

# Single Molecule Observation of Liposome-Bilayer Fusion Thermally Induced by Soluble N-Ethyl Maleimide Sensitive-Factor Attachment Protein Receptors (SNAREs)

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**ABSTRACT** A single molecule fluorescence assay is presented for studying the mechanism of soluble N-ethyl maleimide sensitive-factor attachment protein receptors (SNAREs)-mediated liposome fusion to supported lipid bilayers. The three neuronal SNAREs syntaxin-1A, synaptobrevin-II (VAMP), and SNAP-25A were expressed separately, and various dye-labeled combinations of the SNAREs were tested for their ability to dock liposomes and induce fusion. Syntaxin and synaptobrevin in opposing membranes were both necessary and sufficient to dock liposomes to supported bilayers and to induce thermally activated fusion. As little as one SNARE interaction was sufficient for liposome docking. Fusion of docked liposomes with the supported bilayer was monitored by the dequenching of soluble fluorophores entrapped within the liposomes. Fusion was stimulated by illumination with laser light, and the fusion probability was enhanced by raising the ambient temperature from 22 to 37°C, suggesting a thermally activated process. Surprisingly, SNAP-25 had little effect on docking efficiency or the probability of thermally induced fusion. Interprotein fluorescence resonance energy transfer experiments suggest the presence of other conformational states of the syntaxin•synaptobrevin interaction in addition to those observed in the crystal structure of the SNARE complex. Furthermore, although SNARE complexes involved in liposome docking preferentially assemble into a parallel configuration, both parallel and antiparallel configurations were observed.

## INTRODUCTION

The maintenance of distinct organelles within the eukaryotic cytosol is essential for survival. Within each organelle, conditions can be optimized for disparate biochemical processes, but the exchange of material between these organelles requires the merger of two phospholipid membranes (Ferro-Novick and Jahn, 1994; Rothman, 1994). All known forms of intracellular membrane fusion involve a highly conserved family of proteins termed *Soluble N-ethyl maleimide sensitive-factor Attachment Protein Receptors* (SNAREs) (Chen and Scheller, 2001; Jahn and Südhof, 1999; Südhof, 2000). Numerous biochemical, structural, and genetic studies have lent support to the *zipper model*, which states that SNARE complex assembly begins in *trans*, with separate SNAREs on the donor and acceptor membranes, and ends with formation of a *cis* complex with all proteins residing in the same membrane, and that directional folding of SNAREs into a highly stable parallel four-helix bundle drives membrane fusion (Fiebig et al., 1999; Hanson et al.,

1997; Lin and Scheller, 1997; Sutton et al., 1998; Weber et al., 1998).

The many intracellular trafficking pathways in the cell must be distinctly maintained to allow the specific compartmentalized cellular functions to proceed. Although SNAREs can compensate for one another to some degree, each step in membrane trafficking is governed by a unique subset of SNAREs (Chen and Scheller, 2001; Pelham, 2001). The isolated cytosolic domains of SNAREs from different trafficking pathways can promiscuously form complexes with thermal stability similar to the cognate complexes (Fasshauer et al., 1999; Yang et al., 1999). However, an *in vitro* liposome fusion assay revealed that fusion was limited to combinations that resembled the compartmental localization of intracellular trafficking for yeast SNAREs (Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000, 2002). This suggests that fusion is influenced by more subtle differences than overall complex stability. Although other factors regulate upstream targeting and docking (Ungar and Hughson, 2003), SNAREs from a particular pathway may be optimized to work together under the specific regulatory environment of that pathway.

Neurotransmitter release is one of the most regulated membrane fusion events. Unlike constitutive vesicle trafficking, synaptic vesicles are recruited to the presynaptic membrane, but do not readily fuse. Instead, an average of 10 vesicles is stably docked at a region of the synapse termed *the active zone* awaiting an action potential (Harlow et al.,

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2001; Heuser and Reese, 1977; Rosenmund et al., 2003; Schikorski and Stevens, 1997). Membrane fusion is closely associated with the  $\text{Ca}^{2+}$  influx that follows arrival of an action potential. Exocytosis is triggered within  $\sim 0.2$  ms of the  $\text{Ca}^{2+}$  arrival (Martin, 2003; Südhof, 1995), whereas the background rate of fusion is approximately one per minute per synapse in the absence of action potentials. Although extremely rapid, neurotransmitter release is a probabilistic process, with only one fusion event for every 5–10  $\text{Ca}^{2+}$  signals (Dobrunz and Stevens, 1997). This low release probability means that usually at most one synaptic vesicle per synapse undergoes exocytosis upon depolarization (Südhof, 2000). Thus, regulation of neurotransmission occurs at the level of synaptic vesicle release probability.

The complete inhibition of neurotransmitter release following the specific cleavage of any one of the SNAREs by clostridial neurotoxin proteases supports the fundamental role of the SNARE proteins in synaptic vesicle fusion (Jahn et al., 1995; Schiavo et al., 1994). SNAREs exist in some form of partially assembled “loose” complex before the arrival of the  $\text{Ca}^{2+}$  signal, since the partially assembled complex is still susceptible to neurotoxin protease cleavage whereas conformation-dependent antibodies do not interact with the partially assembled complex (Chen et al., 2001; Xu et al., 1998, 1999). Thus, neuronal SNAREs appear to be optimized not to fuse until the  $\text{Ca}^{2+}$  signal arrives. Patch-clamp capacitance measurements of exocytosis in chromaffin cells triggered by photolysis of caged  $\text{Ca}^{2+}$  suggested that SNARE complex formation is linked directly to  $\text{Ca}^{2+}$  triggering of exocytosis, most likely in conjunction with auxiliary proteins (Sorensen et al., 2002). Although numerous other proteins have been found to be essential for  $\text{Ca}^{2+}$ -dependent neurotransmitter release, such as syntaxin, complexin, Munc18, and Munc13, the molecular mechanism of synaptic vesicle membrane fusion and  $\text{Ca}^{2+}$ -triggering remains unclear (Gerst, 2003).

A serious limitation of bulk membrane fusion assays is the inability to correlate structural and mechanistic details of the SNARE proteins with membrane fusion events. For example, biochemical bulk assays cannot resolve subpopulations of SNARE configurations during docking and fusion. Furthermore, the contribution of SNAREs to membrane fusion has been difficult to isolate *in vivo* due to the complex network of cellular interactions that contribute to the cycle of vesicle targeting, priming, and fusion.

Here we describe an *in vitro* single molecule assay to investigate the role of SNARE proteins and other factors in membrane fusion. Recombinant full-length proteins with site-specific fluorescent labels, and synthetic liposomes capable of retaining small, soluble fluorophores were used for single molecule fluorescence experiments (Michalet et al., 2003; Weiss, 1999; Weninger et al., 2003; Zhuang et al., 2000). By direct observation of the time course of colocalized fluorophores we monitored liposome docking and fusion to deposited bilayers, the configuration of

SNARE complexes, and the release of liposome content. This is the first time that the liposome content is directly monitored in SNARE fusion experiments using single molecule methods.

We find that both syntaxin and synaptobrevin are necessary, and sufficient, in opposing membranes to facilitate liposome docking to supported planar bilayers and thermally induced fusion. Surprisingly, synaptosome-associated protein of 25 kDa (SNAP-25) did not significantly change the docking efficiency or the thermally induced fusion rate, requiring a revision of current models of SNARE-induced vesicle fusion. We estimate that as little as approximately one SNARE interaction between opposing membranes is sufficient for liposome docking and fusion. Increase of the ambient temperature increased the fusion probability, suggesting that fusion is a thermally activated process. Thermally induced fusion occurred on the second timescale, as expected for neuronal SNAREs, since spontaneous neurotransmitter release probability is low in the absence of  $\text{Ca}^{2+}$ -triggering.

## MATERIALS AND METHODS

### Proteins: plasmids, expression, purification, and labeling

Full-length rat syntaxin-1A and SNAP-25A were expressed, purified, and labeled as described earlier (Weninger et al., 2003). Full-length rat synaptobrevin-II containing the mutation Cys<sup>103</sup>Ser was expressed from pet28a (Novagen, Madison, WI) in *Escherichia coli* strain BL21 (DE3) grown in Terrific Broth as described previously (Bowen et al., 2002). Single cysteine substitutions were introduced into this construct to produce a series of proteins for site-specific labeling as described earlier for the cytoplasmic domain of synaptobrevin (Weninger et al., 2003). The mutations in full-length synaptobrevin were Ser<sup>28</sup>Cys and Ala<sup>72</sup>Cys.

Hexahistidine-tagged synaptobrevin was purified using Ni-nitrilotriacetic acid (NTA) agarose (Qiagen, Hilden, Germany) according to manufacturer's instructions as described previously (Bowen et al., 2002). Lysis buffer was supplanted with 5% Triton X-100. Washes and elution buffer contained 0.1% Thesit to allow quantitation by ultraviolet spectroscopy. Although the cytoplasmic domain of synaptobrevin binds to the cation exchange resin monoS (Amersham Biosciences, Piscataway, NJ), the full-length protein failed to bind under similar conditions. The positively charged residues necessary for monoS binding are clustered near the transmembrane domain. These residues may be obscured by the Thesit micelle that solubilizes the transmembrane domain in detergent solution. The protein sample was reverse-purified by passing it over a MonoQ column (Amersham Biosciences) in 20 mM Tris pH 8, 200 mM NaCl, and 0.1% Thesit to remove impurities that bound to this column.

For dye labeling, synaptobrevin was buffer-exchanged into labeling buffer, 25 mM sodium phosphate pH 7.5, 300 mM NaCl with 0.1% Thesit and 0.1 mM Tris(2-carboxyethyl)phosphine. The sample was incubated with a 10-fold excess of the maleimide derivative of either the Cy3 or the Cy5 dye (Amersham Biosciences) for 12 h at 4°C. Labeled protein was purified from free dye using Ni-NTA agarose affinity chromatography (Qiagen) with extensive washing into TBS-BOG (20 mM Tris pH 8.2, 300 mM NaCl, 100 mM  $\beta$ -octyl glucoside, and 1 mM DTT). Labeled protein was eluted in TBS-BOG with 250 mM imidazole and dialyzed against imidazole-free buffer. Thrombin was then added to remove the hexahistidine tag followed by addition of the protease inhibitor PPACK (EMD Biosciences, San Diego, CA) to inhibit residual thrombin activity. Despite attempts to optimize the

labeling reaction complete labeling was not achieved. Variable labeling efficiencies of 55–85% were typical.

## Reconstitution into liposomes

The lipids egg phosphatidylcholine (egg PC) and brain phosphatidylserine (brain PS) (both Avanti Polar Lipids, Alabaster, AL) were used. For experiments using lipid mixtures, lipids were mixed in chloroform at the ratios indicated in the text. In all cases, lipids were dried under flowing argon to a thin film in a glass tube and then placed into vacuum for several hours. Liposomes were formed by hydration of the lipid film in TBS (20 mM Tris pH 8.2, 200 mM NaCl, and 1 mM DTT) to give a final lipid concentration of 30 mg/ml. When content dye was used, lipid films were hydrated in HBS (50 mM HEPES buffer, 150 mM NaCl) containing 50 mM or 200 mM calcein (Molecular Probes, Eugene, OR) also to yield a final lipid concentration of 30 mg/ml. The HBS-calcein buffer was adjusted to a final pH of 8.0 before being added to the lipid films. Liposomes were sized by extrusion using the Avanti Mini-extruder with 50-nm pore size filters according to manufacturer instructions.

Syntaxin and synaptobrevin were reconstituted as described earlier (Weninger et al., 2003). Briefly, protein solutions (1–30  $\mu$ M syntaxin and 100–120  $\mu$ M synaptobrevin) in 100 mM  $\beta$ -octyl glucoside were mixed at a 1:4 ratio with 30 mg/ml lipid samples and allowed to incubate at 4°C for 30 min. Except when noted otherwise, these mixtures were then diluted 1:1 with detergent-free TBS and separated from detergent and unincorporated protein using size exclusion chromatography with Sepharose CL4B (Amersham Biosciences) (Weninger et al., 2003). This chromatography step was also sufficient to remove residual calcein not trapped within the liposome interior.

The reconstitution procedure resulted in liposomes with >95% of the proteins inserted with their cytoplasmic domains facing outward as assayed by susceptibility to chymotrypsin proteolysis (data not shown). At 50–200 mM, the calcein dye at pH 7.5–8.5 is highly self-quenched. The containment of the calcein in the final synaptobrevin liposomes was verified by the intensity and emission wavelength change of the calcein dye in bulk fluorescence observations of the liposome sample before and after addition of Triton X-100 to dissolve the liposomes (data not shown).

## Supported lipid bilayers

Supported lipid bilayers of mixed lipid composition were formed by spontaneous liposome condensation on quartz substrates within rapid flow cells. Supported bilayers are known to maintain a 1-nm gap of water between the membrane and the surface (Groves and Boxer, 2002; Kiessling and Tamm, 2003). Condensation from liposomes reconstituted with SNAREs, as described above, was used to introduce protein into the supported bilayer. The concentration of syntaxin was low in the experiments that pertain to docking and fusion (up to 100 molecules per  $\mu$ m<sup>2</sup>) and intramolecular fluorescence resonance energy transfer (FRET) (0.03/ $\mu$ m<sup>2</sup>), so the effect of syntaxin on the deposited bilayer should be minimal.

The integrity of the supported bilayers was characterized by incorporating  $\frac{1}{2}$ –1 mol % of a lipophilic fluorescent probe. Experiments were conducted using a series of probes: (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; DiI<sub>C18</sub>(3)), 2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine ( $\beta$ -BODIPY 581/591 C<sub>5</sub>-HPC) and Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE) (all Molecular Probes). Supported bilayers prepared with fluorescent labels were generally free of defects or inhomogeneities when observed with the microscope. Occasionally, large isolated defects were observed. The low rate of incorporation of liposomes incubated above protein free, supported bilayers for 40 min at 0.3 mg/ml lipid (see Fig. 1) also suggests a low occurrence of bilayer defects. The lipid mobility was examined with fluorescence recovery after photobleaching (FRAP). Typical measurements found mobile fractions of 50–70% and average diffusivity ( $D$ )

of 1–2  $\mu$ m<sup>2</sup>/s. Single particle tracking of the labeled lipids yielded distributions of  $D$  in the range 1–5  $\mu$ m<sup>2</sup>/s.

Mobility of dye-labeled proteins reconstituted into supported bilayers was also measured with FRAP. For both syntaxin and synaptobrevin incorporated into supported bilayers, the mobile fraction ranged from 3 to 7%. Average diffusivity for synaptobrevin was 0.4  $\mu$ m<sup>2</sup>/s, whereas for syntaxin the diffusivity was 0.07  $\mu$ m<sup>2</sup>/s. The low mobile fraction prevented characterization of diffusivity by single particle tracking for the proteins. Measurement of the emission from dye-labeled syntaxin before and after chymotrypsin cleavage revealed that ~50% of syntaxin in the supported bilayers are oriented such that their cytoplasmic domains point away from the glass (data not shown).

## Content labeling

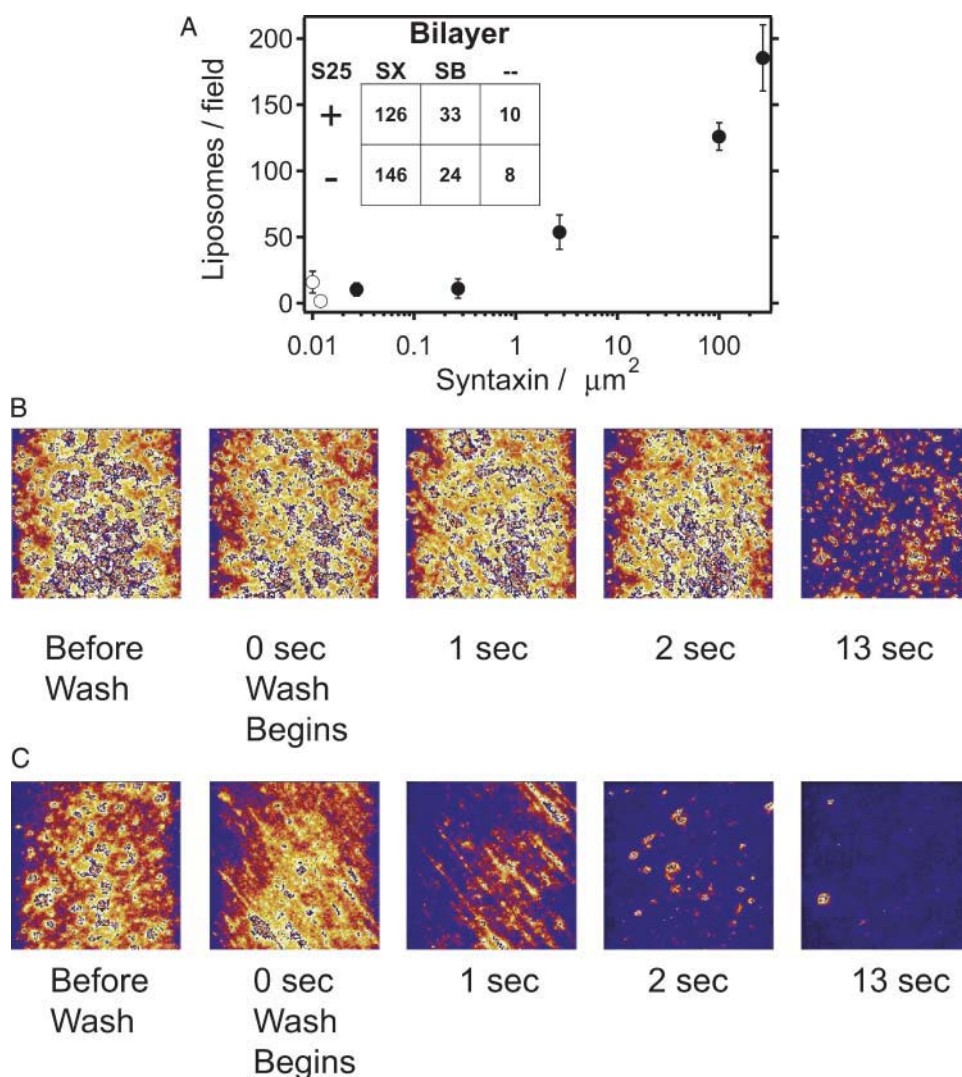
Previous work (Bai and Pagano, 1997) and our experience (unpublished) showed that dye-labeled lipids have the tendency to spontaneously transfer between liposomes and membranes on the second timescale, even in the absence of fusion. The accumulation of highly mobile lipid dyes in the planar bilayer made observation of interprotein FRET problematic. Thus lipid dyes were not used as a sensor for membrane fusion. Previous in vitro fusion systems have lacked a real-time sensor of soluble content mixing (Fix et al., 2004; Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000).

To follow content mixing we relied on the concentration-dependent emission of calcein (Molecular Probes). Our calcein content dye-based assay is measuring soluble content release, which is a relevant and reliable quantity for studying membrane fusion. We conducted bulk measurements of calcein dye quenching with a fluorimeter (Model F-4500, Hitachi Electronic Devices, Norcross, GA). Emission intensity is highest at 20  $\mu$ M, and decreases for both higher and lower concentrations. The emission at 20  $\mu$ M is four orders-of-magnitude higher than at either 10 nM or 10 mM. At concentrations >10 mM or <10 nM, the emission was too low to be detected in bulk by the fluorimeter. However, the fluorescence microscope was capable of detecting emission from 200 mM calcein contained in docked liposomes, and therefore, we conclude that the microscope is capable of detecting the emission from calcein over the concentration range 10 nM to 200 mM. Here we note that bulk fluorescence emission from 174 nM and 12 mM calcein is greater than emission from 200 mM calcein, an observation specifically relevant to the discussion of the bursting of docked liposomes in Results.

## Total internal reflection (TIR) fluorescence microscopy

The data was collected in quartz flow cells with a total internal reflection (TIR) laser fluorescence microscope (Weninger et al., 2003). The illumination from the TIR evanescent field covered a region within a few hundred nanometers of the bilayer. The apparatus could detect the dynamics of single molecules in an 80- $\mu$ m  $\times$  80- $\mu$ m region with 100-ms temporal resolution. Liposomes reconstituted with synaptobrevin were placed in solution above the deposited bilayer along with other soluble components. The circulating water bath connected to the microscope stage, TIR prism, and objective allowed ambient temperature to be controlled. Soluble components could be introduced by rapid buffer exchange with a flow-regulated perfusion system, which did not disrupt the bilayer.

Lasers with emission at 488 nm, 532 nm, and 635 nm were used to excite calcein, Cy3, and Cy5, respectively. The fluorescent image collected by a 60  $\times$  1.2 NA water immersion objective was passed through a 515OG longpass glass filter (Schott Glass, Yonkers, NY) and split by a 650-dclp dichroic mirror (Chroma, McHenry, IL) into a long and a short wavelength path. The two spectrally resolved images were relayed onto separate halves of a charged-coupled device (CCD) detector (PentaMAX, Princeton Instruments, Roper Scientific, Tucson, AZ). For all observations, the longer



**FIGURE 1** SNARE-dependent docking of liposomes to supported bilayers. (A) Number of docked liposomes as a function of protein concentration in the supported bilayer. Supported bilayers were prepared from egg PC with unlabeled syntaxin at the concentration indicated below the graph and exposed to 100 nM SNAP-25 for 40 min. Liposomes reconstituted by dialysis with 10–30 Cy3 labeled Ser<sup>28</sup>Cys synaptobrevin molecules were then introduced above the bilayers for 40 min at a concentration of 0.3 mg/ml (lipid) (corresponding to 10 nM liposome concentration) and rinsed away. Solid circles report the average of the number of docked liposomes per 4050  $\mu\text{m}^2$  field of view sampled at many locations on the bilayer (error bars are the standard deviation of the average). Open circles are the docking results for identical experiments using protein-free supported bilayers. The inset table shows docking results for supported bilayers containing syntaxin, synaptobrevin (100 molecules/ $\mu\text{m}^2$ ), or no protein and with/without SNAP-25 pretreatment of 250 nM for 1 h. For the inset, synaptobrevin liposomes incubation was 0.3 mg/ml for 1 h for the syntaxin and synaptobrevin experiment and 0.15 mg/ml for 100 min for the protein-free control. Membrane protein concentrations are derived from initial lipid/protein ratios during reconstitution. The emission intensity of synaptobrevin liposomes docked to syntaxin-containing bilayers was similar to that measured for spatially resolved liposomes adsorbed to a quartz surface at

sufficient dilution such that bilayers do not form (Johnson et al., 2002). Thus, the emission intensity for a single docked liposome could be determined. The experiment was conducted at 22°C. (B) Real-time washing of liposomes reconstituted with Cy3 labeled synaptobrevin (Ser<sup>28</sup>Cys) docked to syntaxin•SNAP-25 egg PC bilayers. The deposited bilayer contains 200 unlabeled syntaxin/ $\mu\text{m}^2$  in Egg PC incubated with 60 nM SNAP-25 for 20 min before docking. Incubation with liposomes was as in A. Panels are the raw output from the CCD camera showing emission between 550 and 650 nm (Cy3) for a 45  $\mu\text{m} \times 45 \mu\text{m}$  area of the deposited bilayer. The labels along the bottom axis indicate the time, relative to the initial onset of flow in the movie, for the particular movie frame. The first frame shows 10-nM liposomes in solution before rinsing has commenced. By the second frame, an automated buffer exchanger begins to rinse the bilayer with liposome free buffer (flow is from the upper left to the lower right). The last frame shows the view field after washing has concluded. Note the intensity of these docked liposomes is greater than a single Cy3 dye and some photobleaching has occurred by the end of the movie (see supplementary movie S1). C is the same as B, but for a protein-free egg PC bilayer (see supplementary movie S2).

wavelength path included a HQ 700  $\times$  75 nm bandpass emission filter (Chroma). The short wavelength path contained a 580-nm short pass (Coherent Laser Group, Santa Clara, CA) for calcein/Cy5 observation. For simultaneous calcein, Cy3, and Cy5 detection, a 532  $\times$  20 holographic super notch (Kaiser Electro-Optics, Carlsbad, CA) filtered the output of the microscope before the dichroic mirror, and a 550  $\times$  100 bandpass filter (Chroma) was placed in the short wavelength path. Careful selection of high performance optical filters and the alternating color illumination scheme (see next section) were critical factors enabling the single molecule observation of three different dyes.

Observations were conducted in TBS buffer. If TBS oxygen scavenger is indicated the observation was made with the same TBS buffer augmented

with 2% glucose and the enzymatic oxygen scavengers glucose oxidase at 100 units/ml and catalase at 1000 units/ml.

### Correlated measurement of liposome content and FRET using three colors

Single molecule FRET signals from SNARE proteins were observed coincident with the content signal. We used three different illumination colors but only two spectrally resolved detection channels (see filters above). The content fluorescence and the donor (Cy3) emission were both passed into the same, shorter wavelength channel (green trace), whereas the

acceptor (Cy5) emission was detected in the other, longer wavelength channel (*red trace*). The different absorption and emission properties of the dyes were independently measured and were used to determine the signals in the two detection channels.

The bilayer was illuminated with an alternating sequence of the three colors of illumination. The blue light in the first stage should produce measurable emission only from the content dye. The signal of typically 300–800 fluorescence intensity units in this first 1 s stage of the illumination sequence was used to verify the presence of content. In the second stage, the green light caused an emission of the content dye at a signal level approximately equal to its emission signal under blue illumination. Simultaneously in the green illumination period, the donor dye was driven to fluoresce. If it was not quenched by FRET to the acceptor, the donor emission was detected at a signal level in the green trace channel at a level of ~400–800 fluorescence intensity units as an additive emission to the content dye. If the donor was quenched by FRET to the acceptor, emission of the acceptor was detected during the green light stage. Finally, for the last stage, 5 mW of 635-nm laser light was used in order to excite just the acceptor. The acceptor emission was detected in the second channel.

### Botulinum neurotoxin type B cleavage

Botulinum neurotoxin type B (BoNT/B) “light chain” protease cleaves free synaptobrevin at residue 76, i.e., synaptobrevin that is not involved in a fully assembled SNARE complex (Hayashi et al., 1994). A recombinant expression plasmid (pBN13) encoding the protease domain of BoNT/B was kindly provided by Dr. Thomas Binz. BoNT/B light chain protease was expressed in *E. coli* strain BL21 (DE3) grown in Super Broth containing ampicillin. The culture was grown to an  $OD_{600} = 1.0$  at 37°C with shaking at 250 rpm. Then, the temperature was decreased to 30°C and expression was induced by addition of IPTG to 1 mM for 4.5 h. The hexahistidine-tagged BoNT/B light chain protease was purified using Ni-NTA agarose affinity chromatography (Qiagen) according to manufacturer’s instructions. It was then exchanged into 10 mM BisTris buffer pH 6.8, 100 mM NaCl, and 1 mM DTT with a desalting column immediately before use.

We performed control experiments to test the activity of the BoNT/B protease. First, the activity of the BoNT/B protease in solution was tested by SDS-PAGE gel electrophoresis. Second, the ability of BoNT/B protease to cleave and release the labeled synaptobrevin fragment was tested by monitoring the fluorescence from a deposited bilayer with reconstituted labeled synaptobrevin. Upon BoNT/B treatment the fluorescence decreased by 42–47% consistent with the 50:50% mixture of upside-down synaptobrevin molecules in the deposited bilayer. The results were very similar upon more aggressive chymotrypsin treatment (40–54% decrease of fluorescence). Thus, taking into account statistical uncertainties, the effects of chymotrypsin and BoNT/B protease are comparable, and we therefore conclude that nearly all accessible and labeled synaptobrevin molecules will be released from the membrane upon BoNT/B treatment.

Despite the efficient cleavage by BoNT/B protease and release of the labeled fragment from a planar bilayer, there may be somewhat limited accessibility for the protease near the contact site of a liposome docked to the deposited bilayer. Assuming that the phospholipid headgroups of the deposited bilayer and a docked liposome with a diameter of 500 Å are within 20 Å (the approximate width of the folded SNARE complex) apart, and taking into account the minimum dimension of the BoNT/B protease (~40 Å), one can estimate that an area of at least 4% on the surface of the liposome is excluded from the action of the protease (the surface area of a sphere is  $4\pi R^2$  and that of a segment is  $2\pi Rh$  where  $R$  is the radius of the sphere (250 Å) and  $h$  is the height of the spherical segment; the height is the difference between the width of the SNARE complex and the minimum dimension of the protease, i.e.,  $h = 20$  Å). Considering the number of synaptobrevin molecules in the liposomes (20–30), we thus estimate that at most 1–2 synaptobrevin molecules may be affected by topological restrictions near the docking site. The possible incomplete cleavage of 1–2 synaptobrevin molecules by the BoNT/B protease does not affect our

conclusions since the proteolysis is primarily used to reduce background emission from synaptobrevin labels. The only exception is the number of SNARE complexes involved in docked liposomes that may have been overestimated by 1–2 molecules.

### Effect of incomplete labeling

The incomplete labeling of syntaxin and synaptobrevin did not affect our conclusions, but it affects the percentages listed in Table 1 (discussed in Results). We first consider whether an unlabeled synaptobrevin molecule can be involved in a SNARE complex with a labeled syntaxin molecule that may have been colocalized by an uncomplexed labeled synaptobrevin molecule on the same liposome, producing a colocalized  $FRET = 0$  instance. Although BoNT/B cleavage should be nearly complete, 1–2 synaptobrevin molecules residing on the docked liposomes close to the docking site may be inaccessible to the BoNT/B protease. Combined with the incomplete labeling of synaptobrevin of 75%, it is thus possible that an uncomplexed, labeled synaptobrevin molecule may reside near an unlabeled synaptobrevin molecule in complex with syntaxin.

We now consider the reverse situation, involving an unlabeled syntaxin molecule. The syntaxin concentration for the experiments in Table 1 is roughly 100–200 labeled syntaxin molecules per  $4050 \mu\text{m}^2$ . The syntaxin labeling efficiency is typically 50%, so the true protein density doubles to give a typically linear spacing of  $\sim 3 \mu\text{m}$  between molecules. The large signal occurring when multiple dyes are present on an individual liposome can lead to blooming in the image from a multichannel plate, intensified CCD. Due to this effect, the colocalization criterion was defined as  $2 \mu\text{m}$ . Thus, at most 1–2 SNARE complexes were expected for any docked liposome at the dilute syntaxin concentration used. Then, the probability to find an unlabeled syntaxin within the circle of radius  $1 \mu\text{m}$  around a given labeled syntaxin molecule is dominated by the ratio of the available areas and is  $\sim 200 \times (\pi/4050) = 15\%$ . If a liposome is docked to an unlabeled syntaxin and a labeled syntaxin happens to be within the colocalization circle, it will produce another  $FRET = 0$  instance.

In summary, the various colocalized instances break down as follows: 15% of all colocalized spots have  $FRET = 0$  due to liposome docking to an unlabeled syntaxin that is colocalized with a labeled syntaxin. The remaining 85% of colocalized spots correspond to liposomes docked by a labeled syntaxin. The incomplete labeling of synaptobrevin suggests that 25% of the remaining 85% instances (21% of all colocalized spots) will have  $FRET = 0$ . The remaining ~64% of all colocalized spots presumably involve complexes of labeled synaptobrevin and labeled syntaxin. The antiparallel population is approximately one-fifth the size of the parallel population and will also show  $FRET = 0$  when matched N-terminal labels are used. The  $FRET = 1$  population is ~25–30% when parallel labels are used and ~5% with antiparallel labels. The remaining 29–35% of all colocalized spots with  $FRET = 0$  are presumably due to complexes that do not contain SNAP-25. This relatively large population of complexes not recruiting a SNAP-25 is in agreement with single molecule fluorescence experiments that suggested a low rate of assembly of the syntaxin-SNAP-25 binary complex in the supported bilayer geometry. We found typically only 5–10% of syntaxin molecules in a supported bilayer bound a SNAP-25 after being exposed to 200 nM SNAP-25 in solution for 2 h (data not shown).

## RESULTS

Calcium-regulated synaptic vesicle fusion is a millisecond process involving dynamic, complex protein-protein and protein-lipid interactions. As synaptic vesicle release is stochastic (Redman, 1990), single molecule fluorescence observation is well suited to resolve the dynamics of this process as well as the roles of the proteins involved. By constructing an assay from highly purified lipids and

**TABLE 1** Single particle FRET reveals SNARE complex assembly during docking of liposomes to supported bilayers

Labeling scheme			Docked liposomes with <i>FRET</i> = 1		
Syntaxin	Synaptobrevin	SNAP-25	Exp. 1	Exp. 2	Exp. 3
None	N-term	After docking	0	0	
N-term	N-term	None	4%	5%	
N-term	N-term	After docking	26%	23%	28%
N-term	N-term	Before docking	31%	26%	
C-term	N-term	Before docking	6%		

Supported bilayers of 100% egg PC were prepared on quartz slides with no protein or with dye-labeled syntaxin at low enough surface density that individual molecules could be optically separated (typically 100/4020  $\mu\text{m}^2$  field of view). For syntaxin, *N-term* indicates labeling syntaxin at Ser<sup>193</sup>Cys (near the N-terminus of the SNARE motif) and *C-term* indicates labeling at Ser<sup>249</sup>Cys (near the C-terminus of the SNARE motif). Liposomes containing 50 mM calcein and reconstituted with 10–30 synaptobrevin molecules that were dye-labeled at Ser<sup>28</sup>Cys (near the N-terminus of the SNARE motif) were then introduced above the 22°C bilayers for 1–2 h at concentrations of 1–3 mg/ml (lipid), respectively, and rinsed away (the different incubation times and concentrations did not significantly affect the results). SNAP-25 was used where indicated in the table at 100 nM for 2 h and rinsed away. The entry in the SNAP-25 column indicates whether the bilayer was exposed to SNAP-25 before or after the incubation with liposomes for docking. After docking and SNAP-25 exposure were complete, uncomplexed synaptobrevin at docked liposomes was eliminated by treatment with BoNT/B protease at 1 mg/ml for 80 min at room temperature in 10 mM BisTris buffer (pH 6.8), 100 mM NaCl, and 1 mM DTT followed by rinsing into TBS oxygen-scavenger buffer. Single molecule fluorescence microscopy was used to determine the individual docked liposomes that had both a donor and acceptor present and the degree of FRET. Within the subset of docked liposomes having both donor and acceptor dyes present, the level of acceptor emission and photobleaching characteristics under the green emission was used to further divide these liposomes into two sets: *FRET* = 1 (*#FRET*) and *FRET* = 0 (*#COLOC*). Up to three independent experiments were carried out (Exp. 1, Exp. 2, and Exp. 3). The values in the *Exp. 1*, *Exp. 2*, and *Exp. 3* columns report the fractional populations of *FRET* = 1 liposomes as a percentage of all docked liposomes with both donors and acceptors present:  $\#FRET/(\#FRET + \#COLOC)$  where available. Labeling combinations and labeling efficiencies (in parentheses) were: 1, syntaxin (*N-term*) synaptobrevin (*N-term*) SNAP-25 (*none*) = syntaxin Ser<sup>193</sup>Cys Cy5 (57%)/synaptobrevin Ser<sup>28</sup>Cys Cy3 (75%); 2, syntaxin (*N-term*) synaptobrevin (*N-term*) SNAP-25 (*before and after docking*) = syntaxin Ser<sup>193</sup>Cys Cy3 (60%)/synaptobrevin Ser<sup>28</sup>Cys Cy5 (74%); and 3, syntaxin (*C-term*) synaptobrevin (*N-term*) SNAP-25 (*before docking*) = syntaxin Ser<sup>249</sup>Cys Cy5 (42%)/synaptobrevin Ser<sup>28</sup>Cys Cy3 (75%).

recombinant proteins one can incorporate any choice or order of addition of molecular constituents. To investigate the role of SNARE proteins in docking synaptic vesicles to the plasma membrane, we followed the docking of synthetic liposomes to a planar bilayer. This geometry should faithfully reproduce docking of highly curved synaptic vesicles with the plasma membrane, and, when combined with TIR illumination, allows interactions near the bilayer to be selectively observed. To isolate the role of SNAREs in docking and fusion, simple lipid blends from natural sources were used along with the recombinantly expressed neuronal SNARE proteins.

### SNARE-dependent docking

To represent synaptic vesicles, 50-nm diameter liposomes of egg PC were prepared and reconstituted with dye-labeled synaptobrevin. The synaptobrevin concentration was determined from the total fluorescence intensity of individual liposomes, and photobleaching steps observed in the intensity time trace. Approximately 20–30 synaptobrevin molecules were observed per liposome, consistent with the initial protein/lipid ratio used during the reconstitution. This synaptobrevin concentration is close to that reported for synaptic vesicles (Coorssen et al., 2002; Walch-Solimena et al., 1995).

The synaptobrevin-containing liposomes were incubated over a planar-supported egg PC bilayer deposited on a quartz surface and rinsed extensively. Liposomes docked to the

bilayer were visualized by measuring emission of synaptobrevin dyes using fluorescence microscopy (Fig. 1). The docking of synaptobrevin liposomes to the bilayer required the presence of syntaxin in the deposited bilayer. The number of docked liposomes increased as the concentration of syntaxin in the deposited bilayer was increased (Fig. 1 *A*). The docked synaptobrevin liposomes did not move in response to flow imposed over the top of the bilayer (Fig. 1 *B*). To determine the specificity of the docking we observed, we examined docking of synaptobrevin liposomes to a synaptobrevin bilayer. Docking was significantly reduced to 16–26% despite using bilayers containing synaptobrevin at densities equal to the highest surface density of syntaxin used (Fig. 1 *A*, *inset*). As a further control, docking of synaptobrevin liposomes to a protein-free bilayer was also examined (Fig. 1 *A*, *inset*). Nonspecific docking was further reduced to <8% of that seen to the syntaxin bilayer, and the few synaptobrevin liposomes that adhered to protein-free bilayers were susceptible to being swept along with an externally imposed flow over the bilayer (Fig. 1 *C*).

Since our system does not rely on the pre-forming of a binary complex between syntaxin and SNAP-25, we were able to investigate the effect of SNAP-25 on the docking of synaptobrevin liposomes to the syntaxin bilayer. We found that exposure of syntaxin bilayers to SNAP-25 before incubation with synaptobrevin liposomes did not significantly change the number of docked liposomes (Fig. 1 *A*, *inset*). Addition of SNAP-25 after the docking reaction also had no effect on the number of docked liposomes (data not

shown). Thus, the interaction between synaptobrevin and syntaxin in the absence of SNAP-25 (Calakos et al., 1994; Fasshauer et al., 1998) is sufficient to dock liposomes to deposited bilayers. This result is remarkable considering that the regulatory N-terminal domain was included in the syntaxin construct used, so the interaction with synaptobrevin must have shifted the conformation of syntaxin to the “open” form or synaptobrevin must be able to interact with the closed form of syntaxin (Munson et al., 2000) in the context of docked liposomes.

Although attempts have been made to estimate the number of SNARE complexes involved in vesicle fusion, no direct measurements have been possible (Hua and Scheller, 2001). Our experimental setup allows one to determine the number of labeled proteins at a docked liposome from the overall intensity and the number of discrete steps in the photo-bleaching decay. At the bilayer density of syntaxin used, the docked liposome has both free and complexed synaptobrevin, so the BoNT/B light chain protease was used to essentially eliminate the contribution from free synaptobrevin. Synaptobrevin within a fully assembled SNARE complex is resistant to proteolysis by the BoNT/B protease whereas monomeric synaptobrevin in solution is readily cleaved (Hayashi et al., 1994). The BoNT/B cleavage occurs at residue 76 separating the labeled cytoplasmic fragment from the transmembrane domain. We tested the activity of the BoNT/B protease against membrane-reconstituted synaptobrevin and concluded that nearly all fragments of uncomplexed synaptobrevin should be cleaved and released from the membrane, apart from a small fraction of topologically constrained synaptobrevin molecules ( $\sim 4\%$ , corresponding to 1–2 molecules) near the docking site (see Materials and Methods). To estimate the number of synaptobrevin molecules incorporated into SNARE complexes during liposome docking, we exposed the docked liposomes on a syntaxin•SNAP-25 bilayer to the BoNT/B protease and determined the number of synaptobrevin molecules resistant to cleavage (Fig. 2).

Upon BoNT/B proteolysis, most liposomes remained docked ( $74 \pm 18$  compared to  $64 \pm 8$  per  $4050 \mu\text{m}^2$  field before and after BoNT/B treatment, respectively), but a large number of labeled synaptobrevin fragments were removed from the liposomes. The resistance to BoNT/B proteolysis suggests that the liposomes are held to the bilayer by SNARE interactions that cannot be cleaved by the BoNT/B protease. The large bin at the start of the histogram in Fig. 2 *B* indicates that as little as one SNARE complex is sufficient for docking. This is also consistent with the FRET results using a lower syntaxin concentration (discussed below, compare to Table 1) that show SNARE complex formation and consequently liposome docking with as few as one SNARE complex per liposome. On average, approximately 12 complexes are involved in liposome docking at the protein concentrations used in Fig. 2 *B*, with a standard deviation of 11 complexes.

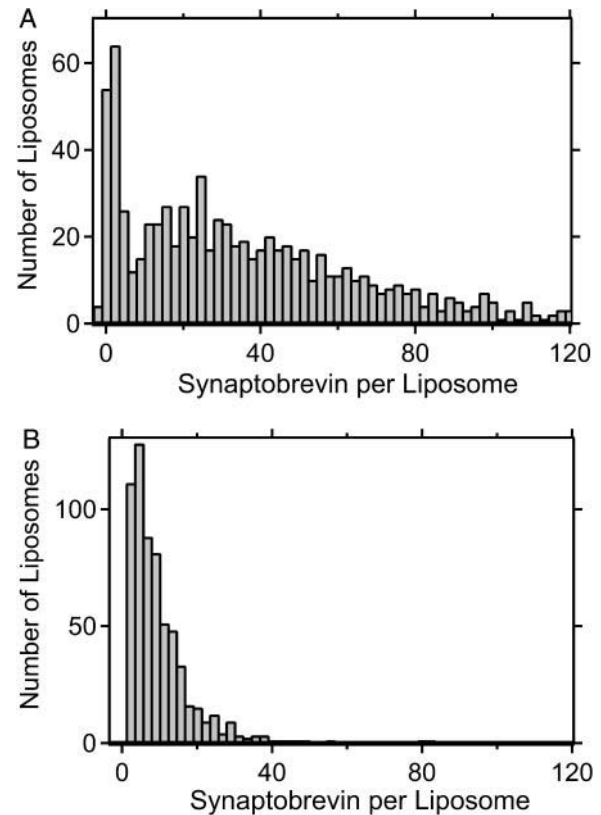
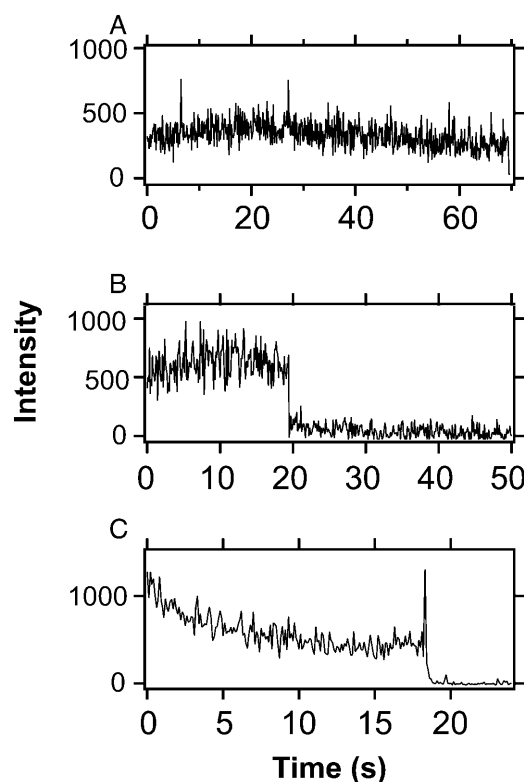


FIGURE 2 The *Botulinum* serotype B light chain can digest excess synaptobrevin from liposomes but does not undock them. (A) Conditions are similar as in Fig. 1 A, but at a concentration of 270 syntaxin per  $\mu\text{m}^2$ , and exposure of the syntaxin•egg PC bilayers with Cy5 labeled Ser<sup>28</sup>Cys synaptobrevin liposomes for 15 min, followed by rinsing with buffer containing 250 nM SNAP-25 for 2 h. The sample was then rinsed with SNAP-25 free buffer and illuminated with blue and red light to observe fluorescence from the content and protein dyes. Shown in A is the intensity distribution (immediately after SNAP-25 is rinsed away) of the synaptobrevin Cy5 dye for the locations with docked liposomes normalized by the independently measured intensity of single Cy5 dyes in this apparatus. The average number of docked liposomes per field of view ( $4050 \mu\text{m}^2$  in the microscope) was  $74 \pm 18$  as measured by content fluorescence as sampled at many locations on the bilayer. (B) BoNT/B protease was then flowed into the chamber at 1 mg/ml in 10 mM BisTris pH 6.8, 100 mM NaCl, and 1 mM DTT for 1 h and rinsed away. The resulting distribution of the intensity of synaptobrevin Cy5 dyes for locations with docked liposomes is shown. After BoNT/B treatment, the density of docked liposomes changed very little ( $74 \pm 18$  per field of view before BoNT/B treatment and  $64 \pm 8$  per field of view after BoNT/B treatment as measured from content spots) but the amount of synaptobrevin present at each docked liposome was significantly reduced. All data were acquired at  $22^\circ\text{C}$  and corrected for the 53% labeling efficiency of synaptobrevin.

### Undocking and bursting of docked liposomes

To assay for fusion and other events, liposomes were loaded with the soluble, self-quenched fluorescent dye calcein and the emission intensity was observed. Four characteristic time-dependent behaviors were observed for synaptobrevin liposomes docked on syntaxin•SNAP-25-supported bilayers (Figs. 3–5): stable docking, undocking, bursting, and fusion.



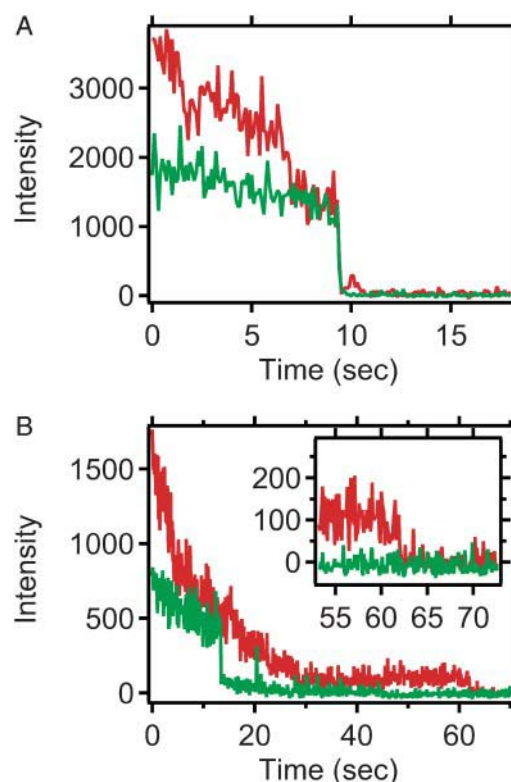


**FIGURE 3** Examples of undocking and bursting events. Supported bilayers of egg PC were prepared with 100 molecules/ $\mu\text{m}^2$  syntaxin (*A* and *B*) or 180 molecules/ $\mu\text{m}^2$  (*C*) and exposed to 250 nM SNAP-25 for 1 h (*A* and *B*) or no SNAP-25 (*C*). Liposomes containing 200 mM calcein were reconstituted with 10–30 Cy5-labeled Ser<sup>28</sup>Cys synaptobrevin molecules. They were then introduced above the bilayers for 80 min (*A* and *B*) or 15 min (*C*) at a concentration of 0.15 mg/ml (lipid) (corresponding to a liposome concentration of 5 nM) and rinsed away. Calcein at 200 mM is highly self-quenched and illumination of docked liposomes loaded with calcein by 488-nm light ( $\frac{1}{2}$  mW/ $0.02$  mm<sup>2</sup>) lead to a number of different behaviors. *A* shows a stable, docked liposome, *B* shows an undocking liposome, and *C* shows a docked liposome that bursts above the bilayer. All data were acquired at 22°C.

Stably docked liposomes show low calcein emission intensity and photobleach very slowly (Fig. 3 *A*).

If a liposome undocks from the bilayer, a simple diffusion calculation suggests that it will diffuse vertically away from the 200-nm-thick illumination layer of the TIR laser beam within 1 ms, much faster than the data acquisition rate of 10 frames per second (based on a diffusion coefficient of  $9 \mu\text{m}^2/\text{s}$  derived from Stokes' drag of a 500-nm diameter liposome in 25°C water). Representative events are shown in Figs. 3 *B* and 4 *A*. The content signal vanishes in a single time step.

Bursting of the liposome above the bilayer is another class of events where the content signal suddenly disappears. Bursting can lead to a pronounced upward spike in the fluorescence intensity (Fig. 3 *C*). Similar spikes are never seen in undocking traces (Figs. 3 *B* and 4 *A*). In movies of bursting events, an expanding dye cloud is visible for several frames due to calcein diffusing in three dimensions, simultaneously



**FIGURE 4** Simultaneous recording of fluorescence emission from liposome content and labeled protein during liposome undocking and bursting events. Supported bilayers of 10% brain PS/90% egg PC were prepared with syntaxin at 100 molecules/ $\mu\text{m}^2$  without SNAP-25. Liposomes containing 200 mM calcein were reconstituted with 10–30 Cy5 labeled Ser<sup>28</sup>Cys synaptobrevin molecules and then introduced above the bilayers at a concentration of 0.3 mg/ml (lipid) (corresponding to a liposome concentration of 10 nM). After 80 min, the bilayers were well rinsed. The data were acquired at 22°C. The bilayer was illuminated with  $\frac{1}{2}$  mW 488-nm light and 5 mW 635-nm light. The green trace is the emission from the calcein dye. The red trace is the emission from the Cy5 synaptobrevin dye. The intensity level for a single Cy5 dye is estimated to be 100–150 (see *B*, inset). The Cy5 dye was photobleaching rapidly because no oxygen scavenger enzymes were used. (*A*) An undocking event where the content and synaptobrevin simultaneously vanish. (*B*) A bursting event where the content signal vanishes but the synaptobrevin molecules remain in the same location. The inset in *B* is a detail of the final Cy5 photobleaching in the full trace presumably corresponding to a single labeled synaptobrevin molecule.

expanding radially away from the release spot and moving vertically out of the illumination layer of the TIR laser.

The interpretation of clouds of calcein from bursting liposomes follows from a simple calculation. When a docked liposome of radius  $R_{\text{initial}}$  bursts or leaks, a spherical expansion model of the dye predicts that the average dye concentration in that volume will decrease from its initial value by a factor of  $(R_{\text{initial}}/R(t))^3$ , where  $R(t)$  is the radius of the expanding sphere from the site of docking as a function of time  $t$ . The presence of the planar-supported bilayer will constrain the dye to move only into a half-sphere and will thus increase this concentration approximately a factor of 2. Analysis of a typical bursting event (see supplementary



movie S3) shows an expanding cloud of dye-emission width-measured radii of 3.2  $\mu\text{m}$ , 8.0  $\mu\text{m}$ , and 11  $\mu\text{m}$  in three consecutive frames at 10 frames/s (data not shown). The diffusion model predicts the concentration of dye at these radii to be 174 nM, 12 nM, and 5 nM, respectively, assuming  $R_{\text{initial}} = 50$  nm and an initial concentration of 200 mM.

Given the sensitivity of the microscope and the emission properties of calcein (described in Materials and Methods), it follows that the expanding cloud of calcein should be visible for a few frames after a bursting event. Furthermore, the signal reaching a pixel of the detector for the expanding cloud is also derived from an  $\sim 100$  times greater volume of dye (175 nm  $\times$  175 nm  $\times$  200 nm using the magnification of the objective, 90  $\mu\text{m}/512$  pixels, and the depth of the TIR illumination field, 200 nm) than it is when the liposome is intact [ $\frac{4\pi}{3}(25 \text{ nm})^3$ ]. This effect suggests that even the lower emission of calcein at the third frame of the expanding cloud (5 nM calcein concentration) can be detected in the microscope.

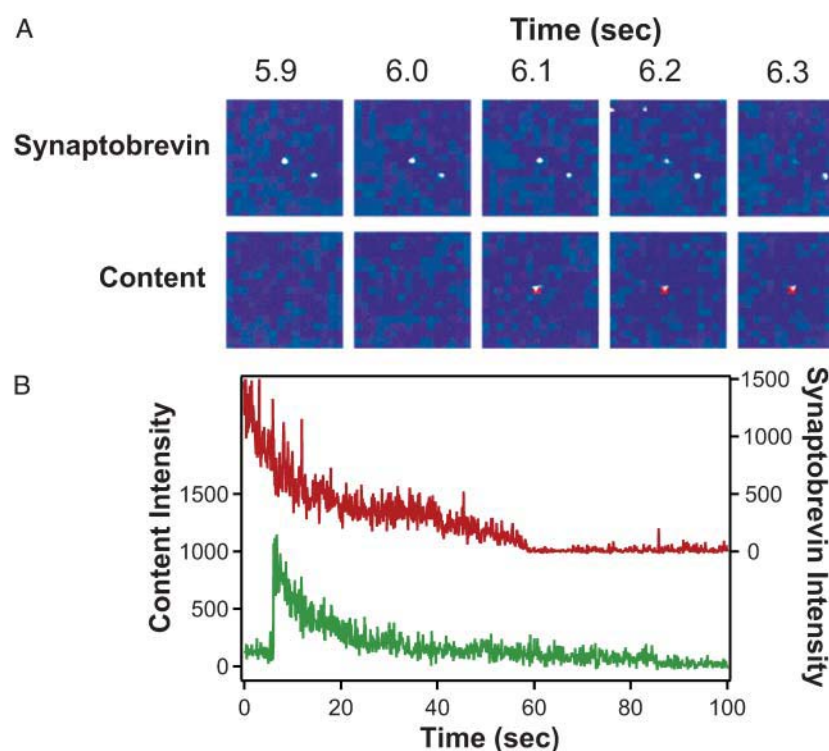
Within 400–500 ms after the bursting event, the signal from the expanding cloud is no longer detectable. Extrapolation from our model predicts that at 400 ms the dye concentration decreases to  $\sim 1$  nM. Our bulk measurements detected no emission at this concentration, and this concentration is probably approaching the minimum level of detection for the TIR microscope. Additionally, we have observed that unquenched calcein photobleaches rapidly during laser illumination in the TIR microscope (data not

shown). The calcein in the illuminated layer will also be photobleaching during this observation period, further decreasing the emission signal.

Further support for our discrimination between undocking and bursting events is seen in the correlation between liposome content emission and labeled synaptobrevin emission (Fig. 4). For undocking events, the disappearance of content fluorescence is typically coincident with the disappearance of any synaptobrevin dye fluorescence and both occur within a single frame (Fig. 4 A). For bursting events, content emission vanishes but emission from labeled synaptobrevin typically remains essentially unchanged, other than the gradual decay caused by photobleaching, indicating a selective loss of content (Fig. 4 B).

### Fusion of docked liposomes

In addition to docking and bursting, events were observed for docked synaptobrevin liposomes that met the criterion for fusion. The liposome content is expected to diffuse into the space below the bilayer upon fusion. The resulting dilution then leads to dequenching of calcein, which should be detectable as a sudden increase in fluorescence intensity. Fig. 5 A shows consecutive frames from a movie of such a fusion event, whereas the corresponding time trace is shown in Fig. 5 B. The content dye emission increased by nearly an order



**FIGURE 5** Simultaneous recording of fluorescence emission from liposome content and labeled protein during liposome fusion events. Supported bilayers of 10% brain PS in 90% egg PC were prepared with 200 syntaxin/ $\mu\text{m}^2$  and exposed to 250 nM SNAP-25 for 1 h. Liposomes containing 200 mM calcein were reconstituted with 10–30 Cy5 labeled Ser<sup>28</sup>Cys synaptobrevin molecules, introduced above the bilayers for 1 h at a concentration of 0.3 mg/ml (lipid), and then were rinsed away. (A) The images represent a single 11  $\mu\text{m} \times 11 \mu\text{m}$  patch of membrane with docked liposomes observed in two different spectral ranges: emission between 515–580 nm (*lower row*) from calcein in liposome content, and emission at wavelengths  $>650$  nm (*upper row*) from Cy5 on synaptobrevin. The times above the frames indicate relative times of extraction from raw data movie (see supplementary movie S4). (B) Time trace for content and protein emission extracted from the same fusion event. Calcein emission shows a rapid increase due to dequenching consistent with liposome fusion, whereas the Cy5 emission shows an exponential decay due to photobleaching. The data were acquired at 22°C.

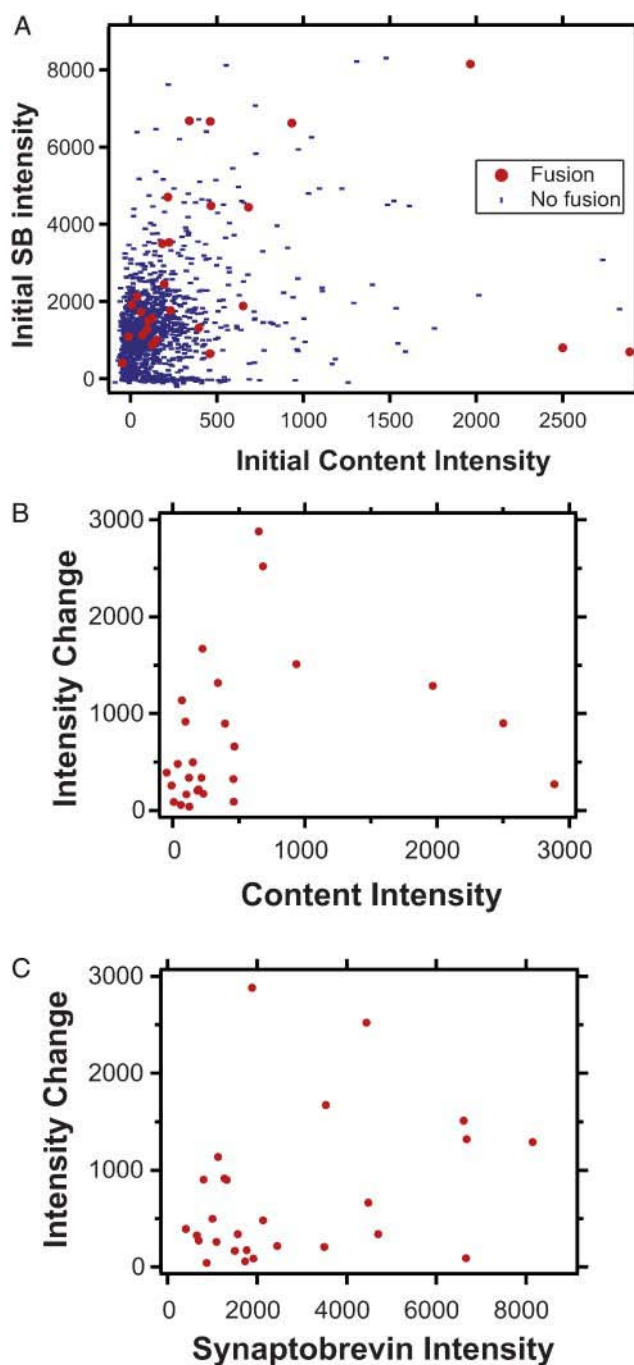
of magnitude within a single frame, whereas the synaptobrevin dye continued to undergo photobleaching.

Studies performed in the absence of a bilayer found that calcein in solution adsorbed strongly to the quartz surface at the buffer conditions of the experiment. Adsorbed calcein showed no FRAP, indicating a lack of diffusion (data not shown). Thus, calcein is not expected to diffuse away from the fusion site. When adsorbed to quartz, calcein photobleaches at a much faster rate than when concentrated inside liposomes (data not shown). The different rates of photobleaching are consistent with the change in photobleaching rate seen after fusion events (Fig. 5 B).

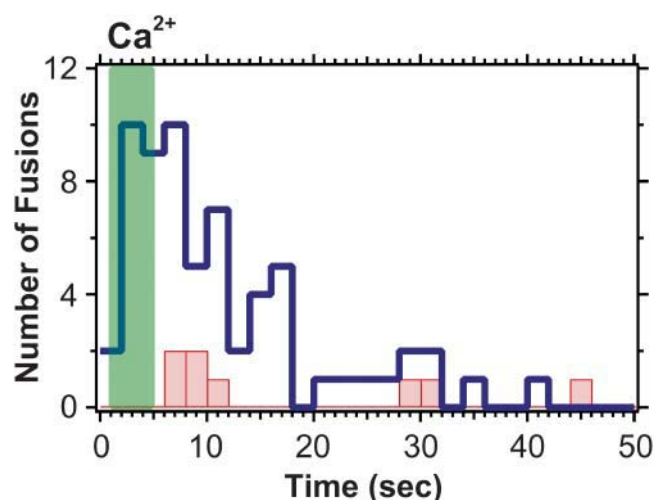
To exclude the possibility that the behavior observed in Fig. 5 could be caused by content leaking and subsequent dequenching, individual 50-nm liposomes were prepared to contain 4 mM calcein adsorbed to quartz, simulating liposomes that suffer >90% leakage from an initial preparation. The dyes in the adsorbed liposomes were found to photobleach in <2 s (data not shown). This behavior is clearly different from the decay observed in Fig. 5 B. The fact that we cannot observe an expanding cloud of dye during a fusion event is further support that these events are fusions and not partial leaking as the TIR microscope has the sensitivity to detect the diffusion of a cloud of leaked dye as discussed above. Additionally, if leakage were involved, one would expect the degree of leakage to be dependent on protein concentration or liposome size (Barry Lentz, personal communication). In contrast, liposomes that gave a fusion signal similar to the one shown in Fig. 5, have a similar initial content intensity distribution (implying the same size distribution) and a similar synaptobrevin concentration to those that do not fuse (Fig. 6 A). Also, no correlation is seen between content intensity or protein concentration and the amplitude of the content intensity increase during fusion (Fig. 6, B and C). Thus, events such as that shown in Fig. 5 are interpreted as fusion events, and cannot be caused by content leaking. This suggests that there are no physiochemical differences in the liposome population that explain the fusion probability of an individual liposome.

### No effect upon $\text{Ca}^{2+}$ influx

In response to  $\text{Ca}^{2+}$  influx, a docked synaptic vesicle exhibits a dramatic increase in fusion probability (Martin, 2003; Redman, 1990; Südhof, 1995). We sought to determine the  $\text{Ca}^{2+}$  sensitivity of fusion between synaptobrevin-containing liposomes docked to syntaxin•SNAP-25-supported bilayers. Specifically, using a rapid buffer exchange system, we introduced buffer containing 2 mM calcium chloride above the bilayer containing 10% PS, while simultaneously observing content fluorescence. In both experiments with and without  $\text{Ca}^{2+}$  ~10–15% of docked liposomes fused. The time distribution of fusion events for three replicate experiments is shown in Fig. 7. No significant



**FIGURE 6** The fusion probability of docked liposomes is independent of their size or synaptobrevin (SB) concentration. Liposomes containing 200 mM calcein were reconstituted with 10–30 Cy5-labeled Ser<sup>28</sup>Cys synaptobrevin, docked to supported bilayers as described in Fig. 8, and observed with ½ mW 488-nm light and 5 mW 635-nm light simultaneously for ~2 min (data at 23°C). The liposomes were divided into two populations: those that fuse and those that do not fuse during the observation period. The value of the initial content intensity and the initial synaptobrevin-Cy5 intensity were measured and plotted in A. The subpopulation of liposomes with a fusion event is shown as large red circles, and that with no fusion event is represented by small blue dots. In B and C the magnitude of the jump in intensity during a fusion event (content intensity postjump minus content intensity prejump) for the subpopulation with fusion was extracted and is plotted against the initial content intensity and the initial synaptobrevin-Cy5 intensity.



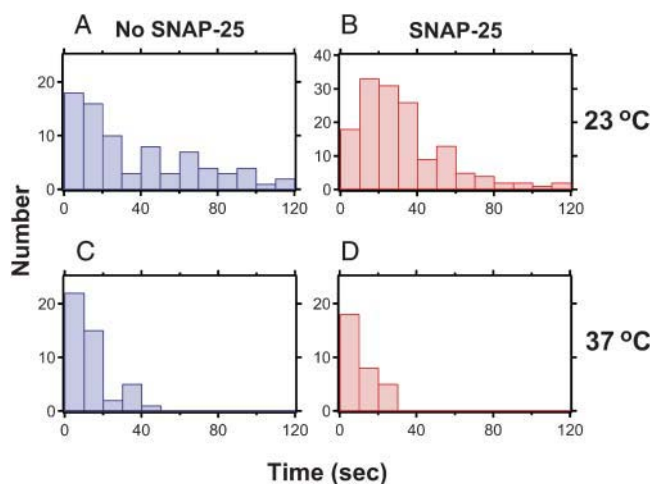
**FIGURE 7** Added  $\text{Ca}^{2+}$  has no effect on the distribution of fusion events over time. Histogram shows the time of the onset of a fusion signal relative to the start of illumination and data recording. Supported bilayers consisting of 10% brain PS in 90% egg PC were prepared with 100 molecules/ $\mu\text{m}^2$  syntaxin and exposed to 250 nM SNAP-25 for 1 h. Liposomes containing 200 mM calcein and reconstituted with 10–30 Cy5-labeled Ser<sup>28</sup>Cys synaptobrevin molecules were then introduced above the bilayers at 22°C for 40 min at a lipid concentration of 0.3 mg/ml (corresponding to a liposome density of 10 nM) and rinsed away. The temperature of the bilayer was then increased to 37°C. Fluorescence emission from the calcein content dye was recorded during excitation with 488-nm laser light. The “skyline” histogram shows fusion events recorded in the absence of  $\text{Ca}^{2+}$ . In three replicate experiments, a total of 75 movies were recorded at different locations of the deposited bilayers before introduction of calcium-containing buffer. A total of 64 fusion events were observed in a population of 640 docked liposomes. The shaded histogram shows fusion events in the presence of 2 mM  $\text{Ca}^{2+}$ . For these movies, after 1 s an automated buffer exchange apparatus began to pump TBS buffer augmented with 2 mM calcium chloride over the membrane (pump active for duration of the green bar). The experimental chamber held  $\sim 25 \mu\text{l}$  of fluid and the pump maintained flow at  $\sim 25 \mu\text{l/s}$  for the subsequent 4 s to insure complete buffer exchange. Three replicate experiments yielded three movies because each bilayer could only be flushed with  $\text{Ca}^{2+}$  once. A total of eight fusion events was observed in a population of 57 docked liposomes. The distributions are similar to the  $\text{Ca}^{2+}$ -free population. No fusion events were observed within 5 s of the onset of  $\text{Ca}^{2+}$  addition, and fusion persisted throughout the observation period.

increase of fusion probability is observed upon  $\text{Ca}^{2+}$  exchange and no fusion events were observed within 5 s of the onset of  $\text{Ca}^{2+}$  exchange, nor were late fusion events eliminated. The difference in magnitude of the histograms with and without  $\text{Ca}^{2+}$  is due to the larger number of observations that were carried out in the absence of  $\text{Ca}^{2+}$ . We conclude that the introduction of 2 mM calcium chloride buffer does not increase the probability of fusion for liposomes docked to the membrane by *trans* SNARE complexes alone.

### Fusion is a thermally activated process

Correlation analyses suggested that there are no physiochemical differences in composition between liposomes that

fuse and those that do not (Fig. 6, A and B). Additionally, fusion of docked liposomes was not affected by  $\text{Ca}^{2+}$ . To determine the factors affecting fusion, the role of laser illumination and temperature were examined. After docking liposomes to the supported bilayer, followed by incubation with SNAP-25 and BoNT/B protease cleavage to eliminate the contribution from uncomplexed synaptobrevin molecules, the sample was illuminated after variable delay times. Histograms in Fig. 8 were compiled from different locations on the same supported bilayer with the zero time corresponding to the initiation of laser illumination of each location. Fusion events were clustered toward the zero time (Fig. 8). Thus, there is a high correlation between the commencement of the laser illumination and fusion events. A plausible explanation of this observation is that the self-quenching of the content dye provides a channel for the conversion of laser illumination into heat inside the docked liposomes (Bialkowski, 1996). The characteristic clustering of fusion events at zero time followed by a slow decay is



**FIGURE 8** Probe laser and ambient temperature stimulate fusion with no requirement for SNAP-25. Supported bilayers of 10% brain PS, 90% egg PC were prepared with syntaxin at 100 molecules/ $\mu\text{m}^2$  and exposed to 250 nM SNAP-25 for 1 h where indicated. Liposomes containing 200 mM calcein were reconstituted with 10–30 Cy5-labeled Ser<sup>28</sup>Cys synaptobrevin molecules and then introduced above the bilayers at a lipid concentration of 0.3 mg/ml (corresponding to liposome concentration of 10 nM). After 80 min, the bilayers were well rinsed. The bilayers were maintained at room temperature throughout the docking process. Separate fields on the same bilayer were viewed with  $\frac{1}{2}$  mW 488-nm light for 2 min. (A) Time distribution, relative to onset of illumination, for fusion events (see Fig. 5 B) occurring at 23°C with a syntaxin bilayer lacking SNAP-25. (B) Time distribution, relative to onset of illumination, for fusion events occurring at 23°C with a syntaxin bilayer preincubated with SNAP-25. (C) Time distribution, relative to onset of illumination, for fusion events at 37°C with a syntaxin bilayer lacking SNAP-25. (D) Time distribution, relative to onset of illumination, for fusion events at 37°C with a syntaxin bilayer preincubated with SNAP-25. The temperature of the slides used for A and B was increased to 37°C and the observations were repeated on unobserved areas of the bilayer to yield histograms C and D. The total fraction of docked liposomes observed to fuse was 15% for the experiment without SNAP-25 at 23°C, and 5% for all other cases.



due to the finite number of docked liposomes that undergo fusion. Increasing the ambient temperature of the sample to 37°C resulted in the fraction of fusion-competent liposomes to fuse more quickly after laser illumination (Fig. 8, *C* and *D*). The rate of spontaneous fusion for docked liposomes during delay periods of 1–3 h without illumination was estimated to be low since the variable delay periods did not significantly affect the observed number of fusing liposomes. Thus, we conclude that the SNARE-dependent liposome fusion we observe is thermally induced. The effect of temperature on fusion was largely kinetic since the percentage of liposomes that undergo fusion was not affected by temperature (Fig. 8). The majority of the docked liposome population did not fuse during the observation period even at higher temperature. This argues against accumulation of photodegradation products as a potential cause of membrane fusion since these should be continuously accruing during the illumination period.

### Thermally induced fusion is SNAP-25-independent, but requires both syntaxin and synaptobrevin in opposing bilayers

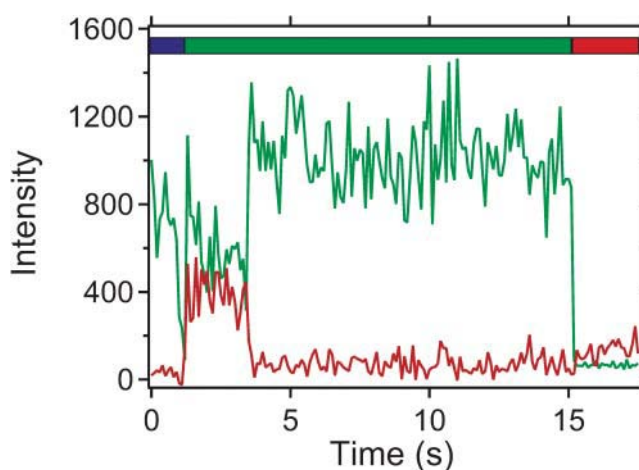
Surprisingly, the thermally induced fusion events did not require SNAP-25 since the distributions were similar for experiments conducted with and without SNAP-25 (Fig. 8, *A* and *B*). Thus, synaptobrevin and syntaxin are sufficient for thermally induced fusion. Vesicle fusion without SNAP-25 has been reported previously using modified synaptic vesicles and black lipid membranes (Woodbury and Rognien, 2000). To establish that a specific SNARE interaction is required for the fusion events that we observe, we carried out a control with only synaptobrevin. Although both synaptobrevin in the liposome and syntaxin in the deposited bilayer are required for efficient docking (Fig. 1 *A*), a few synaptobrevin liposomes can be docked to a synaptobrevin bilayer (*Table inset* in Fig. 1). No fusion events were observed out of 395 instances of synaptobrevin liposomes docked to synaptobrevin bilayers in the presence of SNAP-25, involving multiple view fields. Without SNAP-25, there was one fusion event out of 569 instances. Thus, for liposomes docked by synaptobrevin-synaptobrevin interactions, the fusion rate is <0.1%. This experiment clearly demonstrates that the fusion events observed in Fig. 8 are protein-specific processes that require both syntaxin and synaptobrevin.

### Configuration of SNARE complexes in a membrane environment

The structure of the neuronal SNARE complex revealed a parallel helix bundle (Sutton et al., 1998). However, single molecule studies showed that assembly of SNAREs in solution results in a mixture of both parallel and antiparallel configurations (Weninger et al., 2003). To investigate if such

mixtures exist in the membrane environment of docked liposomes, combinations of labeling sites in syntaxin and synaptobrevin were used that placed fluorescent dyes at the same or opposite ends of the SNARE complex. The configuration of the SNARE complexes involved in docking liposomes to the bilayer was determined using single molecule FRET.

Supported bilayers containing very dilute dye-labeled syntaxin (0.03 per  $\mu\text{m}^2$ ) were incubated with liposomes containing dye-labeled synaptobrevin and rinsed to remove unbound liposomes. In different experiments, SNAP-25 was added to the syntaxin bilayer before liposome addition, after docking of liposomes, or left out all together. The specific donor-acceptor dye combination was also switched between syntaxin and synaptobrevin to control for bias from specific labeling choices. No effects of specific dye or labeling choice were seen.



**FIGURE 9** Single molecule FRET observation of the SNARE complex configuration for a single docked liposome. Cy3-labeled Ser<sup>193</sup>Cys syntaxin and SNAP-25 were associated with the deposited bilayer. Cy5-labeled Ser<sup>28</sup>Cys synaptobrevin was reconstituted in the liposomes. Supported bilayers of egg PC were prepared with labeled syntaxin at low enough surface density that individual molecules could be optically separated (typically 100 per field of view or 4020  $\mu\text{m}^2$ ). Liposomes loaded with 50 mM calcein and reconstituted with 10–30 labeled synaptobrevin molecules were then introduced above the bilayer for 2 h at a concentration of 1 mg/ml (lipid) and rinsed away. SNAP-25 was added at 100 nM for 2 h, and then rinsed away. BoNT/B protease was added at 1 mg/ml for 80 min at room temperature in 10 mM BisTris buffer (pH6.8), 100 mM NaCl, and 1 mM DTT followed by rinsing into TBS oxygen-scavenger buffer. The bar at the top of the graph indicates the laser illumination sequence. For the first 1.1 s, ½ mW 488-nm light was used, which excited the content dye but not the Cy3 or Cy5 dyes. Between 1.1 s and 15.1 s, 8 mW of 532-nm light is used. 532-nm light excites the Cy3 dye directly, the Cy5 dye very little, and calcein to about the same intensity as with the ½ mW of 488-nm light. After 15.1 s, 635-nm light is used to directly excite the Cy5 dye. In this graph, the green trace shows emission in the short wavelength detection path (calcein and Cy3 dye emission are passed) and the red trace shows emission in the long wavelength (emission of the Cy5 dye only; see Materials and Methods). The experiment was carried out at 22°C. Note that the individual liposome is docked by one labeled SNARE complex.

Spots were identified that simultaneously contained quenched content dye, acceptor dye, and donor dye, presumably corresponding to unfused liposomes docked by *trans* SNARE complexes. Fig. 9 shows a representative example of a content-loaded liposome docked by a *trans* SNARE complex involving N-terminally labeled synaptobrevin and N-terminally labeled syntaxin. The bilayer was incubated with unlabeled SNAP-25 to form binary syntaxin•SNAP-25 complexes before docking. The blue laser stage indicated that content emission is present since only calcein and not Cy3 was excited with the blue laser. At the beginning of the green laser stage, the donor channel intensity was consistent with content emission only since calcein was excited to a similar extent by both the blue and green lasers. The measured acceptor emission was consistent with  $FRET = 1$ . Since only content emission was visible in the lower wavelength channel, the donor dye must have been completely quenched by the acceptor. This interpretation was confirmed after  $\sim 3$  s when the acceptor underwent photobleaching and the donor emission recovered. This type of anticorrelated intensity change is a hallmark of single molecule FRET. The photobleaching of the acceptor was confirmed by the lack of acceptor emission during the final red light stage. Interprotein  $FRET = 1$  coincident with quenched liposome content dye was also observed when labeling sites in the C-terminal ends of the syntaxin and synaptobrevin SNARE motifs were used (not shown).

FRET measurements from many individual docked liposomes were acquired to determine the corresponding population distributions for the SNARE complexes involved in docking. When SNAP-25 was included, either before or after liposome docking,  $FRET = 1$  was observed for 23–31% of the colocalized spots (Table 1). These instances correspond to SNARE complexes with syntaxin and synaptobrevin in the parallel configuration as observed in the crystal structure of the core complex (Sutton et al., 1998). Fusion events were also observed under these conditions, although the results discussed above (Fig. 7) made use of a higher syntaxin concentration.

The parallel reporting labeling sites produce  $FRET = 0$  for an antiparallel complex, so to confirm the existence of SNARE complexes in the antiparallel configuration, an N-terminal labeling site in synaptobrevin was used with a C-terminal site in syntaxin. In liposomes docked by these SNAREs only 4–5% of colocalized spots showed  $FRET = 1$  consistent with an antiparallel configuration (Table 1). The fact that the size of the high FRET population observed when using two N-terminal labeling sites is approximately fivefold greater than is seen when using a combination of an N-terminal with a C-terminal label indicates that liposome docking to a supported bilayer favor the assembly of SNAREs into the parallel configuration compared to our previous studies of assembly in solution where the majority of complexes were found in the antiparallel configuration. Using labeling pairs that identify the parallel and antiparallel

configurations of the SNARE complex we identified 23–31% as parallel and 4–5% as antiparallel, respectively. How can the remaining 64–73% colocalized instances with  $FRET = 0$  be explained? There is a certain population of syntaxin-synaptobrevin interactions with  $FRET = 0$  that may occur in the absence of a SNAP-25 molecule (see next section) since SNAP-25 is not always incorporated when syntaxin and synaptobrevin interact. Other possibilities of colocalized instances with  $FRET = 0$  are caused by incomplete labeling of synaptobrevin or syntaxin (see Materials and Methods).

### Conformation of the syntaxin-synaptobrevin binary complex

In the absence of SNAP-25,  $FRET = 1$  was observed between the N-terminal ends of synaptobrevin and syntaxin for 4–5% of colocalized spots (Table 1). In addition, we noticed a 1–3% population of intermediate FRET instances (not shown). No such instances were seen in any of the other experiments that include SNAP-25. These intermediate FRET instances suggest the existence of partially folded syntaxin-synaptobrevin complexes in the absence of SNAP-25, consistent with the reduced stability of this binary interaction compared to the ternary complex. However, our results also indicate the existence of syntaxin-synaptobrevin interactions with  $FRET = 0$  that are sufficient to promote liposome docking since the liposomes stay docked in the absence of SNAP-25. In these experiments, the syntaxin concentration in the deposited bilayer is so low that we expect at most two syntaxin molecules per docked liposome. These instances could involve an antiparallel syntaxin-synaptobrevin configuration or they could involve a partially folded syntaxin-synaptobrevin complex where the N-terminal parts of the core SNARE domains are sufficiently far apart to result in  $FRET = 0$ .

### DISCUSSION

SNARE proteins play a fundamental role in vesicle trafficking between cellular compartments and the plasma membrane (Chen and Scheller, 2001; Pelham, 2001). The key roles commonly attributed to SNAREs are: lending specificity for vesicle trafficking, and mediating the membrane fusion process (Ferro-Novick and Jahn, 1994; Rothman, 1994). Previous in vitro liposome fusion assays, performed in bulk, have demonstrated that SNAREs are sufficient to catalyze membrane fusion (Fix et al., 2004; Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000; Schuette et al., 2004). However, these experiments did not reveal the underlying molecular mechanism of SNARE-mediated membrane fusion (Duman and Forte, 2003; Ungar and Hughson, 2003). Permeabilized PC-12 cell experiments suggested that full SNARE complex assembly occurs downstream of docking, and strongly links SNAREs to

fusion (Chen et al., 1999). In contrast, it has been proposed that *trans* SNARE assembly is necessary for docking but not for fusion for yeast vacuolar fusion (Ungermann et al., 1998). Although the molecular roles that SNAREs play for synaptic vesicle fusion and homotypic yeast vacuole fusion could be quite different, these potentially conflicting results reveal the uncertainty about the role of SNAREs.

We have developed a single molecule fluorescence assay to investigate the role of SNARE proteins in membrane fusion reactions that should overcome many of these limitations. Our assay combines sufficient sensitivity to determine the structural details of SNARE complex assembly with the ability to diagnose the dynamics of membrane fusion. The single molecule approach avoids the difficulties of synchronizing membrane fusion events in bulk experiments. Single molecule observations also allow one to resolve subpopulations of the different configurations that occur during unregulated SNARE complex assembly as liposomes spontaneously dock to membranes through SNARE-mediated interactions.

We used purified lipids and recombinant proteins with site-specific labeling sites for FRET studies. Liposomes reconstituted with SNAREs were incubated above a planar lipid bilayer, reconstituted with complementary SNAREs that had been deposited on a quartz surface. Using single molecule fluorescence detection and three-color, TIR illumination of the deposited bilayer, SNARE complex configurations were monitored while simultaneously recording dynamic information about the degree of content containment and release for an individual liposome.

Synaptobrevin liposomes docked to a syntaxin•SNAP-25 bilayer were not released by proteolysis with the synaptobrevin-specific BoNT/B protease. By counting the number of synaptobrevin molecules after BoNT/B proteolysis, we found that 1–2 SNARE interactions are sufficient for docking (Fig. 2 B and Table 1). Using a self-quenched content dye, we directly observed thermally induced SNARE-dependent liposome fusion. The observed fusion events occurred on the second timescale, a trait similar to the physiological fusion rate in unstimulated neurons (Südhof, 2000). Docked liposomes could be stimulated to fuse by the illumination with laser light, and raising the temperature further enhanced this effect. The percentage of liposomes that underwent fusion did not correlate with protein content or initial content intensity (Fig. 6). This suggests that the fusion competence of the liposomes arises from protein conformational states rather than physical or chemical properties of the liposomes.

Using a rapid perfusion system to introduce  $\text{Ca}^{2+}$ -containing buffers while performing simultaneous monitoring of SNARE complex configuration and liposome content, we found that the minimal system of neuronal SNAREs did not provide the  $\text{Ca}^{2+}$  dependence associated with neurotransmitter release in response to action potentials in neurons. This differs from a recent report using TIR of deposited bilayers that found fusion catalyzed by the neuronal

SNAREs alone was sensitive to both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Fix et al., 2004). Our experimental system differs from this report in too many ways to readily explain this discrepancy. However, our findings are in agreement with the observation of the numerous cofactors that are necessary to impart  $\text{Ca}^{2+}$  sensitivity (Bennett, 1999; Rettig and Neher, 2002).

Previous *in vitro* fusion studies have relied on reconstitution of a preformed syntaxin•SNAP-25 binary complex (Fix et al., 2004; Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000), so the role of SNAP-25 in docking and fusion was unknown. Surprisingly, we found that SNAP-25 had little effect on the efficiency of docking of synaptobrevin liposomes to a syntaxin bilayer, or on the rates of thermally induced fusion. Although surprising, fusion without SNAP-25 has been reported previously (Woodbury and Rognien, 2000). Studies of SNAP-25 knockout mice showed that vesicle docking and stimulus-independent fusion persisted although  $\text{Ca}^{2+}$ -triggered release was abolished (Washbourne et al., 2002). In a followup study, it was shown that overexpression of a SNAP-25 homolog did rescue calcium-dependent fusion (Sorensen et al., 2003). Thus, it is very unlikely that the residual stimulus-independent fusion observed in the SNAP-25 knockouts could utilize a SNAP-25 homolog without also displaying stimulus-dependent release.

We interpret these results to mean that the interaction between syntaxin and synaptobrevin (Calakos et al., 1994; Fasshauer et al., 1998) is sufficient to promote docking and thermally induced fusion. Additionally, the synaptobrevin-syntaxin binary complex does not have to be in a conformation that produces  $\text{FRET} = 1$ , corresponding to the crystal structure of the synaptic core complex. Although there is a small population of syntaxin-synaptobrevin complexes with  $\text{FRET} = 1$  in the absence of SNAP-25 (Table 1), the large majority of these binary complexes shows no FRET. These instances could involve an antiparallel configuration or a partially folded complex. Note that we observed a small population of intermediate FRET instances in the absence of SNAP-25, suggesting the existence of SNARE conformations in the syntaxin-synaptobrevin binary complex that are significantly different from those found in the ternary complex. Another possibility might be a putative interaction between the closed form of syntaxin (Munson et al., 2000) and synaptobrevin in the membrane proximal part of the complex that would leave the N-terminal, labeled end of synaptobrevin unstructured. Our observations also show that 18–23% of syntaxin-synaptobrevin complexes with  $\text{FRET} = 0$  can be converted to  $\text{FRET} = 1$  by adding SNAP-25 after docking indicating some structural plasticity (Table 1). This result also suggests that SNARE complexes can still form after a vesicle is stably docked.

We conclude that the binary syntaxin-synaptobrevin interaction is both necessary and sufficient for liposome docking and thermally induced fusion. Our observations suggest the existence of a variety of new conformational states and configurations of SNAREs in addition to those



observed in the crystal structure of the ternary core complex (Sutton et al., 1998). Clearly, the role of these states and configurations remains to be established in the physiological environment of the neuron.

## SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at <http://www.biophysj.org>.

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