposomes. At this ratio, only a small fraction (~5%) of the liposomes contained more than one SNARE, and only a proportion of those SNARES were correctly oriented (**Fig. 1e**,**f**). Hence, the number of SNAREs that could participate in fusion was effectively reduced to a single SNARE per liposome. We used these liposomes to determine the effect of the SNARE density on the fusion kinetics.

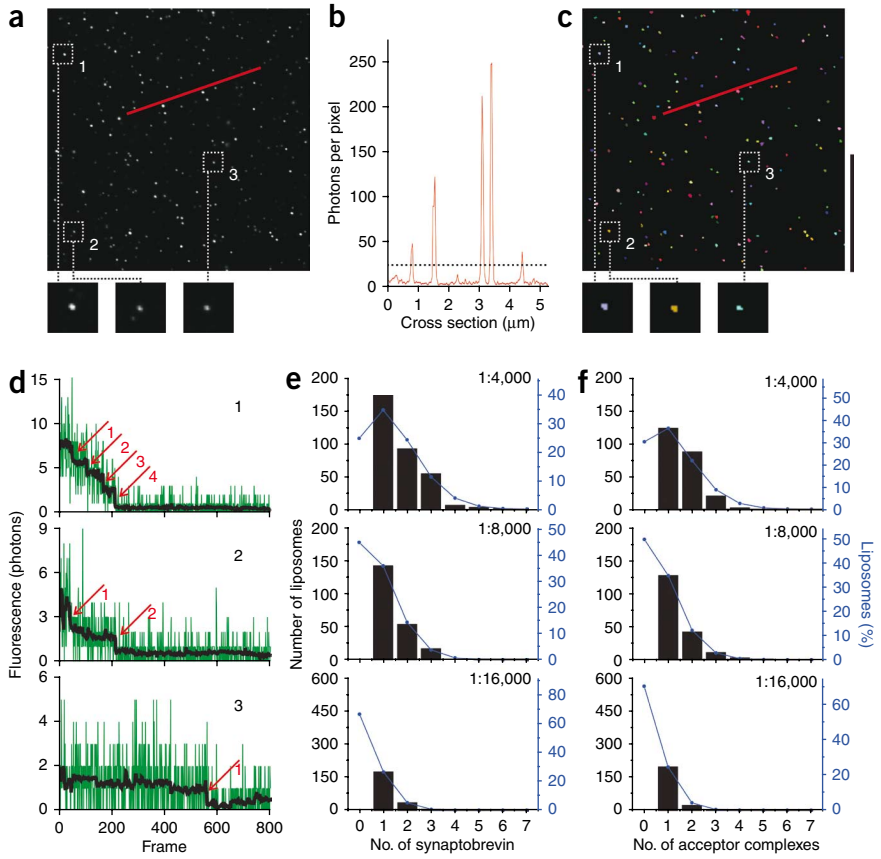

Figure 2 SNARE distribution over the liposomes. (**a**) Two-photon excitation microscopy image of liposomes containing Texas red–labeled synaptobrevin. (**b**) Cross-section of the image (red line in **a**). The liposomes were selected using an offset (dotted line) as described[18]. (**c**) Liposome recognition; each liposome was assigned a random color. Scale bar, 5 μm. (**d**) Sequential photobleaching of the liposomes indicated in **c** (green). We used a Chung-Kennedy nonlinear filtering technique[38] to correct for fluorescence intermittency (black). The three liposomes contained four, two and one synaptobrevin molecules, respectively (red arrows). (**e**,**f**) Distribution of synaptobrevin (**e**) and syntaxin (**f**) for the p/l ratios indicated (bars; left axis). Fitting the data with Poisson distributions (blue curves; right axis), we found that the SNARE molecules were randomly distributed over the liposomes, with p/l ratios of 1:4,000, 1:8,000 and 1:16,000 yielding averages of 1.4, 0.8 and 0.4 synaptobrevin and 1.2, 0.7 and 0.35 syntaxin molecules per liposome, respectively.

A single SNARE complex is sufficient for membrane fusion

First, we employed classical cuvette-based lipid-mixing (FRET) experiments to measure membrane fusion. In these experiments, one population of liposomes is labeled with Oregon green–phosphatidylethanolamine (PE; donor fluorophore) and the other with Texas red–PE (acceptor fluorophore). Upon membrane fusion, these spectrally separated fluorescent lipid analogs mix, and this results in a FRET signal (**Fig. 3a**). We measured the effect of lowering the concentration of the stabilized acceptor complex and that of synaptobrevin via the quenching of the Oregon green donor fluorophore (**Fig. 3b–d**). The fluorescent cross-talk of Texas red in the Oregon green channel was negligible (<1%), and therefore the quenching of the Oregon green donor fluorescence could be used immediately as a measure of lipid mixing. We varied the p/l ratio from 1:1,000 to 1:16,000 and observed lipid mixing in all cases, even at the lowest SNARE density. SNAP-25 enhanced lipid mixing, although with syntaxin only (in the absence of SNAP-25) we observed approximately 10% of the amount of lipid mixing seen in the presence of the acceptor SNARE complex (**Fig. 3e**). Membrane fusion without SNAP-25 has been previously reported[4,20]. Notably, in all cases, lipid mixing was SNARE specific and could be blocked by competitive inhibition using the soluble SNARE domains: the addition either of the cytoplasmic domain of synaptobrevin-2 (residues 1–96) or of a combination of SNAP-25 and the soluble

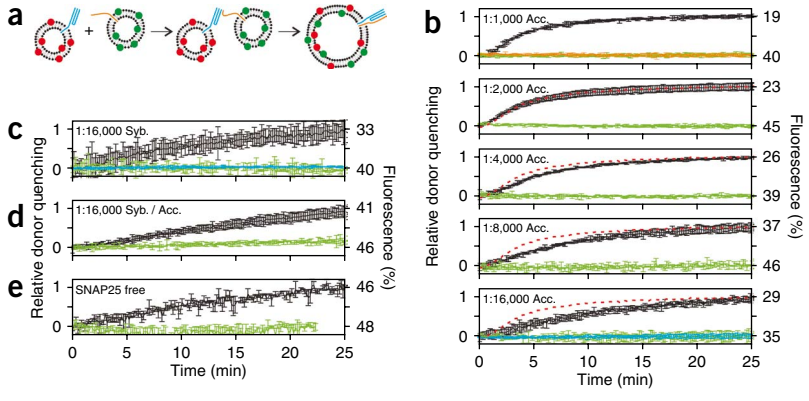

Figure 3 Bulk lipid mixing as a function of SNARE density. (**a**) Scheme of the lipid-mixing experiment in which liposomes containing Texas red–PE (red) and the acceptor SNARE complex (blue) fuse with liposomes containing Oregon green–PE (green) and synaptobrevin (orange), resulting in quenching of the Oregon green donor fluorophore. (**b**) Lipid mixing of 1:1,000 synaptobrevin liposomes with acceptor-complex liposomes (Acc.) at the p/l ratios indicated. The total lipid concentration in the cell was 50 μM, corresponding to approximately 4 nM liposomes, as determined by fluorescence correlation spectroscopy. Recordings were made at 20.0 °C. (**c**,**d**) Fusion of 1:16,000 synaptobrevin (Syb.) to 1:1,000 (**c**) and 1:16,000 (**d**) acceptor-complex liposomes; in all cases lipid mixing was observed. (**e**) Lipid mixing of 1:1,000 synaptobrevin with 1:1,000 syntaxin-$1_{183–288}$ liposomes. In the absence of SNAP-25, 10% fusion compared to the acceptor complex (**b**) was observed. Curves are normalized (left axis); real Oregon green fluorescence is indicated as a fraction of Triton X-100 controls (right axis). For reference, the shape of the highest-concentration curve is shown in all subsequent panels (red). Lipid mixing was SNARE specific and could be inhibited with 10 μM synaptobrevin-$2_{1–96}$ (green curves) or with a combination of 10 μM SNAP-25 and the soluble SNARE domain of syntaxin-$1_{183–263}$ (orange). Moreover, liposomes containing no acceptor SNARE complex or no synaptobrevin did not show membrane lipid mixing (blue curves in **b** and **c**, respectively). Error bars indicate s.d. from triplicates.

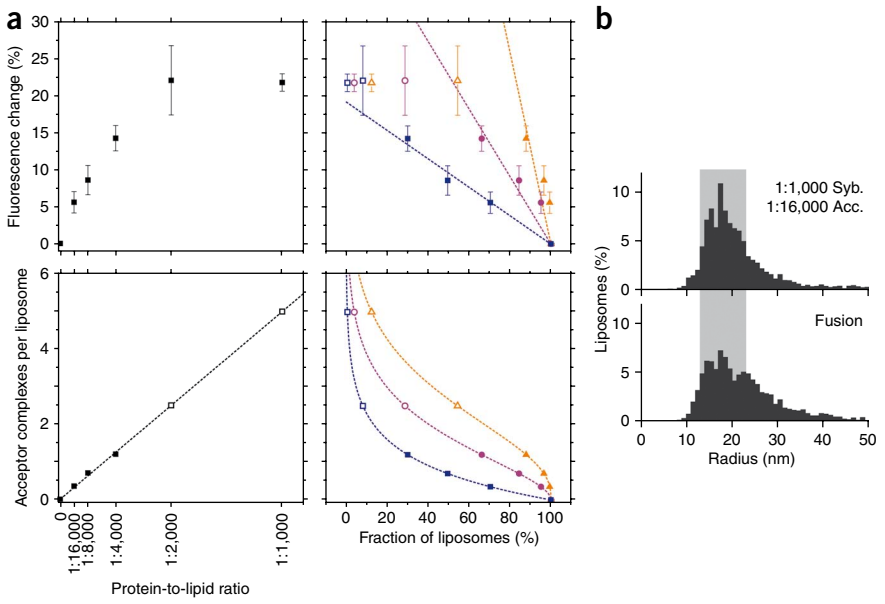

Figure 4 Liposomes containing a single SNARE participate in membrane fusion. (**a**) Top left, the fluorescence change after 25 min for the lipid-mixing reactions of **Figure 3b**. Bottom left, the number of acceptor complexes from **Figure 2f** as a function of the protein-to-lipid ratio. Bottom right, the number of acceptor complexes relative to the fraction of liposomes containing less than one (blue), two (purple) or three (orange) SNAREs (data from **Fig. 2f**). The number of SNAREs for the 1:1,000 and 1:2,000 acceptor-complex liposomes could not be determined with sequential photobleaching because at these high protein-to-lipid ratios the error of the sequential photobleaching increases progressively. Therefore, these were estimated using Poisson distributions (bottom right graph, dotted lines) with the averages linearly extrapolated from the 1:4,000 liposomes (bottom left graph, dotted line). Closed symbols indicate measured data points and open symbols indicate extrapolated data points. Top right, the fluorescence change as a function of the fraction of liposomes containing no (blue), one or fewer (purple) or two or fewer (orange) SNAREs. The data for multiple SNAREs (purple and orange) did not scale linearly (dotted lines; fits of measured data), whereas the data for empty liposomes (blue) scaled relatively well. Thus, the fluorescence change decreased linearly (dotted line) with the fraction of empty liposomes, indicating that all liposomes that contained one or more SNARE molecules participated in fusion. (**b**) Size distribution of a 1:1 ratio of 1:1,000 synaptobrevin (Syb.) and 1:16,000 acceptor-complex liposomes (Acc.) determined with negative-staining electron microscopy (n = 2,887). A slight increase in size was observed upon fusion (n = 1,774). The increase was small because the radius increases only 1.4-fold ($\sqrt{2}$) upon fusion and because only 30% of the acceptor-complex liposomes contained SNARE proteins (**Fig. 2f**).

SNARE domain of syntaxin-1 (residues 183–263) resulted in a complete blocking of fusion. In addition, we observed no lipid mixing with empty liposomes in the absence of either the acceptor complex or synaptobrevin.

The observed lipid mixing was not caused by the small fraction of liposomes that contained multiple SNAREs, because the increase in FRET decreased linearly with the fraction of empty liposomes containing no SNARE molecules, whereas it did not scale linearly to the fraction containing multiple SNAREs (**Fig. 4a**). We further confirmed this by electron-microscopic analysis of the fusion product, observing a slight increase in size upon fusion of 1:1,000 synaptobrevin to 1:16,000 acceptor-complex liposomes (**Fig. 4b**). The increase in size was relatively small because only 30% of the acceptor-complex liposomes contained SNARE proteins and were capable of fusion, and because the radius increases only 1.4-fold upon fusion. Moreover, we can exclude the possibility that lipid mixing is a consequence of liposome aggregation, because no aggregates were observable using either electron microscopy or fluorescence correlation spectroscopy[11]. In addition, at a p/l ratio of 1:16,000, sequential photobleaching experiments indicated that the distribution of fluorophores over the liposomes did not change substantially upon fusion. Together, these results show that a single SNARE complex is sufficient to induce lipid mixing.

We next asked whether single SNARE complexes are also sufficient to mediate fusion with native biological membranes. In contrast to proteoliposomes, such membranes are crowded with a large variety of proteins and probably have a complex, asymmetric lipid composition. We have shown previously that synaptic vesicles isolated from rat brain are capable of fusion with liposomes containing syntaxin and SNAP-25 in a SNARE-dependent manner[21]. Here we measure fusion of purified synaptic vesicles with liposomes containing increasing dilutions of the acceptor SNARE complex. The liposomes contained membrane lipids labeled with Oregon green and Texas red, and fusion was recorded by donor fluorophore dequenching. At a p/l ratio of 1:16,000 for the acceptor SNARE complex, specific lipid mixing with synaptic vesicles was still evident (**Fig. 5a**,**b**). These results demonstrate that a single SNARE complex is sufficient to propagate lipid mixing not only of liposomes but also of complex native biological membranes.

Lipid mixing does not distinguish between fusion and hemifusion, and it is thus conceivable that with a single SNARE complex, the reaction does not progress beyond the hemifusion state. To distinguish between fusion and hemifusion, we employed a content-mixing assay in which liposomes with encapsulated calcein were fused with empty (calcein free) liposomes, resulting in calcein dequenching (**Fig. 5c**)[22,23]. At p/l ratios for the acceptor SNARE complex of both 1:1,000 and 1:16,000, SNARE-specific content mixing was observed (**Fig. 5d**). The kinetics of the calcein dequenching overlapped reasonably well with that of the lipid mixing (**Fig. 3b**), indicating that the progression from hemifusion to full fusion occurred faster (<10 s) than could be resolved with our time resolution. To exclude the possibility that the signal was caused by leakage of calcein from liposomes, we quenched external calcein with cobalt ions (5 μM). Only a small amount of leakage was detectable when liposomes at a p/l ratio of 1:1,000 were used (4.1 ± 1.4%; s.d.), whereas no leakage was observed during fusion of liposomes with a p/l ratio of 1:16,000. Because we observed full fusion at p/l ratios for which the average number of correctly oriented SNARE molecules per liposome is well below 1 (**Fig. 2**), we conclude that a single SNARE complex is sufficient to mediate full membrane fusion.

No cooperativity in SNARE complex formation

If one SNARE complex is sufficient for membrane fusion, the question arises of why fusion is faster at higher SNARE densities in the membrane (**Fig. 3b**). As docking precedes fusion, it is conceivable that the rate of docking (**Fig. 5e**, step A) increases with the SNARE density, causing faster fusion (step B). If this were the case, one would expect the liposome concentration to affect the fusion kinetics.

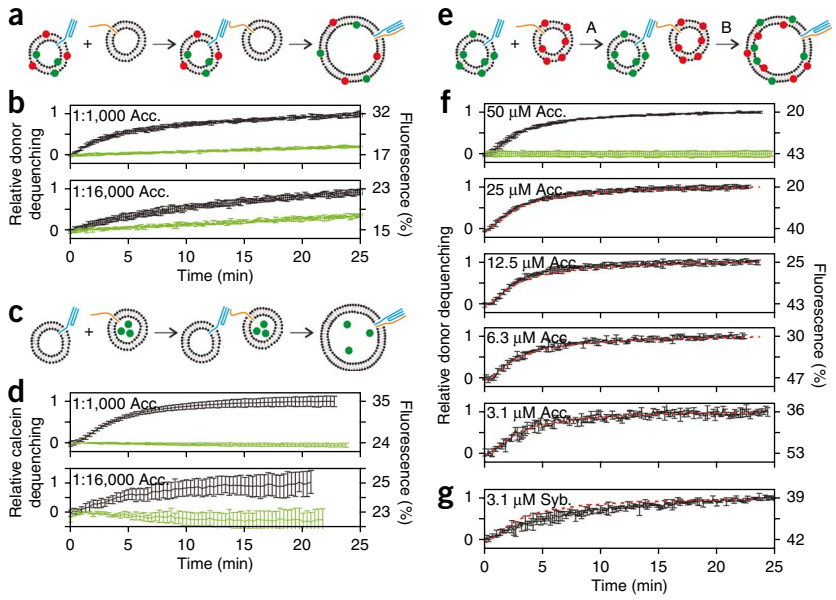

Figure 5 Fusion with purified synaptic vesicles, content mixing and lipid mixing as a function of liposome concentration. (**a**,**b**) Schematic (**a**) and results (**b**) of lipid mixing experiments in which 1:1,000 or 1:16,000 acceptor–SNARE complex liposomes (Acc.; 50 μM total lipid) containing both Texas red–PE and Oregon green–PE were fused to purified synaptic vesicles (8.5 μg total protein). Fusion results in lipid mixing and dequenching of the Oregon green donor fluorophore. The results indicate that a single SNARE complex is sufficient to drive fusion of native biological membranes. (**c**) Schematic of the content-mixing assay based on calcein (green) fluorescence dequenching upon membrane fusion. (**d**) At p/l ratios of 1:1,000 or 1:16,000 of the acceptor SNARE complex and 1:1,000 of synaptobrevin, content mixing was observed. The results show that a single SNARE complex is sufficient not only for lipid mixing but also for content mixing. (**e**,**f**) Schematic (**e**) and results (**f**) of lipid-mixing experiments in which 50 μM (total lipid) of Texas red–PE synaptobrevin liposomes were fused with Oregon green–PE acceptor SNARE complex liposomes at the lipid concentrations indicated in the figure. In **e**, A marks docking and B marks fusion. (**g**) Fusion of 50 μM Oregon green–PE acceptor complex liposomes to 3.2 μM Texas red–PE synaptobrevin liposomes. These results indicate that the rate of membrane fusion is only weakly dependent on the liposome concentration. For reference, the shape of the highest-concentration curve is shown in all subsequent panels (red). Curves are normalized (left axis); real fluorescence is indicated as a fraction of Triton X-100 controls (right axis). Fusion was inhibited with 10 μM synaptobrevin-$2_{1–96}$ (green curves). Error bars indicate s.d. from triplicates.

However, when using a p/l ratio of 1:1,000 for both the acceptor complex and synaptobrevin, we observed no effects or only minor effects on the lipid mixing rate when the liposome concentrations were varied almost 20-fold (**Fig. 5f**,**g**). This indicates that under these conditions, membrane fusion is slow relative to the docking of the liposomes. Consequently, the results show that the sigmoidal fusion kinetics (for example, in **Figs. 3**, **5** and **6**) are caused not by slow membrane docking, but rather by multiple steps progressing after the docking, probably the slow dissociation of the synaptobrevin$_{49–96}$ fragment[10,11].

Subsequently, we checked whether the correlation between the fusion rate and the SNARE density is caused by cooperativity in SNARE complex formation—specifically, whether increased SNARE densities would promote the formation of *cis* complexes. We labeled syntaxin and synaptobrevin with Alexa Fluor 488 and Texas red at their respective C-terminal ends. Upon complex formation, the respective C-terminal ends come in close proximity, and this results in a FRET signal. We measured FRET via the emission of the Texas red acceptor fluorophore (sensitized emission; **Fig. 6a–c**). The sensitized emission was used because the initial levels of fluorescence were proportional

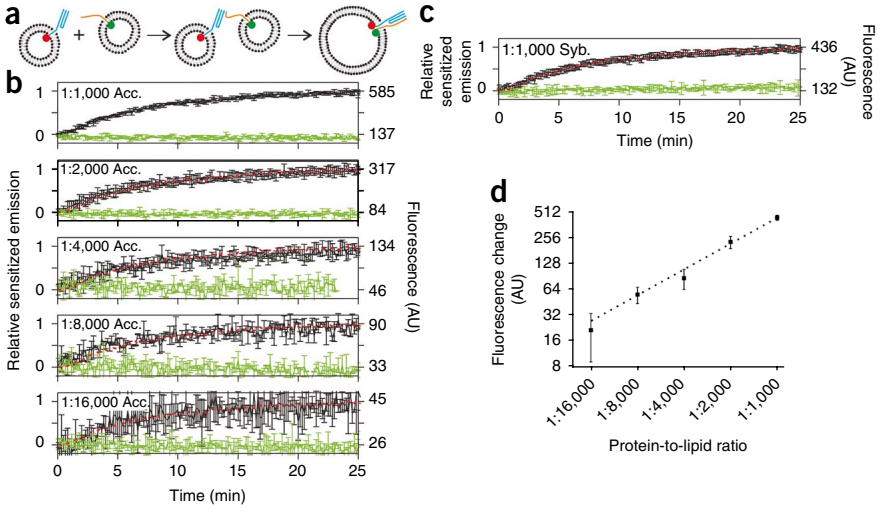

Figure 6 Formation of SNARE complexes, monitored using C-terminal FRET. (**a**) Schematic of the FRET experiment to measure complex formation of Texas red–labeled syntaxin with Alexa Fluor 488–labeled synaptobrevin. (**b**) Complex formation of 1:1,000 synaptobrevin liposomes with the stabilized acceptor complex (Acc.) at the p/l ratios indicated in the figure (50 μM total lipid). Data is presented as emission of the Texas red acceptor fluorophore (sensitized emission), so initial fluorescence is proportional to the acceptor-complex levels. (**c**) Reversing the label did not influence the curves. The experiment shows that the rate of complex formation is not dependent on the SNARE density and indicates that there is no cooperativity in SNARE complex formation. Curves are normalized (left axis); real fluorescent signals of the acceptor fluorophore are indicated (right axis). Syb., synaptobrevin. Fusion was inhibited with 10 μM synaptobrevin-$2_{1–96}$ (green curves). For reference, the shape of the highest-concentration curve is shown in all subsequent panels (red). (**d**) The increase in the Texas red emission after 25 min for the reactions in **b** as a function of the p/l ratio. The linear correlation (dotted line) indicates that the total amount of core complex formed is directly dependent on the concentration of SNAREs in the cuvette. Error bars indicate s.d. from triplicates. AU, arbitrary units.

to the concentration of acceptor complex and the increase in signal corresponds to *cis*-complex formation (**Fig. 6d**). Upon changing the p/l ratios, we found no effect on the rate of complex formation, indicating that there is no cooperativity in the formation of the core complex. Moreover, this shows that docking of the liposomes proceeds faster than fusion at low p/l ratios as well as high ones. Together, these results indicate that higher SNARE densities lead to faster fusion via a simple stochastic mechanism in which the chance of a fusion event increases owing to a greater population of docked liposomes and multiple SNAREs participating in the docking events.

DISCUSSION

Our results show that liposomes containing a single SNARE molecule are capable of propagating membrane fusion, both with their cognate partner liposomes and with purified synaptic vesicles. This is an unexpected finding, considering that previous estimates of the necessary number of SNARE molecules have been much higher (3–15)[6–9]. Notably, our findings suggest the need for a reconsideration of popular models that invoke ring-shaped arrangements of multiple SNARE complexes surrounding a central patch of membranes[9] or that propose the existence of fusion pores that are lined by multiple SNARE transmembrane domains[8]. If multiple SNARE complexes interact at the site of fusion, our data suggest that interaction is stochastic—that is, the resulting complexes may be randomly distributed across the interaction plane, with single SNARE complexes being sufficient to cause the opening of a fusion pore. Our findings extend previous observations showing that a single complex suffices for membrane attachment[4,5]. They are also compatible with energetic considerations suggesting that the free energy estimated to be released during core-complex formation (33–43 k_BT)[4,24–27] may suffice for membrane fusion[24,25]. It should be pointed out that the activation energy needed to fuse two membranes is not known, with estimates ranging from 40 to 200 k_BT (refs. 28,29). A further consideration is that the average radius of the liposomes used in this study was 17 nm, which is slightly smaller than that of synaptic vesicles (20 nm). Larger membranes may have a higher energy barrier for fusion and may consequently require multiple SNARE complexes for fusion or, alternatively, may use chaperones such as synaptotagmin to locally change the curvature of the membrane and facilitate fusion[30].

Our findings have several implications for biological fusion events. First, they explain the fact that in many intracellular membranes undergoing frequent and rapid fusion, the density of SNAREs is notably low. For instance, it has recently been shown that in recycling endosomes, the density of the relevant SNAREs is more than 100-fold lower than that of the synaptic SNAREs in nerve terminals, and even if the concentration of the endosomal SNAREs is further downregulated by 90%, the residual concentrations suffice to maintain fusion at nearly normal rates[31]. Considering that SNARE pairing is preceded by the cooperation of tethering and docking factors that recruit the SNAREs to the prospective fusion site[32], fusion mediated by single complexes can thus remain highly efficient.

In contrast, specialized membranes such as synaptic vesicles and synaptic plasma membranes contain extraordinarily high densities of SNAREs. Synaptic vesicles contain, on average, 70 active synaptobrevin molecules[33], and the 25-nm fusion sites on the plasma membranes of PC12 cells contain, on average, 75 clustered syntaxin molecules each[34]. What are the advantages of such high SNARE densities, given that a single complex is sufficient to execute fusion? First, SNAREs are dynamic and can diffuse through the membrane[35], thereby moving away from the fusion site. Therefore, a high density of SNAREs will increase the likelihood of a SNARE being present when external signals trigger the assembly of the fusion machinery. Second, SNAREs are known to form promiscuous *cis* complexes in the plane of the membrane[21] that need to be reactivated by the disassembly ATPase NSF, thus lowering the effective concentration of active SNAREs. Consequently, a large redundancy might act as a safety mechanism to ensure that there are always active SNAREs available at the fusion site irrespective of NSF activity.

In addition, it needs to be considered that neuronal docking and calcium-dependent secretion are tightly regulated, both spatially and temporally, by a multitude of proteins. Thus the complexity is vastly increased compared to our *in vitro* system. Many of the regulatory proteins, such as Munc18, complexin and synaptotagmin, interact with the SNAREs[1,2]. It is conceivable that binding of these proteins siphons away some of the energy of SNARE assembly and that under these conditions one SNARE complex may no longer suffice to bring about fusion. However, there are some indications that the number of SNARE complexes required for neuronal exocytosis is low. For instance, experiments with a temperature-sensitive NSF mutant (comatose) in *Drosophila melanogaster* have shown that synaptic transmission in the absence of active NSF is blocked only over a slow time course, while core complexes accumulate on the synaptic vesicles[36]. This indicates that only a small number of the synaptobrevins available on the synaptic vesicle participate in the fusion event. More recently, the number of synaptobrevin molecules required for exocytosis has been directly measured using cultured hippocampal neurons from synaptobrevin-knockout mice. Here, the reintroduction of only two synaptobrevin molecules per vesicle was sufficient for full recovery of stimulation-dependent synaptic vesicle fusion (R. Sinha (Max-Planck Institute for Biophysical Chemistry) and J. Klingauf (Univ. of Münster), personal communication). In summary, we cannot exclude the possibilities that under *in vivo* conditions more than one SNARE complex is needed to effect fusion and that cell types and trafficking reactions may differ in the number of SNAREs they require. However, in a 'bare bones' minimal system, a single SNARE complex is unequivocally sufficient for membrane fusion. This observation is of fundamental importance for researchers' understanding and calls for a re-evaluation of current models of SNARE-dependent membrane fusion.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

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AUTHOR CONTRIBUTIONS

G.v.d.B. purified the proteins, designed, performed and analyzed the FRET and sequential photobleaching experiments, and programmed the software for the data analysis. M.G.H. purified the synaptic vesicles and performed the partial proteolysis and flotation experiments. G.B. and F.S.W. assisted with the microscopy. D.R. performed the electron microscopy. G.v.d.B. and R.J. designed the study and wrote the paper. All authors discussed the results and commented on the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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ONLINE METHODS

Protein purification and labeling. We expressed and purified synaptobrevin-2 117Cys, synaptobrevin-$2_{1–96}$, cysteine-less SNAP-25, syntaxin-$1_{183–288}$, syntaxin-$1_{183–263}$, and the stabilized complex consisting of syntaxin-$1A_{183–288}$ with an additional cysteine at the C terminus (289Cys), cysteine-less SNAP-25A and synaptobrevin-$2_{49–96}$ (all from rat) as described[10], except that buffers contained 2% (w/v) CHAPS as detergent and 0.2 mM Tris-(2-carboxyethyl)phosphine instead of DTT. For fluorescent labeling, we incubated a two-fold molar excess of Alexa Fluor 488 C_5-maleimide (485/515 nm) or Texas red C_2-maleimide (596/604 nm, Invitrogen) for 2 h and then performed gel filtration. The endogenous cysteines of synaptobrevin (Cys103) and syntaxin (Cys91, Cys92), all located in the transmembrane helices, are inaccessible for maleimide labeling. We determined labeling efficiencies with UV-visible spectroscopy.

Protein reconstitution in liposomes. We prepared proteoliposomes as described[37] in 20 mM HEPES, pH 7.4, with 150 mM KCl. We used lipids in a 5:2:1:1 ratio of brain L-α-phosphatidylcholine, L-α-phosphatidylethanolamine (PE), L-α-phosphatidylserine and cholesterol (Avanti). For the lipid-mixing experiments, we substituted PE with 1.5% of Oregon green–1,2-dihexadecanoyl-*sn*-glycero-3-PE (496/524 nm) or Texas red–1,2-dihexadecanoyl-*sn*-glycero-3-PE (Invitrogen). We encapsulated calcein (495/515 nm) as described[23]. We measured fusion on a Fluorolog (Horiba) at 20.0 °C with 1-nm bin width at a final volume of 1.2 ml. For microscopy, we mixed liposome suspensions with three volumes of 5% low–melting temperature agarose (Sigma). Synaptic-vesicle protocols have been described[33].

Sequential photobleaching. We carried out microscopy on an FV1000 laser scanning microscope with a UPlanSApo ×60 1.35-NA objective (Olympus) and a Chameleon-Ultra II tunable infrared laser (Coherent) running at 890 nm, 80 MHz and 15 mW. We recorded fluorescence with a Micro Photon Devices PDM avalanche photodiode coupled to a Picoharp-300 (Picoquant). For the sequential photobleaching, we recorded a time series of images (516 × 516 pixels), with a pixel step of 200 nm and a dwell time of 2 μs. For the Chung-Kennedy filtering[38], we used 60 equally probable forward-moving unbiased predictors, an analysis window of 20 data points, and 20 as a weighting factor.

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