

Determinants of liposome fusion mediated by synaptic SNARE proteins

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Synaptic exocytosis requires the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins syntaxin 1, SNAP-25, and synaptobrevin (VAMP). Assembly of the SNAREs into a stable core complex is supposed to catalyze membrane fusion, and proteoliposomes reconstituted with synaptic SNARE proteins spontaneously fuse with each other. We now show that liposome fusion mediated by synaptic SNAREs is inhibited by botulinum neurotoxin E (BoNT/E) but can be rescued by supplementing the C-terminal portion of SNAP-25. Furthermore, fusion is prevented by a SNAP-25-specific antibody known to block exocytosis in chromaffin cells, and it is competed for by soluble fragments of the R-SNAREs synaptobrevin 2, endobrevin/VAMP-8, and tomosyn. No accumulation of clustered vesicles is observed during the reaction. Rapid artificial clustering of SNARE-containing proteoliposomes enhances the fusion rate at low but not at saturating liposome concentrations. We conclude that the rate of liposome fusion is dominated by the intrinsic properties of the SNAREs rather than by the preceding docking step.

Exocytosis of synaptic vesicles requires the *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins syntaxin 1 and SNAP-25 on the synaptic plasma membrane, and synaptobrevin (also referred to as VAMP) on the vesicle membrane. Syntaxin 1 and synaptobrevin possess a single transmembrane domain at the C-terminal end, whereas SNAP-25 is membrane-anchored by palmitoyl side chains attached in the middle of the molecule. Although the essential function of the SNAREs for neurotransmitter release is well established, it is still debated whether these proteins operate as fusion catalysts or whether they act upstream of the actual fusion reaction (for review, see refs. 1–3).

Each SNARE-protein contains one (syntaxin, synaptobrevin) or two (SNAP-25) characteristic stretches of 60–70 aa arranged in heptad repeats, referred to as SNARE motifs (4). Although isolated SNARE motifs are unstructured, they spontaneously assemble into stable core complexes consisting of four helix bundles. Each helix is contributed by a different SNARE motif (5, 6) representing a separate subfamily, referred to as Qa-, Qb-, Qc-, and R-SNARE motif (4, 7). Disassembly requires ATP and the action of the AAA-ATPase the *N*-ethylmaleimide-sensitive factor in conjunction with cofactors (8). Because membrane fusion requires that SNAREs are initially present on both membranes, assembly of the core complex would pull the membranes closely together, resulting in fusion, with the energy being provided by the assembly reaction (9, 10).

Although studies on soluble recombinant SNAREs have been instrumental in developing our current thinking about how SNAREs fuse membranes, it still needs to be clarified how the speed and efficiency of biological fusion reactions is brought about at the molecular level. As a step toward this goal, it is necessary to reconstitute exocytic membrane fusion by using purified proteins and artificial membranes. Recently, Rothman and colleagues (11–13) have studied the fusion of liposomes reconstituted with SNARE proteins by using a lipid dequenching assay. When liposomes containing appropriate sets of SNAREs are mixed with each other, they spontaneously fuse (11–13).

Replacement of the transmembrane domains with lipid anchors (14) or insertion of additional amino acids between the SNARE motif and the transmembrane domain (15) inhibited or attenuated fusion, supporting the idea that during SNARE assembly force is exerted on the membrane that leads to fusion.

Although these studies have provided a useful starting point toward reconstitution of exocytosis, the reported properties of liposome fusion mediated by neuronal SNAREs are still very different from that of exocytosis. Most importantly, the rate of fusion is slow and resembles that of *in vitro* assembly of soluble SNARE motifs. However, a rigorous investigation of the factors responsible for *in vitro* fusion kinetics has not been performed. Fusion of liposomes in solution requires random collisions of freely diffusing vesicles, and thus fusion rate will critically depend on collision frequency and the reactivity of the SNAREs under such conditions. Furthermore, it has been reported that, during preincubation of liposomes at low temperature, clustering (“docking”) occurs that accelerates fusion, but it is not known to which extent and under which conditions clustering determines the rate of fusion, i.e., whether clustering or the fusion reaction itself are rate limiting. Because the comparison of reaction rates forms the basis of virtually all functional studies of exocytosis and membrane fusion, it is necessary to investigate the influence of such parameters on the reaction rate.

Materials and Methods

Preparation of Recombinant Proteins. The following constructs were described (all from rat): SNAP-25a (all cysteines replaced by serines; ref. 16), SNAP-25 C-terminal fragment (amino acid positions 120–206) (17), SNAP-25 N-terminal fragment (amino acids 1–180) (18), endobrevin N-terminal fragment (amino acids 1–76) (16), and tomosyn C-terminal fragment (amino acids 1051–1116) (19). Synaptobrevin 2 (amino acids 1–116; ref. 20) was subcloned into pET15 (Novagen) via the *Nde*I/*Xho*I sites.

N-terminally shortened syntaxin (syxH3) (amino acids 183–288) was prepared (20) and cloned into pET28a via the *Nde*I/*Xho*I sites. The following mutant constructs were generated by site-directed mutagenesis (21): syxH3 with a C-terminally added cysteine (amino acids 183–289C) and synaptobrevin with a C-terminally added cysteine (amino acids 1–117C).

BoNT/E and TeNT light chains were gifts from H. Niemann (Medizinische Hochschule, Hannover, Germany). All proteins were expressed as His-6-tagged fusion proteins and purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography.

Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; syxH3, syntaxin fragment of residues 183–288; BoNT/E, botulinum neurotoxin E; FRET, fluorescence resonance energy transfer.

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For proteins containing transmembrane domains, 1.5% Na-cholate (wt/vol) was added to each buffer. Fluorescent labeling of proteins was performed as described (22) except that buffers containing 1.5% sodium cholate were used.

Preparation and Purification of Proteoliposomes. Lipids (Avanti, Alabaster, AL) were mixed in chloroform to yield (molar ratios): phosphatidylcholine (5), phosphatidylethanolamine (2), phosphatidylserine (1), phosphatidylinositol (1), cholesterol (1). After drying, they were resuspended in HB100 containing 5% (wt/vol) cholate at a total lipid concentration of 13.5 mM. SNARE-proteins in 1.5% cholate were added (lipid to protein ratio of 100:1 *n/n*), followed by chromatography on Sephadex G-50 superfine equilibrated in HB100 buffer by using a sample-to-column volume ratio of 1:30. For the preparation of liposomes containing syxH3 and SNAP-25, the proteins were preincubated for 1 h before addition to the phospholipid mixture unless indicated otherwise.

For purification, 500 μ l of the liposome fraction were mixed with an equal volume of 80% Nycodenz in HB100, overlaid with 500 μ l of 30% Nycodenz and 150 μ l of HB100. The gradient was centrifuged at 165 000 $\times g$ for 4 h. Liposomes were retrieved from the top of the gradient.

To determine protein orientation, proteoliposomes were incubated with trypsin at a molar ratio of 25:1 (protein/trypsin) at 37°C for 4 h. Parallel incubations were performed in the presence of 0.2% Triton X-100. Reacted samples were separated on a 10% SDS gel. Labeled proteins were visualized in a LAS-Reader (Fuji) using the appropriate filters.

Fusion Assay by Particle Counting and by Fluorescence Resonance Energy Transfer (FRET). Liposomes were mixed to yield a 1:1 molar ratio of synaptobrevin (labeled with Alexa594) and syntaxin/SNAP-25 complex (syntaxin labeled with Alexa488) with a final lipid concentration of \approx 3 mM. For particle counting, the mixture was incubated at 37°C for 3 h (standard assay) or for the indicated time and then diluted 1:50,000 in HB100 buffer containing TetraSpeck microspheres (0.22 nm, Molecular Probes). A total of 200 μ l of this sample was pipetted on a coverslip and left to settle for 15 min at 4°C. The coverslips were then washed with 4 ml of HB100 buffer and mounted on slides by using SlowFade Light antifade reagent A (Molecular Probes). The samples were observed under a Zeiss Axiovert 100 TV Fluorescence microscope with a \times 100 1.4 numerical aperture plan achromate objective using appropriate filters. Pictures were acquired by using a back-illuminated frame transfer charge-coupled device camera (2 \times 512 \times 512 EEV chip, 13 \times 13 μ m pixel size; Princeton Instruments, Princeton). Object positions were determined by using METAMORPH software (version 4.1.7, Universal Imaging Corporation, West Chester, PA). The position of a TetraSpeck bead was used for alignment of the channels. Liposomes were counted as colocalized if the position of their intensity maxima did not differ by $>$ 1 pixel (130 nm). Colocalization was calculated as percent of donor liposomes colocalizing with an acceptor liposome. All values represent averages of at least five frames.

For FRET measurements, the liposomes were mixed in a microquartz-cuvette to yield a final volume of 30 μ l and analyzed in a FluoroMax II Fluorometer (Yvon Jobin) equilibrated to 37°C, using an excitation wavelength of 496 nm and an emission wavelength of 615 nm.

Streptavidin–Biotin-Mediated Docking of Liposomes. Liposomes were prepared containing 0.2% (mol/mol lipid) of biotinylated phosphatidylethanolamine, prepared as described in *Supporting Text*, which is published as supporting information on the PNAS web site. One population of liposomes was saturated with streptavidin by a 15-min incubation with a 5-fold molar excess of

streptavidin over biotin. Excess streptavidin was removed by density gradient centrifugation (see above).

Electron Microscopy. Liposome solutions were applied to perforated carbon coated grids, plunge frozen in liquid ethane (23), and transferred to a Phillips CM120 BioFilter electron microscope by using Gatan cryostage. Images were recorded on a 512 \times 512 pixels Gatan slow scan charge-coupled device camera at a magnification of \times 31,000 by using DIGITAL MICROGRAPH 3.4 software (Gatan) at a constant defocus.

For negative staining of liposomes, a solution containing 1:10 diluted liposomes were applied to a glow discharged carbon-coated grid and stained with 1% uranyl acetate.

Other Methods. Liposome fusion by lipid dequenching was measured as described (11, 24). SDS/PAGE was performed according to ref. 25. Protein determination was performed according to ref. 26. The figures show representative examples of experiments that were repeated several times with consistent results.

Results

Characterization of SNARE-Mediated Liposome Fusion by Using Complementary Fusion Assays. For the preparation of SNARE containing proteoliposomes, we generated full-length synaptobrevin 2 and a shortened version of syntaxin 1a (residues 183–288, SyxH3). We omitted the N-terminal domain of syntaxin because it is known to interact with the SNARE-motif, significantly reducing its reactivity (12, 27, 28). The same proteins were also prepared with a cysteine added C-terminally for labeling. The proteins were labeled with the fluorescent dyes Alexa488 or Alexa594, yielding labeling efficiencies ranging from 65 to 95%. No significant labeling was obtained in control incubations by using variants lacking the C-terminal cysteine (data not shown) showing that the only other cysteines that are located in the transmembrane domain are inaccessible for the negatively charged dyes. For SNAP-25, mutant proteins were used in which the cysteine residues in the loop region were replaced by serines.

Proteoliposomes were formed from cholate micellar solutions of proteins and lipids by gel filtration, yielding small unilamellar liposomes (average diameter, 30 nm, see Fig. 3) containing about one copy of protein per 100 phospholipid molecules. This ratio is within the range estimated for synaptobrevin in purified synaptic vesicles (ref. 29 and unpublished observations). To determine the orientation of the proteins, liposomes were treated with trypsin in the presence and absence of detergent. Approximately 80% and 65% of syntaxin and synaptobrevin, respectively, were correctly oriented (Fig. 1).

For measuring SNARE-mediated liposome fusion, we used three complementary assays, each with specific advantages and disadvantages. The first assay is based on particle counting by fluorescence microscopy after adsorption of the liposomes to a glass surface. When donor and acceptor liposomes contain SNAREs labeled with different fluorescent dyes, the percentage of docked/fused vesicles can be measured by determining the percentage of vesicles containing both dyes.

Because fusion results in the formation of *cis*-SNARE-complexes, we used complex formation as second readout, which was measured by fluorescence resonance energy transfer between synaptobrevin and syntaxin labeled C-terminally with fluorescent dyes. Finally, we monitored lipid mixing by fluorescence dequenching (24).

Fig. 2 shows the time course of liposome fusion between donor liposomes containing a preformed complex of SyxH3 and SNAP-25, and acceptor liposomes containing synaptobrevin. The time course of fusion measured by all three assays was similar, with an estimated half-rise time of \approx 20 min and reaching a plateau at \approx 2 h. Particle counting revealed that at the end of the reaction the percentage of double-labeled particles was $>$ 80%. Because

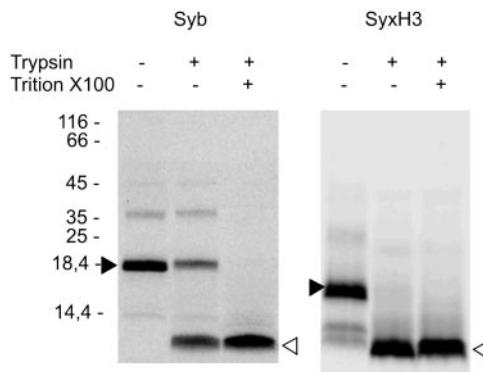


Fig. 1. Orientation of the SNAREs in proteoliposomes. Liposomes reconstituted with either synaptobrevin labeled with Alexa594 (syb) or a preformed complex of syntaxin H3 (labeled with Alexa488, SyxH3) and SNAP-25 were incubated with trypsin in absence or presence of Triton X-100 and analyzed by SDS/PAGE and fluorescence imaging. Filled and open arrowheads mark the position of undigested protein and protease-resistant fragments, respectively.

the size of individual liposomes is below the resolution limit of the light microscope, particle counting reports the sum of docking and fusion, in contrast to lipid dequenching that only reports fusion. Thus, it appears that, during the reaction, there is no significant accumulation of vesicle clusters that precedes fusion. Moreover, large vesicle clusters were not observed at any time during the reaction. Fusion depended on the presence of syntaxin and SNAP-25 in the donor and of synaptobrevin in the acceptor liposome fraction, because no fusion was observed when either of the proteins was omitted (not shown). Preincubation of synaptobrevin liposomes with the light chain of tetanus neurotoxin (which cleaves synaptobrevin) largely abolished fusion (Fig. 2). Fusion was associated with formation of SDS-resistant core complexes (Fig. 2 Lower). No “homotypic” fusion was observed between syntaxin or synaptobrevin-containing liposomes, regardless of whether SNAP-25 was present (data not shown).

Next, we performed electron microscopy of shock-frozen liposomes to check whether larger liposomes are generated during the reaction. A significant shift to larger sizes was observed at the end of the fusion reaction indicating repetitive fusion events (Fig. 3). Again, vesicle clusters were only rarely observed.

Effects of SNARE Perturbation on Liposome Fusion. Truncation of the C-terminal SNARE motif of SNAP-25, as effected by botulinum neurotoxin E, is known to result in an inhibition of exocytosis. To test whether the toxin exerts a similar inhibition on liposome fusion, SNAP-25 was either toxin-treated before being added to syntaxin-containing liposomes, or the toxin was added to liposomes reconstituted with a preformed syntaxin-SNAP-25 complex. As shown in Fig. 4*a*, preincubation of SNAP-25 with BoNT/E light chain largely abolished fusion, whereas no inhibition was observed with toxin-treated liposomes containing preformed syntaxin-SNAP-25 complexes. Analysis by SDS/PAGE revealed that SNAP-25 in complex with syntaxin is resistant to toxin cleavage (Fig. 4*b*). As shown in Fig. 4*c*, fusion is significantly reduced when liposomes are coreconstituted with truncated SNAP-25 corresponding to the BoNT/E-cleaved fragment (E-frag).

It was previously reported that inhibition of exocytosis by BoNT/E in permeabilized PC12 cells was partly rescued by the addition of the isolated C-terminal SNARE-motif of SNAP-25 (30). To test whether this is also the case for liposome fusion, we

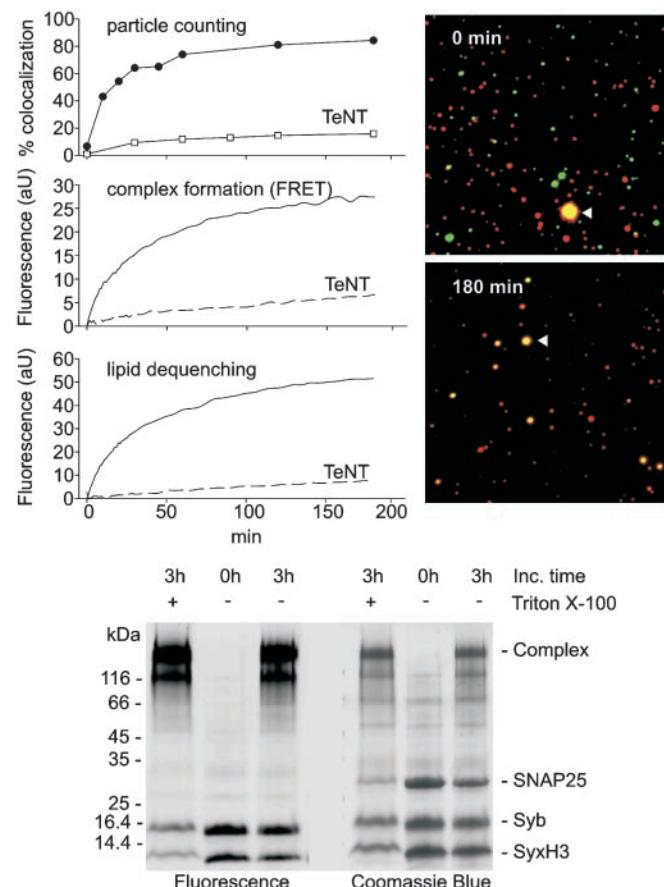


Fig. 2. (Upper Left) Comparison of fusion kinetics using particle counting (Top), FRET (Middle), and lipid dequenching (Bottom). Donor and acceptor liposomes were coreconstituted with syntaxin H3/SNAP-25 and synaptobrevin, respectively. For particle counting and FRET, C-terminally labeled variants of syntaxin-H3 and synaptobrevin were used. Fusion was performed at 37°C. As control, synaptobrevin liposomes were preincubated for 30 min with 3 μM tetanus toxin light chain (TeNT) at 37°C. (Upper Right) Representative microscopic pictures of a fusion assay at the begin (Upper) and end (Lower) of the incubation. The pictures acquired in the red and green channel were aligned by using TetraSpeck beads (arrowheads) as reference. (Bottom) Assembly status of SNAREs at the beginning (0 h) and end (3 h) of the fusion reaction using fluorescently labeled syntaxin and synaptobrevin. As control, one sample was incubated for 3 h at 37°C in the presence of 2% (vol/vol) of Triton X-100 to allow for maximal complex formation. The samples were separated by SDS/PAGE and visualized either by fluorescence imaging using a filter set consisting of HQ470/40 excitation filter and a HQ645/75 emission filter (Left) or by Coomassie staining (Right).

added the C-terminal SNARE motif of SNAP-25 or of full-length SNAP-25 to liposomes containing truncated SNAP-25. Under both conditions, fusion was partially restored, with full-length SNAP-25 being more effective (Fig. 4*c*).

As an independent means to interfere with SNAP-25 we used monoclonal antibody Cl 71.1 that binds to the N-terminal SNARE motif. This antibody blocks chromaffin cell exocytosis and prevents the formation of SNARE complexes (31). Two experiments were carried out. First, SNAP-25 was preincubated with the antibody before addition of syntaxin proteoliposomes. Second, proteoliposomes containing a preformed complex of syntaxin and SNAP-25 were incubated with the antibody. As shown in Fig. 4*d*, fusion was only inhibited if the antibody was added before complex formation with syntaxin. Thus, it appears that both SNARE motifs of SNAP-25 are inaccessible to perturbation in the binary complex.

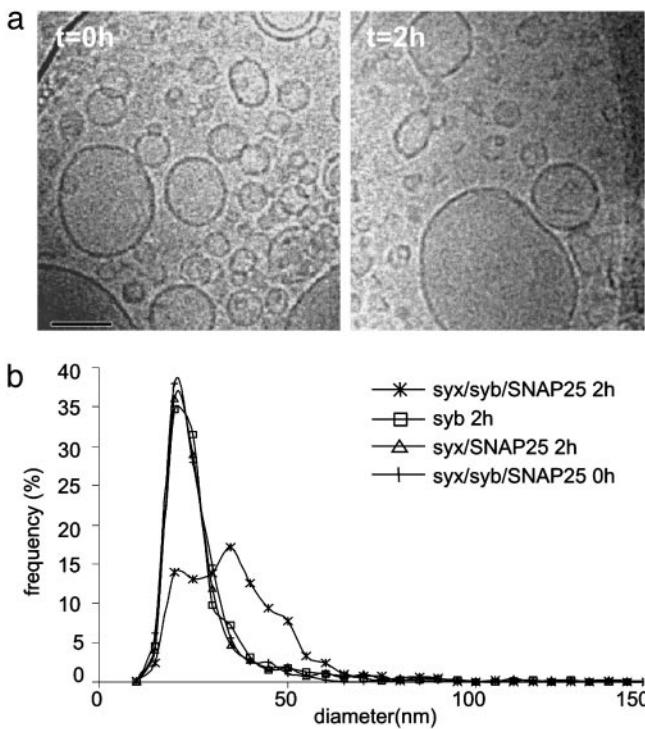


Fig. 3. Increase of liposome size after fusion, analyzed by cryo-electron microscopy. (a) Images of a fusion reaction shock-frozen either immediately ($t = 0\text{ h}$) or 2 h ($t = 2\text{ h}$) after the begin of the reaction. (Bar = 100 nm.) (b) Distribution of the liposome diameters ($n \approx 1,000$) at the beginning (0 h) and end (2 h) of the reaction, in comparison with the diameter distribution of separately incubated donor and acceptor liposomes.

Finally, we examined whether recombinant soluble R-SNARE motifs interfere with the fusion reaction. Such R-SNARE motifs are expected to compete with liposome-bound synaptobrevin for complex formation with syntaxin and SNAP-25. Such an inhibition was indeed observed (Fig. 4e). Interestingly, the R-SNARE motifs of endobrevin/VAMP 8, an R-SNARE operating in the fusion of late endosomes (32), and of tomosyn, a soluble protein of unknown function (33), were as efficient as that of synaptobrevin in inhibiting fusion.

Determinants of Fusion Kinetics. The experiments described so far have shown that neuronal SNAREs are capable of fusing liposomes in a manner that depends on the appropriate interaction of their respective SNARE motifs. However, the question arises why liposome fusion proceeds with such slow kinetics. Because liposome fusion depends on random collisions between donor and acceptor liposomes in solution, it is conceivable that fusion kinetics is dominated by the frequency and/or duration of the collision events, particularly when considering that no major accumulation of vesicle clusters form during the reaction. In this case, the measured reaction rate would primarily depend on the incubation conditions (e.g., liposome concentration, viscosity of the media) and would not necessarily reflect the intrinsic reaction rate of the SNAREs.

First, we estimated how often on average liposomes need to collide with each other for a successful fusion event. For this purpose, we determined the diffusion coefficient of SNARE-containing liposomes by using fluorescence correlation spectroscopy analysis, resulting in an average of $33 \times 10^{-9} \text{ cm}^2/\text{s}$. Liposome concentration was then calculated by using the amplitude of the diffusion term in the FCS equation (see *Supporting Text*), resulting in $7.3 \pm 1.5 \times 10^{16}$ liposomes per mg of

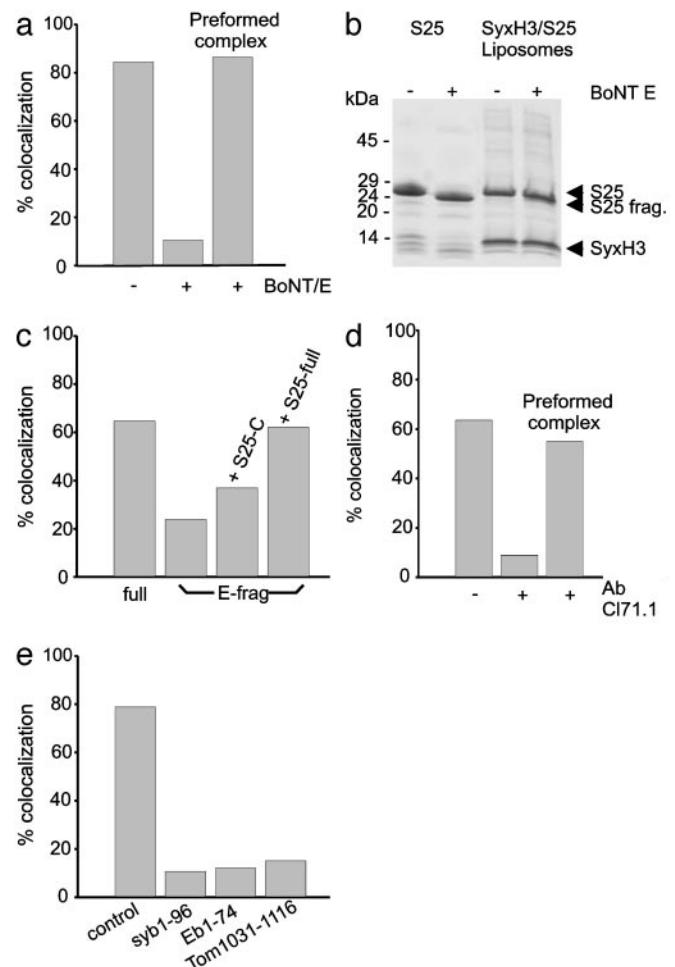


Fig. 4. Perturbation of SNARE assembly leads to a reduction of fusion. All assays were analyzed by particle counting. (a) Effect of BoNT/E on liposome fusion. Toxin treatment was carried out either with isolated SNAP-25 that was then added to syntaxin-containing liposomes, or with liposomes coreconstituted with preformed syxH3/SNAP-25 complexes. All preincubations were for 30 min at 37°C, and fusion was analyzed after 3 h. (b) SNAP-25 is resistant to BoNT/E cleavage in preformed syxH3/SNAP-25 complexes. Liposomes containing preformed complexes were incubated with BoNT/E light chain in a 1:50 molar ratio of toxin to SNAP-25 for 1 h at 37°C. As control, free SNAP-25 was incubated in parallel. Samples before and after treatment were separated by SDS/PAGE and visualized by Coomassie blue staining. S25, SNAP-25, S25 frag., SNAP-25 fragment caused by BoNT/E cleavage. (c) Both intact SNAP-25 (S25-full) and the C-terminal SNARE motif of SNAP-25 (S25-C) rescue fusion of liposomes containing C-terminally truncated SNAP-25 [BoNT/E fragment (E-frag), amino acid 1–180]. Liposomes containing syxH3 were preincubated for 30 min with E-frag (control: full-length SNAP-25, Left) and then either directly used for the fusion assay or incubated for additional 30 min in the presence of S25-full or S25-C before fusion. (d) Effect of monoclonal antibody CI 71.1 (directed against the N-terminal SNARE-motif of SNAP-25) on fusion. Liposomes containing syxH3 were incubated with SNAP-25 either in the presence or absence of antibody 71.1 for 30 min at 37°C. In parallel, liposomes containing a preformed complex of syxH3 and SNAP-25 were incubated with antibody 71.1 for 30 min at 37°C. Fusion was analyzed after 2 h. (e) Soluble R-SNARE motifs inhibit liposome fusion. To liposomes containing a preformed complex of syxH3 and SNAP-25 the soluble SNARE-motifs of synaptobrevin, endobrevin, and tomosyn were added in a 2-fold molar excess over syntaxin, and the mixture was incubated at 37°C for 30 min before starting the fusion reaction. Fusion was analyzed after 3 h.

phospholipids. This number amounts to $\approx 1.8 \times 10^{14}$ per ml each of donor and acceptor vesicles at the begin of the reaction. We then calculated the collision frequency between liposomes in solution, using a diffusion model in which the particles moved

independently by Brownian motion and in which no repulsive forces prevented particle collision (see *Supporting Text*). Using the data obtained in two independent particle counting experiments, the fusion probability/liposome collision was then calculated to be $\approx 10^{-7}$, i.e., only one in 10 million collisions results in fusion.

These data suggest that the low fusion probability per collision event is primarily responsible for the low reaction rate. To overcome the dependence on the collision frequency, we determined the fusion rate under conditions where donor and acceptor vesicles were stably attached to each other by using an avidin-biotin system. Biotinylated phosphatidyl ethanolamine was incorporated into both donor and acceptor liposomes. Donor liposomes were then saturated with streptavidin and combined with acceptor liposomes after removal of unbound streptavidin. To avoid the formation of large aggregates, pilot experiments were carried out in which the concentration of the biotinylated phospholipid was varied, and the cluster size was determined by electron microscopy. In these experiments, both sets of liposomes contained synaptobrevin to avoid fusion. Under optimized conditions (0.2% of total phospholipid biotinylated, i.e., ≈ 20 molecules per vesicle), the majority of liposomes were clustered, but only small clusters containing few liposomes were formed (Fig. 5b). Furthermore, clustering was rapid and occurred at a time scale of <5 min (Fig. 5a).

Fusion reactions were carried out with preclustered liposomes at two liposome concentrations. To determine the influence of clustering, parallel incubations were performed with the same liposomes in which the streptavidin loading step was omitted. As shown in Fig. 5c, a moderate acceleration of the fusion rate was observed under standard assay conditions. When liposomes were incubated at 100-fold dilution, however, preclustering led to a dramatic increase in the overall reaction rate. The normalized time curves showed perfect overlap under all conditions, documenting that the slow reaction rate is not due to a rate-limiting step preceding vesicle docking but rather reflects the properties of the reconstituted fusion machine. No fusion was observed when donor and acceptor liposomes were clustered that both contained synaptobrevin (not shown).

Discussion

In the present study we have used complementary assays to explore the fusion of liposomes reconstituted with neuronal SNARE proteins. Our results show that fusion requires the formation of core complexes because it is inhibited by reagents that block assembly or by truncation of synaptobrevin or SNAP-25. Furthermore, we show that the rate of fusion is relatively low even if liposomes are preclustered.

We introduced assays for monitoring SNARE-mediated liposome fusion to complement the lipid dequenching method that is based on the dilution of two species of fluorescently labeled phospholipids with unlabeled lipids upon fusion (24). Although convenient, dequenching is nonlinear with respect to surface increase, complicating the interpretation of fusion kinetics. For the assays, we added fluorescent labels to the C-terminal end of the SNAREs' transmembrane domain, allowing to monitor the formation of core complexes by FRET. Because donor and acceptor vesicles are thus labeled by different dyes, it is possible to perform particle counting to obtain a direct and quantitative measure of the proportion of interacting vesicles. Particle counting does not distinguish between docking and fusion, however, and it also cannot be differentiated whether a double-labeled vesicle results from single or multiple fusion events.

The basic features of liposome fusion catalyzed by the neuronal SNAREs reported here are in good agreement with those previously reported (12, 13). Fusion requires both syntaxin and SNAP-25 to be present on the donor, and synaptobrevin to be present on the acceptor vesicle population. Fusion is associated

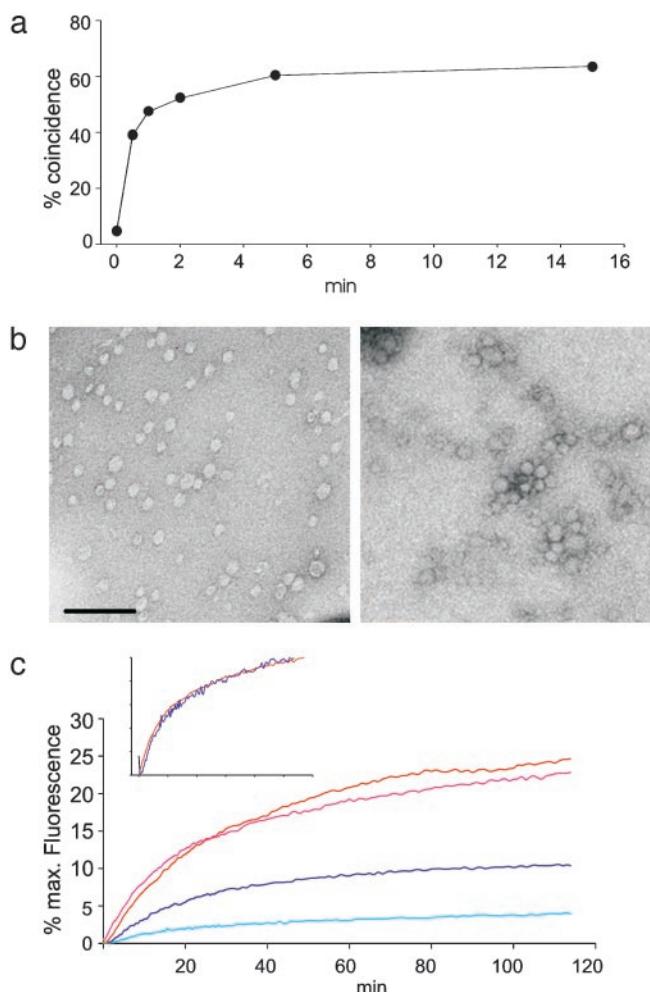


Fig. 5. Influence of preclustering on the liposome fusion rate. (a) Determination of the clustering rate by particle counting. Streptavidin-saturated and -free biotinylated liposomes containing synaptobrevin labeled with Alexa594 and Alexa488, respectively, were mixed, and the reaction was terminated by 1:10 dilution in 50 mM biotin. (b) Electron microscopy of negatively stained vesicles after artificial docking (*Right*). For comparison, streptavidin saturated liposomes adjusted to the same liposome concentration are shown (*Left*). (Bar = 200 nm.) (c) Effect of docking on fusion kinetics under standard conditions and at 100-fold dilution of liposomes. Donor liposomes containing syxH3/SNAP-25 were either saturated with streptavidin or streptavidin was omitted so that docking could not occur. Acceptor liposomes contained synaptobrevin and free biotin-phycerythrin. Red, standard conditions with streptavidin; pink, standard conditions without streptavidin; dark blue, 100-fold dilution with streptavidin; light blue, 100-fold dilution without streptavidin. Fusion was monitored by lipid dequenching. (*Inset*) Normalized fusion rates of predocked vesicles show almost identical fusion kinetics.

with the generation of core complexes and is prevented by preincubation with clostridial neurotoxins cleaving synaptobrevin (11). However, there are also differences with respect to the previous reports. For instance, we obtain saturation kinetics with all assays. Furthermore, in our hands it is not necessary to form a syntaxin-SNAP-25 complex before reconstitution. Some of these discrepancies may be due to differences in the liposome composition. We use a lipid mixture that contains various membrane lipids including cholesterol to approximate native membrane composition, and our SNARE/phospholipid ratio is lower than that used in previous studies to be as close as possible to native concentrations. In addition, it was reported previously that only cognate SNAREs are capable of fusing proteolipo-

somes (14). Although we have not systematically explored SNARE specificity, it is noteworthy that fusion is blocked not only by adding the cytoplasmic portion of synaptobrevin (that competes with the liposome-bound protein for core complex formation), but also by the only distantly related R-SNARE motifs of endobrevin/VAMP-8 and tomosyn. These SNARE motifs were previously shown to form core complexes with syntaxin and SNAP-25 in solution at an efficiency similar to that of synaptobrevin (16, 19, 34), suggesting that the ability of sets of SNAREs to fuse liposomes is determined primarily by the ability of their respective SNARE motifs to form stable core complexes.

Interestingly, loss of fusion caused by truncating the C-terminal SNARE motif of SNAP-25 can be rescued, at least partially, by substituting the C-terminal SNARE motif as soluble protein. This observation agrees perfectly with the observation that in permeabilized PC12 cells exocytosis blocked by BoNT/E can be rescued in a similar manner (30). Apparently, all that is needed is supplementing the missing/truncated α -helix of the C-terminal SNARE motif of SNAP-25 with an intact version. These findings lend further support to the notion that the formation of SNARE core complexes is the critical step in catalyzing membrane fusion.

At lower liposome concentrations, the kinetics of fusion is limited by the frequency of diffusive collisions. In fact, our mathematical model shows that the probability of constructive interactions (docking/fusion) between colliding liposomes is very low. At higher liposome concentrations, however, the reaction cannot be accelerated anymore by artificial clustering of

the vesicles. Together with the fact that, during the course of the reaction, no major clusters are observable as reaction intermediates, the following conclusions can be drawn. First, when a constructive interaction occurs, it proceeds toward fusion and does not involve a prolonged docked state. This result is surprising because it is conceivable that incomplete assembly, or the assembly of only one or two SNARE complexes, may suffice to attach the vesicles but may not suffice to fuse them. These findings argue against the view that SNAREs are docking or tethering factors whose function precedes fusion.

Second, even when vesicles are preclustered, the fusion kinetics is rather slow. At present, it cannot be distinguished whether this is due to the lack of activating proteins such as complexins or synaptotagmins, or whether the conformation of the SNAREs on the liposome surface does not correspond to that in an intact cell. We have shown previously that SNAREs are clustered in cholesterol-dependent microdomains (35), creating hotspots of high local concentration. Furthermore, *in vitro* SNAP-25 and syntaxin 1 form a complex with 1:2 stoichiometry (36), which also forms a four-helix bundle (37), albeit of less stability than the core complex. For synaptobrevin to bind, one of the syntaxin molecules needs to be displaced, which may be rate-limiting *in vitro* (18). Thus, it is possible that the reaction rate depends on the conformation and composition of the syntaxin-SNAP-25 acceptor complex.

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