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SNAREpins: Minimal Machinery for Membrane Fusion

Thomas Weber, Boris V. Zemelman,‡
James A. McNew,‡ Benedikt Westermann,†‡
Michael Gmachl, Francesco Parlati,
Thomas H. Söllner, and James E. Rothman*
Cellular Biochemistry and Biophysics Program
Memorial Sloan-Kettering Cancer Center
1275 York Avenue
New York, New York 10021

Summary

Recombinant v- and t-SNARE proteins reconstituted into separate lipid bilayer vesicles assemble into SNAREpins—SNARE complexes linking two membranes. This leads to spontaneous fusion of the docked membranes at physiological temperature. Docked unfused intermediates can accumulate at lower temperatures and can fuse when brought to physiological temperature. A supply of unassembled v- and t-SNAREs is needed for these intermediates to form, but not for the fusion that follows. These data imply that SNAREpins are the minimal machinery for cellular membrane fusion.

Introduction

What is the minimal protein machinery for the fusion of cellular membranes? To propagate and maintain compartmental organization, a machinery that pairs specific membranes must be coupled to a machinery that fuses bilayers. The simplest possibility is that the two machineries are one and the same.

Considerable evidence now implies that SNARE proteins (Söllner et al., 1993b), a family of compartmentally specific and cytoplasmically oriented integral membrane proteins, provide a core mechanism that specifically pairs membranes. This "SNARE hypothesis" was originally based upon the discovery of a stoichiometric complex (Söllner et al., 1993b) consisting of a v-SNARE and a t-SNARE, together with the general cytosolic fusion proteins SNAP (Clary et al., 1990) and NSF (Block et al., 1988). It was soon established that the v- and t-SNAREs form their own stable complex even in the absence of SNAP and NSF (Söllner et al., 1993a). Many v- and t-SNAREs have now been characterized in yeast, plants, and animals (Rothman, 1994; Bennett, 1995; Linial, 1997). Family members are selectively localized to cellular compartments such as ER and nuclear envelopes, Golgi, endosomes, lysosomes, secretory storage vesicles, and apical and basolateral plasma membranes, and are required for fusion events that involve the compartments in which they are localized (Pelham et al., 1995; Rothman and Wieland, 1996; Bock et al., 1997; Lewis et al., 1997). The v- and t-SNAREs bind each other in a pairwise, cognate fashion (Bennett et al., 1993; Protopopov et al., 1993; Pevsner et al., 1994; Søgaard et al., 1994) and must reside in opposite membranes for fusion to occur (Nichols et al., 1997).

Whereas free cognate SNAREs in solution spontaneously assemble into stable complexes (i.e., assembly is favored energetically), the assembly of v-t-SNARE complexes in cells appears to be under kinetic control by regulatory proteins (Rothman and Söllner, 1997). Among these are various members of the Rab GTPase family, the Sec1 family, fibrous "string" proteins (Orci et al., 1998) such as p115 and GM130 (Nakamura et al., 1997), and exocyst complexes (TerBush et al., 1996; Kee et al., 1997). By modulating the rate of SNARE complex assembly at local sites either directly or indirectly, by concentrating vesicles locally, regulatory proteins can potentially add pairing specificity to compartments or even create regional domains within compartments.

Despite their prevalence, there are clear indications that such regulatory pathways and pre-SNARE docking steps may not be essential. Deletion of a regulatory gene can be compensated by alterations of related regulatory genes (without transport bypassing the compartments that the gene products normally regulate). This has been shown to occur at the ER–Golgi step. Overexpressing local v-SNAREs (Dascher et al., 1991) or a mutation of a Sec1 family regulatory protein (Lupashin and Waters, 1997) bypasses deletion of an otherwise essential *Rab* gene. Along the same lines, expression of a single hybrid protein of the Rab family bypasses the need for two distinct Rab proteins along the entire secretory pathway (Brennwald and Novick, 1993; Dunn et al., 1993).

Altogether, the evidence suggests that the core specificity of transport (accounting for correct vesicle targeting to the principal cellular compartments), as distinct from the regulation of either the rate of transport or of localized delivery at an acceptor compartment, does not rely on these regulatory proteins. By contrast, the same evidence implies that cognate v- and t-SNARE proteins are necessary as core transport machinery, and by elimination, are most likely sufficient as the minimal recognition machinery for pairing specific membranes as partners for subsequent fusion.

Could the SNARE proteins also be the minimal machinery that fuses paired lipid bilayers? It is known that SNARE proteins must be intact during the last few milliseconds prior to the completion of fusion, as judged from the persistent sensitivity of calcium-dependent exocytosis to proteolytic cleavage of SNARE proteins by neurotoxins following ATP-dependent priming (Bruns and Jahn, 1995; Parsons et al., 1995; Banerjee et al., 1996a, 1996b). The simplest interpretation of this is that SNAREs are, at the very least, a part of the minimal machinery for lipid bilayer fusion. But if they are necessary for specific pairing and for subsequent bilayer fusion, are they also sufficient?

Together with SNAREs, SNAPs and the ATPase NSF (or homologs) are required quite generally in intracellular membrane fusion. NSF and SNAPs dissociate the otherwise stable complexes of v- and t-SNAREs, utilizing

*To whom correspondence should be addressed.
†Present address: Institute for Physiological Chemistry, Ludwig-Maximilians University Munich, Goethestrasse 33, 80336 Munich, Germany.
‡These authors contributed equally to this work

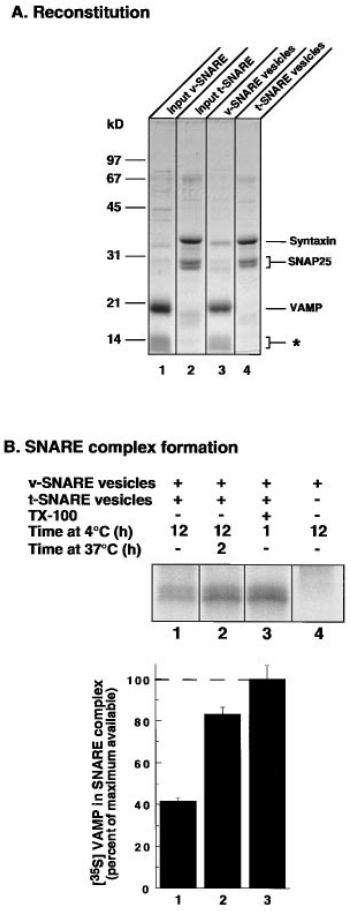

Figure 1. Functional Reconstitution of SNAREs into Vesicles

(A) Purified SNARE proteins can be reconstituted into vesicles. The purified v-SNARE VAMP (lane 1) and the t-SNARE complex, consisting of syntaxin 1 and SNAP25 (lane 2), were separated by SDS-PAGE and stained with Coomassie blue-R250. VAMP and t-SNARE complex that copurified with vesicles after reconstitution are shown in lanes 3 and 4, respectively. The lower band of the SNAP25 doublet is a C-terminal degradation product. Bands labeled by the asterisk are N-terminal degradation products of VAMP. Removal of these degradation products by Mono-S Sepharose chromatography prior to reconstitution (see Experimental Procedures) did not affect experimental results. The band in lane 3, migrating at a position close to that of syntaxin in lane 4, is a dimer of VAMP that becomes apparent after reconstitution.

(B) Reconstituted SNAREs form complexes with their cognate SNARE partner. Fluorescent donor vesicles containing [^{35}S]-labeled VAMP were incubated in the presence or absence of a 3-fold molar excess of t-SNAREs in vesicles. Botulinum neurotoxin D (BoNT D) was used to monitor SNARE complex formation as described in the Experimental Procedures. Fusion of these vesicles, monitored in parallel (not shown) using the lipid mixing assay, was indistinguishable from reactions of vesicles with nonradioactive VAMP. Following BoNT D digestion, the samples were subjected to SDS-PAGE electrophoresis and analyzed by autoradiography (above). Quantitative analysis by phosphorimaging represents the average of two independent experiments (below). (Lane 1) v-SNARE and t-SNARE vesicles incubated for 12 hr at 4°C before toxin treatment led to SNARE complex formation but did not result in fusion (see Figure 2A, zero time point). (Lane 2) Incubation for 2 hr at 37°C following 12 hr at 4°C increased the extent of [^{35}S]-labeled VAMP complexed to t-SNAREs and resulted in fusion (see Figure 2A, 2 hr). (Lane 3) Triton X-100 was used to dissolve a mixture of [^{35}S]-labeled VAMP donor vesicles and t-SNARE acceptor vesicles to determine the maximum fraction of [^{35}S]-labeled VAMP that could partner with excess t-SNAREs under the conditions used (30 ± 2% of input). The data shown in the bar graph are percentages relative to this maximum. (Lane 4) Toxin digestion of free VAMP was complete, since no [^{35}S]-labeled VAMP could be detected when donor vesicles were digested with botulinum neurotoxin D in the absence of t-SNARE vesicles. This resulting trace background was subtracted from every sample before normalization to lane 3.

energy provided by the hydrolysis of ATP (Söllner et al., 1993a; Hayashi et al., 1995). In at least some cell-free systems that reconstitute only one round of fusion, SNAPs and NSF are not essential for fusion after SNARE complexes pair between membranes; rather, their core role in these cases appears to be to separate v- from t-SNAREs after a round of fusion to allow the next round to take place (Mayer et al., 1996; Nichols et al., 1997; Ungermann et al., 1998). In light of this, NSF and SNAPs do not seem likely to be part of the minimal machinery for the process of membrane pairing and lipid bilayer fusion, though they are core machinery that is needed for reuse of SNAREs in repeated rounds of fusion.

This leaves as the simplest hypothesis the possibility that the SNARE proteins are both necessary and sufficient for membrane pairing and for lipid bilayer fusion, therefore comprising in and of themselves the minimal machinery for both steps (and thus the overall process of membrane fusion). By its very nature, this proposition cannot be tested in cells, or even in cell-free systems using native membranes, but only with synthetic lipid bilayer vesicles incorporating only v- and t-SNAREs.

Results

Reconstitution of SNARE Proteins

Recombinant SNARE proteins were expressed in *Escherichia coli* and purified as described in the Experimental Procedures. The v- and t-SNARE proteins known to be required for exocytosis at synapses, the synaptic vesicle membrane protein VAMP2 (also termed synaptobrevin2; Trimble et al., 1988; Baumert et al., 1989), and the plasma membrane proteins syntaxin1A (Bennett et al., 1992) and SNAP25 (Oyler et al., 1989) were selected for this study because they are the best characterized SNARE proteins at a physical chemical level. Syntaxin 1A and SNAP25 (synaptosome-associated protein of 25 kDa), which serve as a t-SNARE when complexed with each other, were coexpressed in the same bacterial cells; the syntaxin/SNAP25 complexes were purified by taking advantage of glutathione S-transferase (GST) attached to the amino terminus of SNAP25. These t-SNAREs were released from glutathione beads by cleavage with thrombin. VAMP2 was tagged with a His_6 tag at its C terminus and purified by Ni-NTA agarose affinity chromatography, and served as the v-SNARE. The full-length SNAREs were obtained in a nonionic detergent solution and analyzed by SDS-PAGE for purity (Figure 1A, lanes 1 and 2).

To reconstitute the v- or t-SNAREs into vesicles, phospholipids were mixed with protein in the presence of the detergent octyl-β-D-glucopyranoside. After rapid dilution below the critical micellar concentration of the detergent followed by dialysis, the resulting vesicles

were isolated by flotation in a density gradient. The recovery of phospholipids (monitored with trace amounts of radio-labeled phospholipid) varied from 50% to 80%. Of the added proteins, about 40% of the VAMP typically comigrated with the assembled lipid vesicles (Figure 1A, lane 3), while only about 10% of the syntaxin/SNAP25 complex was typically incorporated into vesicles (Figure 1A, lane 4). The ratios of protein to lipid and detergent to phospholipid in reconstitutions were typically 0.5 and 3.0 (w/w), respectively. The phospholipids for both v- and t-SNARE vesicles consisted of a mixture of phosphatidylserine (PS) (between 15 and 25 mol%, depending on the experiment), the balance made up with phosphatidylcholine (PC).

The majority of the v- and t-SNAREs reconstituted with their cytoplasmic domains on the outside of vesicles, as determined by the accessibility of 70%–80% of VAMP, syntaxin, or SNAP25 to digestion by proteases (data not shown) and, more qualitatively, by the ability of membrane-bound v- or t-SNARE to bind the soluble cytoplasmic domains of its cognate t- or v-SNARE (data not shown). All of the VAMP (in v-SNARE vesicles) and syntaxin and SNAP25 (in t-SNARE vesicles) resisted extraction with sodium carbonate (pH 11.5), as expected for integral membrane proteins that are properly inserted into a lipid bilayer (data not shown).

Electron microscopy using negative staining confirmed that vesicles, ranging from about 35–100 nm diameter, had been reconstituted, with a mean diameter and standard deviation of 45 $\pm$ 15 nm, with no significant size difference between v-SNARE and t-SNARE vesicles. This is in the same size range as synaptic vesicles and many transport vesicles. The molar ratio of lipid to protein was typically about 20 for v-SNARE and 300 for t-SNARE–containing vesicles as determined from direct measurement using tracer radiolabeled phospholipids and an amido black protein assay (Schaffner and Weissmann, 1973). From these findings (assuming 65 Å^2/phospholipid), we estimate that the average v-SNARE vesicle of 45 nm contains about 750 copies of VAMP, while each t-SNARE vesicle of similar size contains about 75 copies of an equimolar complex of syntaxin and SNAP25. For comparison, a native synaptic vesicle has between 20 and 150 copies of VAMP, as estimated from data reported in the literature (Jahn and Südhof, 1994; Walch-Solimena et al., 1995).

Synaptic v- and t-SNAREs rapidly and efficiently assemble into a stable SNARE complex when they (or their cytoplasmic domains) are mixed in detergent solution (Söllner et al., 1993a; Chapman et al., 1994; Hayashi et al., 1994; Fasshauer et al., 1997a). To test whether the reconstitution into lipid bilayer vesicles had been successful, we incubated v-SNARE vesicles together with an excess of t-SNAREs (in separate vesicles) at 4°C and determined the amount of the v-SNARE VAMP that was rendered resistant to cleavage by botulinum toxin D, a hallmark of SNARE complex formation (Hayashi et al., 1994). A significant fraction of the available VAMP became resistant to botulinum toxin D treatment (Figure 1B, lane 1) after incubation at 4°C, and an even larger fraction after incubation at 37°C (Figure 1B, lane 2). Toxin treatment was complete under the conditions employed, as shown by the fact that when v-SNARE vesicles were treated with toxin in the absence of t-SNARE vesicles, all of the VAMP was cleaved by the toxin (Figure 1B, lane 4).

Successful reconstitution of t-SNARE vesicles required the use of a preformed complex of syntaxin and SNAP25, produced by coexpression in the same bacterial cells. While syntaxin alone was incorporated into vesicles, as judged by flotation with phospholipid, this syntaxin did not bind SNAP25, and much of the syntaxin could be extracted with carbonate. Even when SNAP25 and syntaxin (expressed separately) were mixed in octyl-β-D-glucopyranoside solution before reconstitution, SNAP25 was not recovered to any significant extent with the syntaxin in the vesicle fraction after flotation (not shown).

Fusion Mediated by Cognate SNAREs Measured by Lipid Mixing

To test whether SNARE-containing vesicles could, in and of themselves, fuse their membranes, we employed a well-characterized lipid mixing assay (Struck et al., 1981). The v-SNARE vesicles contained a quenched mixture of fluorescent phospholipids, NBD-PE (N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-phosphatidylethanolamine) and rhodamine-PE (N-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine), each included as 1.5%–2% of the total phospholipid during reconstitution of the v-SNARE vesicles. When these fluorescent "donor" vesicles fuse with other nonfluorescent "acceptor" vesicles, the concentrations of both NBD-PE and rhodamine-PE in the combined bilayer are reduced. Fusion of a donor and an acceptor vesicle results in dilution of the fluorescent lipid in bulk phospholipid by a factor of 2. This, in turn, results in a decrease in quenching and a consequent increase in NBD fluorescence at 538 nm.

In a typical reaction, donor v-SNARE vesicles (final concentration of 5 μM of v-SNAREs) were mixed with a 15-fold excess (on a lipid basis) of acceptor t-SNARE vesicles (final concentration of 8 μM of t-SNAREs), corresponding to about 1.5 t-SNAREs per v-SNARE. A progressive increase in NBD fluorescence occurred over the course of about 2 hr at 37°C (Figure 2A). This parallels the increased formation of SNARE complexes observed during this time period (Figure 1B, compare lanes 1 and 2). To provide an internal standard for normalized comparison of different fusion reactions, and for gauging absolute efficiency, detergent (Triton X-100) was added at 2 hr to dissolve the vesicles and thereby maximally dequench the fluorescence of NBD-PE (Figure 2A, following the 2 hr time point).

Fusion was temperature-dependent. Little fusion occurred even after 15 hr at 4°C (zero time points in Figure 2A). However, as a result of a preincubation of v-SNARE with t-SNARE vesicles for the same time at 4°C, the initial rate of subsequent fusion upon warming to 37°C was notably increased as compared to when vesicles were simply mixed and immediately incubated at 37°C (Figure 2A, compare open and closed circles).

Because of this kinetic advantage, all subsequent experiments (unless stated otherwise) employed the 4°C preincubation period. The raw data of a typical experiment is shown in Figure 2A. In later figures, the data are

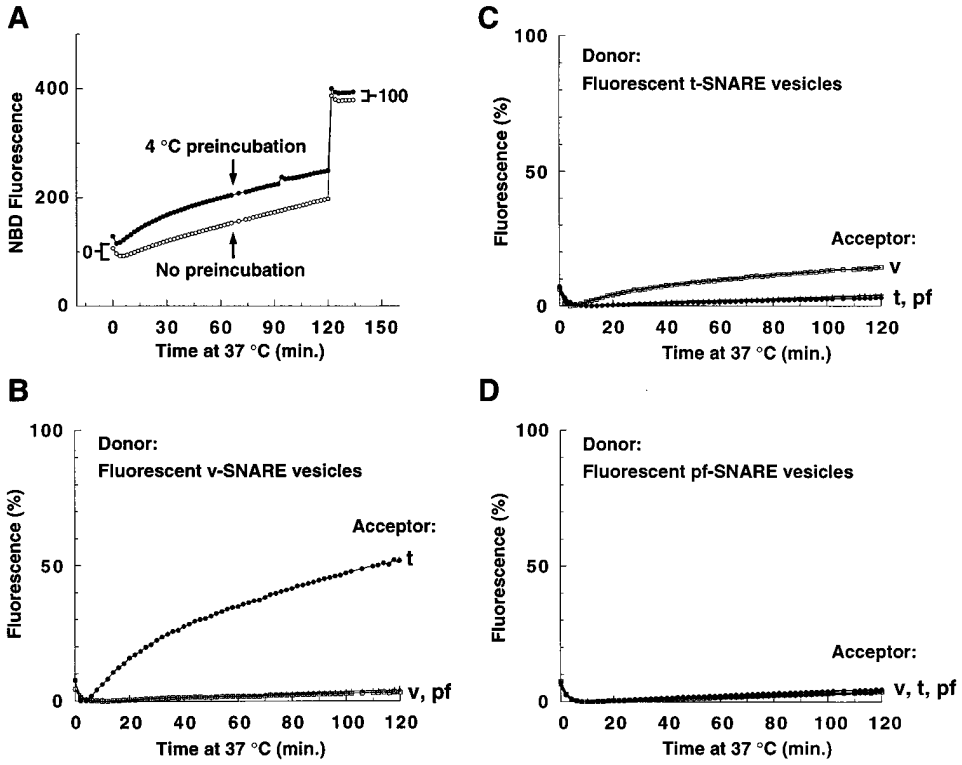

Figure 2. Fusion between Vesicles Containing v- and t-SNAREs

(A) Donor vesicles containing the v-SNARE VAMP were mixed with an excess (15-fold on a lipid basis) of acceptor vesicles containing a 1.5-fold molar excess of the t-SNAREs complex of syntaxin 1 and SNAP25, and NBD fluorescence was monitored at 37°C as described in the Experimental Procedures. One sample (closed circles) had been preincubated at 4°C for 15 hr prior to the temperature shift to 37°C whereas the other (open circles) was not preincubated. Note that preincubation resulted in only a small increase in NBD fluorescence (zero time point), indicating that essentially no fusion had occured after 15 hr at 4°C. After 2 hr at 37°C, Triton X-100 was added to mix the lipids completely and dequench the fluorescence of NBD. To normalize the experiments, in (B)–(D) and in all other figures (unless otherwise stated), the lowest NBD fluorescence signals were set to 0%, and the maximal signals reached after detergent addition were set to 100% fluorescence (as marked).

(B) Donor vesicles containing the v-SNARE VAMP were preincubated with acceptor vesicles containing either no protein (pf, protein free, crosses), the t-SNARE complex of syntaxin 1 and SNAP25, (t, closed circles), or the v-SNARE VAMP (v, open squares) at 4°C for 15 hr. After shifting the temperature to 37°C, NBD fluorescence was monitored as described in the Experimental Procedures.

(C) Donor vesicles containing t-SNAREs were incubated with different acceptor vesicles containing either v-SNAREs (open squares), or t-SNARE complexes (closed circles), or no proteins (crosses) as described in the Experimental Procedures.

(D) Protein-free donor vesicles were incubated with different acceptor vesicles (symbols as in [B] and [C]) as described in the Experimental Procedures.

normalized to a standard baseline, obtained by setting to zero the lowest level of NBD fluorescence following the start of the 37°C incubation (to account for the slight decline in intrinsic fluorescence that invariably occurs as a result of the temperature shift [Chapman et al., 1995]) and by setting to 100% the NBD fluorescence that results after Triton X-100 is added after 2 hr at 37°C (as outlined in Figure 2A).

While v-SNARE vesicles readily fuse with t-SNARE vesicles, they do not fuse with other v-SNARE-containing vesicles or with protein-free phospholipid vesicles (Figure 2B). For these experiments, the fluorescent donor vesicles contained v-SNAREs while the nonfluorescent acceptor vesicles contained either t-SNAREs, v-SNAREs, or were free of protein. When the reciprocal experiment is performed, such that the fluorescent donor vesicles contain t-SNAREs and the nonfluorescent acceptor vesicles contain v-SNAREs, fusion is also observed, but not when the acceptor vesicles contain t-SNAREs or are free of protein (Figure 2C). Protein-free donor vesicles do not fuse with any of the potential acceptor vesicles (Figure 2D).

The less extensive increase in fluorescence that occurs when t-SNARE vesicles (Figure 2C) rather than v-SNARE vesicles (Figure 2B) are used as the donor is readily explained by the 10-fold larger number of v- than t-SNAREs per vesicle. When a donor t-SNARE vesicle fuses with an acceptor v-SNARE vesicle, the product will have no free t-SNAREs; all its t-SNARES will be complexed by excess v-SNAREs. Consequently, no further fusion with v-SNARE-containing acceptor vesicles can take place, so no further increase in fluorescence can occur. However, fusion with the donor t-SNARE population can now occur via the free v-SNAREs; such fusion events will consume donor vesicles with little or no dequenching, further limiting the assay signal.

Fusion experiments were conducted in a buffer consisting of 25 mM HEPES (pH 7.4), 100 mM KCl, 10%

(w/v) glycerol, and 1 mM dithiothreitol. The ionic strength is not critical, since fusion also occurs up to a concentration of at least 0.6 M KCl. Furthermore, fusion occurs equally well in the presence of 1 mM EDTA or 10 mM magnesium ion. Proteolytic N-terminal degradation products of VAMP, present as minor contaminants in routine preparations (Figure 1A), could be removed by ion exchange chromatography (Mono S column) without affecting the fusion reaction (described in the Experimental Procedures).

Complete Mixing of Lipid Bilayers

While lipid mixing is a necessary event in membrane fusion, documentation of lipid mixing between vesicle populations does not formally establish a complete fusion event involving both monolayers. Hemifusion, involving only the outer leaflets of the vesicle bilayers, could also result in the increase in NBD fluorescence in Figure 2. Evidence for hemifusion has been reported in certain instances with mutant forms of viral fusion proteins (Kemble et al., 1994; Bagai and Lamb, 1996). To determine whether a complete fusion reaction involving the inner and outer leaflet of vesicles takes place, we eliminated NBD fluorescence on the outer leaflets after fusion and examined whether NBD lipids on the inner leaflets were also dequenched as a result of fusion and to what extent.

Dithionite was used to eliminate NBD fluorescence from the outer leaflets selectively. This compound reduces NBD to nonfluorescent ABD (N-(7-amino-2,1,3-benzoxadiazole)) (McIntyre and Sleight, 1991), but does not itself cross lipid bilayers (Langner and Hui, 1993) and does not affect rhodamine fluorescence (data not shown). Therefore, the NBD-containing head groups of phospholipids present in the outer leaflet are modified, but the NBD-labeled head groups of phospholipid molecules present in the inner leaflet of vesicles are spared. For this experiment, v-SNARE-containing donor vesicles were prepared with head group-labeled NBD-PS instead of NBD-PE. The PE will flip-flop substantially across the bilayer during the second part of our experiment, while the PS flips much more slowly due to its additional negative charge (Lentz et al., 1997).

When the NBD-PS-labeled donor v-SNARE vesicles were incubated with unlabeled t-SNARE acceptor vesicles, NBD fluorescence promptly increased (as in earlier experiments with NBD-PE), showing that lipid mixing with the acceptor population had occurred (Figure 3A, closed circles). There was, as expected, no increase in fluorescence when the labeled donor v-SNARE vesicles were incubated with unlabeled protein-free acceptor vesicles (Figure 3A, open circles).

To determine whether the observed increase in NBD fluorescence was due to both NBD-PS that remains in the inner leaflet of the vesicles after fusion as well as to NBD-PS in the outer leaflets, we used dithionite reduction (Figure 3B). After the fusion reaction in Figure 3A (2 hr at 37°C), samples were diluted with ice-cold buffer and dithionite was added. NBD fluorescence was reduced to a new stable level (Figure 3B, closed circles, from level a to level b). The slightly disproportionate extent of reduction by dithionite could be explained by the previously reported observation that NBD-labeled phospholipids can reconstitute asymmetrically with a preference to the outer leaflet (Van Meer and Simons, 1986). When detergent (Triton X-100) was added to allow dithionite to reduce NBD-PS present on the inner leaflet, where it had been protected from reduction by the permeability barrier of vesicles, NBD fluorescence was completely eliminated. For comparison, when fusion had not taken place (the control incubation with protein-free vesicles), virtually the same degree of reduction of NBD fluorescence occurred upon addition of dithionite (Figure 3B, open circles, level a′ to level b′). The difference in fluorescence between fused (v + t) and unfused (v + pf) vesicles following dithionite treatment (level b relative to level b′ between 130 and 150 min in Figure 3B) results from the dequenching of NBD-PS in the inner leaflet of donor vesicles when this leaflet mixes with the unlabeled phospholipids of the inner leaflet of an acceptor vesicle. The extent of dequenching of inner leaflet NBD-PS (b/b′) is indistinguishable from that of the total NBD-PS in both the inner and outer leaflet (the ratio a to a′ in Figure 3B). This directly establishes that both leaflets participate in the fusion process to the same extent.

It was not practical to demonstrate fusion by content mixing because of the small trapped volume of the reconstituted vesicles. Also, electron microscopy did not provide statistically unambiguous evidence for fusion, its usefulness limited by the wide dispersion in diameters of the starting vesicles.

Assembly of SNARE Complexes between Vesicles Is Required for and Precedes Fusion

The data presented so far imply that v-SNAREs and t-SNAREs, present in separate bilayers, are the minimal fusion machinery. The requirement for cognate v- and t-SNAREs suggests that SNARE complexes bridging two bilayers must form for fusion to occur. However, the fact that both v- and t-SNAREs are required does not by itself establish this principle. Alternatively, but much less likely, v-SNAREs and t-SNAREs in two different lipid bilayers, which collide randomly, could somehow synergize in a complementary fashion during collisions to provide sufficient driving force for fusion without forming SNARE complexes. It could even be that while interbilayer SNARE complexes are needed to pair the membranes prior to fusion, additional uncomplexed (free) v- or t-SNAREs then proceed to trigger bilayer fusion.

Several experiments distinguish among these possibilities and together suggest not only that interbilayer SNARE complexes assemble prior to fusion and represent a critical prefusion intermediate, but also that free SNAREs are apparently not needed thereafter for fusion to take place.

First, complexes can form between v-SNAREs and t-SNAREs residing in different vesicles at 4°C (Figure 1B, lane 1) under conditions where significant fusion does not occur (corresponding to the preincubation before fusion in Figure 2A). This implies that interbilayer SNARE complexes can assemble before fusion.

Second, preincubation of v-SNARE with t-SNARE vesicles for several hours on ice can accumulate unfused

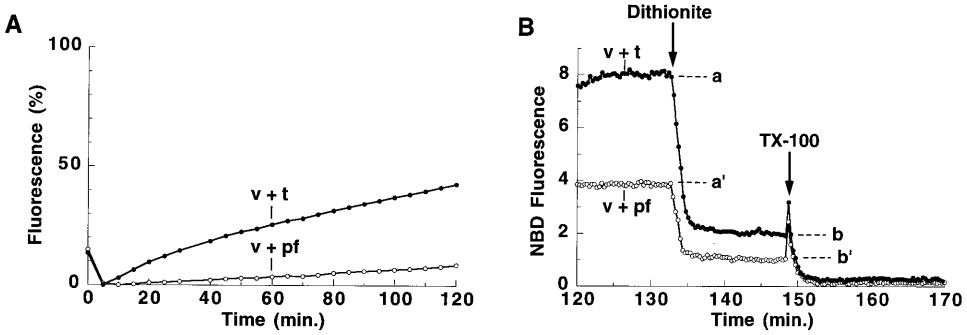

Figure 3. SNARE Mediated Fusion Leads to Complete Mixing of Lipid Bilayers

(A) VAMP-containing, donor vesicles containing fluorescent NBD-DOPS instead of NBD-DPPE were incubated with a 15-fold lipid excess of either protein-free (v + pf, open circles) or t-SNARE-containing acceptor vesicles (v + t, closed circles) at 4°C for 15 hr. Following the temperature shift to 37°C, fusion was monitored in a fluorescence plate reader (see Experimental Procedures), and signals were normalized as described in Figure 2A.

(B) A parallel set of samples, fused under identical conditions to (A), was chilled to 4°C by dilution with ice-cold buffer, and NBD fluorescence was monitored in a quartz cuvette in a conventional fluorimeter. After ~10 min, sodium dithionite was added to abolish the signal resulting from NBD-PS located in the outer leaflet. Vesicles were lysed with Triton X-100 20 min later, allowing the dithionite access to the NBD that had been in the inner leaflet. The small background of autofluorescence in control samples prepared without any NBD-PS was subtracted.

intermediates from which subsequent fusion at 37°C is more rapid (Figure 4A, closed circles) than when the vesicles are initially mixed at 37°C (Figure 4A, open circles). The preincubation results in an increased initial rate of fusion (Figure 4A, closed circles), presumably representing the consumption of prefusion intermediates accumulated during the preincubation at 4°C. After about 20 min, the rate of fusion in the preincubated sample returns to the same level as in a sample without a preincubation, presumably representing the time needed for the prefusion intermediates that had accumulated to fuse.

Third, the initial rate of fusion following preincubation is only slightly reduced when botulinum toxin D (which cleaves free VAMP but not VAMP in a SNARE complex; Hayashi et al., 1994) is added after the low temperature preincubation (Figure 4B, closed circles). However, fusion is almost completely prevented when the donor v-SNARE vesicles are toxin-digested before the preincubation (Figure 4B, open circles), demonstrating the completeness of the treatment used. This difference implies that while free v-SNAREs are needed to assemble functional prefusion intermediates, they are no longer needed for fusion after assembly. This is independently confirmed by a similar differential inhibitory effect of the soluble cytoplasmic domain of the t-SNARE (Figure 4D, closed circles versus open circles).

Fourth, the initial rate of fusion is largely resistant to inhibition by excess soluble cytoplasmic domain of the v-SNARE VAMP when this inhibitor is added after the preincubation (Figure 4C, closed circles), but fusion is almost completely prevented when the t-SNARE acceptor vesicles are treated with the same protein before the preincubation (Figure 4C, open circles). This "soluble" v-SNARE forms complexes with t-SNAREs in vesicles within 5 min (data not shown). Therefore, free t-SNAREs (like v-SNAREs) are needed during the preincubation to assemble prefusion intermediates but are not needed for fusion itself.

Fifth, implicit in the above, when vesicle-bound v- or t-SNAREs are blocked from forming SNARE complexes, fusion cannot occur. This observation provides additional evidence of the specificity of reconstituted fusion.

In sum, free v- and t-SNAREs seem to be needed for fusion only for the purpose of forming SNARE complexes between bilayers; fusion following interbilayer complex formation can occur in the absence of additional free SNAREs. This does not necessarily mean that as soon as the first SNARE complex forms to link two vesicles that these two vesicles can now fuse; rather, a number of SNARE complexes may be needed for fusion. Indeed, preliminary studies (not shown) suggest that even though many SNARE complexes form within minutes of preincubation, much longer preincubations at 4°C are needed to observe the prefusion intermediates that result in the increased initial rate at 37°C and resistance of fusion to elimination of free SNAREs.

A simple possibility for this slow "priming" would be that the SNARE complexes must topologically rearrange (perhaps a very slow process in this minimal system) to form a ring-like structure from which fusion then occurs, giving rise to the well-documented initial opening of a "fusion pore" (Lindau and Almers, 1995; Monck and Fernandez, 1996). In addition, or instead, it may also be that the last SNARE complexes assemble more slowly than the first ones.

Whatever the precise explanation, understanding the kinetics and mechanism of this minimal but still multistep fusion reaction will require a detailed analysis that includes the effects of surface density of the different SNAREs, temperature, and many other parameters, including the size of the vesicles and their lipid composition. Some quantitative (but not qualitative) variations in fusion results were observed among the many vesicle and SNARE protein preparations studied over a 6 month period, as could be expected for a multicomponent system involving natural products whose mechanism has not yet been fully characterized.

The overall time required for fusion by SNAREs alone (hours) is slow as compared to the seconds or minutes

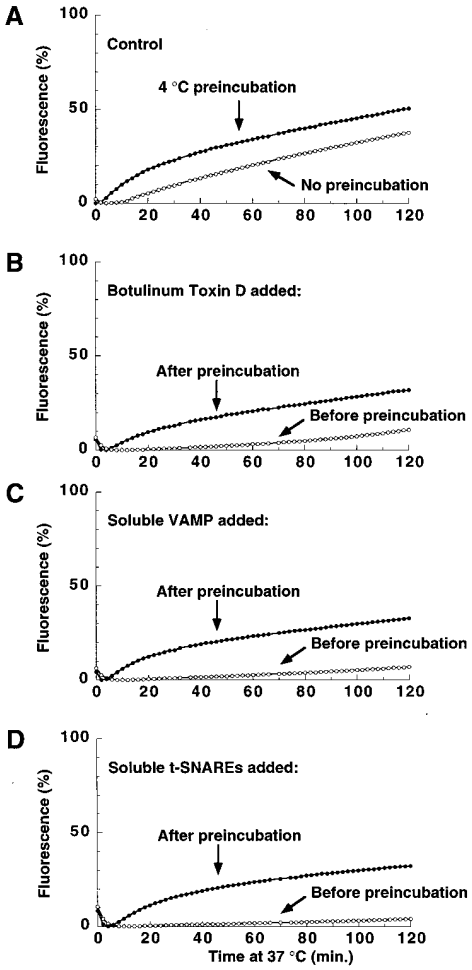

Figure 4. Assembly of SNARE Complexes between Vesicles Is Required for and Precedes Fusion

(A) Standard amounts of donor vesicles containing v-SNAREs and acceptor vesicles containing t-SNAREs were preincubated at 4°C (closed circles) and then incubated at 37°C, as described in Figure 2A, or combined and incubated immediately at 37°C (open circles). Fusion was monitored by the increase in NBD fluorescence, and signals were normalized as described in Figure 2A. Only the time course of the 37° incubation is shown. Zero time marks the start of the 37°C incubation in all cases.

(B) Standard amounts of donor vesicles containing v-SNAREs (see Experimental Procedures) were incubated at 37°C for 1 hr either in presence (open circles) or in absence (closed circles) of recombinant botulinum neurotoxin D light chain (2.1 μg of protein). Samples were chilled on ice, acceptor vesicles containing t-SNAREs were added, and the reactions were incubated at 4°C for 15 hr (the "preincubation"). Botulinum neurotoxin D was then added to the sample that had not been pretreated with this toxin. Next, both samples were warmed up to 37°C and vesicle fusion was followed by NBD fluorescence. Normalized results are shown.

(C) Standard amounts of acceptor vesicles containing t-SNAREs were incubated either in absence (closed circles) or in presence (open circles) of the cytoplasmic domain of VAMP (amino acids 1–94, added in approximately equimolar amounts to t-SNAREs) on ice for 1 hr. After addition of the donor vesicles containing v-SNAREs to each reaction, the samples were incubated for 15 hr at 4°C (the preincubation). Cytoplasmic VAMP was then added to the sample that had not been pretreated with this protein domain. Samples were warmed up to 37°C and vesicle fusion was followed by NBD fluorescence. Normalized results are shown.

(D) Standard amounts of donor vesicles containing the v-SNARE VAMP were incubated with soluble t-SNAREs (amino acids 1–265, approximately 7-fold molar excess over VAMP) on ice for 1 hr. After addition of the acceptor vesicles containing t-SNAREs, the samples were preincubated for 15 hr at 4°C. Cytoplasmic t-SNAREs were added to the sample that had not been pretreated with this protein complex and reactions were further processed as in (C).

typically required for physiological fusion (excluding the extremely rapid fusion that occurs when prefusion intermediates are stockpiled as at neuronal synapses). The various controls we present speak forcefully for the specificity of the reaction in spite of its rate, and it is not unexpected that in a minimal reconstituted system, these reactions should proceed more slowly than in vivo. At present, we do not know which step is rate-limiting in what is likely to be a complex multistep process. Fusion following completion of SNARE complex assembly may be complete within 20 min at 37°C (i.e., half-time of less than 10 min) as judged from duration of the transient increase in rate following the 4°C preincubation (Figure 4A, closed circles). It might reasonably be concluded that cells contain other components, absent when SNAREs alone are present, that facilitate the spatial reorganization of SNARE complexes for fusion.

Discussion

Compartmental identity and function rely on specific membrane fusion processes, whether membrane is transferred between functionally different compartments by transport vesicles (heterotypic fusion), or the membrane delimiting a compartment is maintained by fusion with identical copies (homotypic fusion). With the specificity of their enveloping membranes established, the differential content of subcellular organelles and compartments naturally follows, by virtue of a combination of signal-dependent import systems and sequential vesicle transfers (Blobel, 1980).

The data presented here suggest that cognate v- and t-SNAREs, when present in different membranes, are the minimal fusion machinery needed to achieve this goal. Recombinant v- and t-SNARE proteins can be reconstituted into lipid bilayer vesicles in a functional state, as shown by their ability to assemble SNARE complexes between two vesicles. When this occurs, it leads to spontaneous, but relatively slow fusion of the docked membranes at a physiological temperature, probably requiring a half-time of about 10 min. Docked, unfused intermediates can accumulate at lower temperatures, fusing when brought to a physiological temperature. A supply of unassembled v- and t-SNAREs is needed for such intermediates to form, but not for the fusion that follows. Evidently, fusion is due to intervesicular SNARE complexes already assembled in the intermediates. It is interesting that certain influenza strains also form prefusion complexes at low temperature and acidic pH that can only fuse at elevated temperature (Stegmann et al., 1991; Tsurudome et al., 1992). In that system, the viral fusion peptide is inserted into the target membrane, implying that the fusion protein transiently links viral and target membranes much in the same way SNARE

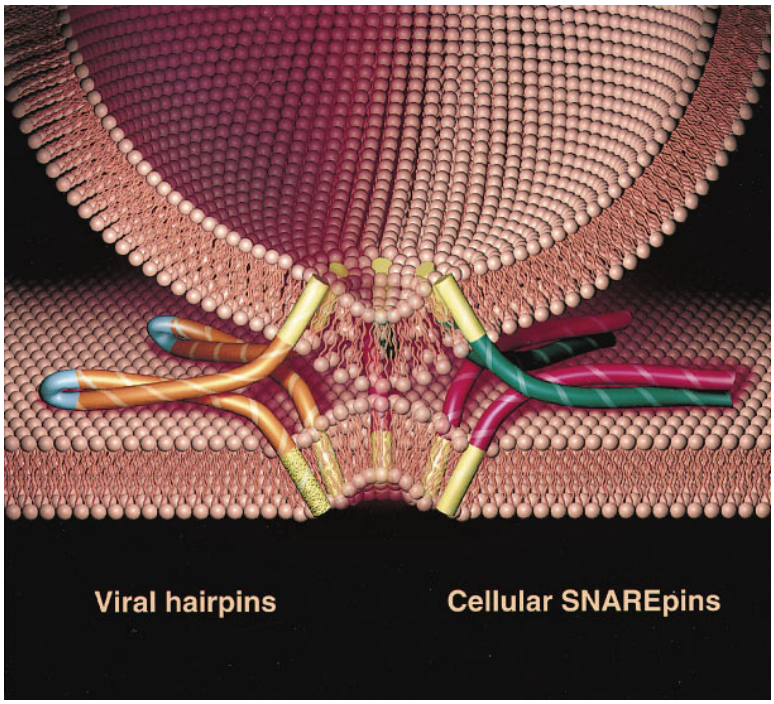

Figure 5. Cellular SNAREpins and Viral Hairpins

v-t SNAREpins (at right) are complexes of cognate v-SNAREs (in green, in the transport vesicle above) and t-SNAREs (in red, in the planar target membrane below) bridging two membranes. Analogous t-t SNAREpins may also be possible. Membrane anchors are highlighted in yellow. The core of certain viral fusion proteins (at left, in orange) is diagrammed in a simplified fashion. The membrane anchor of the fusion protein (in yellow) is inserted into the viral membrane, represented by the spherical lipid bilayer. The fusion peptide (textured yellow) is inserted into the planar target membrane below. Viral fusion proteins generally consist of continuous polypeptides (indicated conceptually by the blue polypeptide loop), within which oppositely oriented (i.e., antiparallel) helical hairpin-like structures assemble in a helical bundle and are proposed to link up the two membranes for fusion (Lu et al., 1995; Chan et al., 1997; Weissenhorn et al., 1997). A SNAREpin (whose precise internal structure is not yet known) consists of a 13–14 nm long helix-rich core rod of 2 nm width, which most likely contains the membrane-proximal helices of VAMP and syntaxin oriented parallel to each other (Hanson et al., 1997; Lin and Scheller, 1997). In contrast to viral hairpins, cellular SNAREpins are formed from separate polypeptides that reside in different membranes before fusion. It is likely that multiple copies of viral hairpins or SNAREpins are needed to trigger fusion, and these are likely arranged in a ring-like structure at a contact point along the lines illustrated. The striking similarity between SNAREpins and viral hairpins suggests that extracellular and lumenally oriented viral fusion proteins, as well as intracellular membrane fusion proteins, all employ a fundamentally similar mechanism to coalesce lipid bilayers.

complexes do in vesicle (v-SNARE) and target (t-SNARE) membranes.

It would appear that cells utilize the simplest possible means to link a mechanism for specifically pairing membranes to a mechanism for fusing their bilayers: a pair of cognate proteins that does both. This principle would not only embody the crux of how subcellular compartments are maintained and propagated, but would also suggest in a natural way how the many and diverse compartments in eukaryotic cells could have evolved in the first place. It is easy to imagine how a primitive pair of *v-* and *t-SNARE* genes could give rise to a family of such genes by duplication and mutational variation, each v-SNARE evolving in tandem with its cognate t-SNARE because they are structurally and functionally linked.

Cognate v- and t-SNAREs bind each other via membrane-proximal heptad repeat regions that are predicted to form coiled-coils or closely related helical bundles. In the electron microscope, SNARE complexes are seen to be rods, 13–14 nm long and approximately 2 nm wide, as is appropriate for such a coiled-coil structure (Hanson et al., 1997; and see the cover of this issue). The membrane anchors of v- and t-SNAREs emerge at the same end of the rod (Hanson et al., 1997; Lin and Scheller, 1997), implying that the rod must lie approximately in the plane of contact between the vesicles paired by a complex of a v-SNARE in one with a t-SNARE in the other, a structure that we term a "*SNAREpin*" (Figure 5).

Isolated SNARE complexes are unusually stable, remaining intact up to 90°C (Fasshauer et al., 1997b) and resisting denaturation by SDS (Hayashi et al., 1994), suggesting a ready source of energy to help overcome energy barriers for fusion. This energy could well be made available when SNAREpins assemble. Put differently, v- and t-SNAREs exist separately in a thermodynamically metastable state but are in a lower energy-stable state when combined. This means that separate v- and t-SNAREs constitute a reservoir of potential energy that can be drawn upon, when required, to overcome the activation energy needed for fusion. This energy could be made available when cognate SNAREs link up to form a SNAREpin between bilayers, representing the means by which this currency of energy for fusion would be tapped. Like energy released from ATP (a molecule that, like SNAREs, is kinetically stable but thermodynamically unstable), the release of energy for fusion from SNAREs is likely to be a catalyzed and tightly regulated process.

How, more precisely, could energy made available from the assembly of SNAREpins be harnessed to cause fusion? One possibility is that binding energy is simply used to bring two lipid bilayers into intimate approximation, perhaps displacing boundary layers of water, and that this, in and of itself, is sufficient to trigger fusion, given appropriate lipid composition and enough time. This mechanism would require no mechanistic coupling between SNARE complex assembly and energy-requiring perturbations in local lipid bilayer structure needed for the transition to fusion. By contrast, another possibility (which we favor because it necessarily requires that the SNARE complexes be exceptionally stable) is that

the conformation of SNAREpins and the local lipid bilayers are indeed mechanistically coupled and therefore thermodynamically linked. In this type of mechanism, energy made available from SNARE complex assembly does work on the local lipid bilayers, advancing the bilayers toward fusion by helping them to overcome the activation energy barrier for this process. Here, some kind of tight mechanical coupling between the cytoplasmic domains of the SNARE complex and their membrane anchors would be needed to provide energy to the bilayer. Put differently, in this hypothesis the energy invested in the virtual process of separating the v- and t- ends of a SNAREpin to insert each in a different bilayer would be released as the SNAREpin relaxes during bilayer fusion, providing a driving force for fusion and a source of energy to exceed that needed for a transition state. By contrast, cognate SNAREs complexing in the same bilayer would dissipate as heat the portion of binding energy that might have been stored in the bilayer, had they assembled as a SNAREpin.

By either mechanism, the principle of fusion by SNAREpins may have considerable generality for other hairpin-like structures. Certain viral fusion proteins have been found to possess a hairpin-like core in which a C-terminal α helix adjacent to the viral membrane pairs antiparallel (in a helical bundle) with another α helix located near the N terminus, adjacent to the hydrophobic fusion peptide. Indirect evidence suggests that these helical hairpins only assemble when the protein is activated for fusion (Lu et al., 1995; Rabenstein and Shin, 1996; Chan et al., 1997; Weissenhorn et al., 1997). Like SNAREpins, viral hairpins (Figure 5) are remarkably stable, dissociating only at temperatures above 90°C (Lu et al., 1995). In their dormant, nonfusogenic state, the hairpins of the viral proteins are most likely prevented from assembling by intra- or intermolecular structures. When this block is removed upon binding an entry receptor at the cell surface, or when the local pH becomes acidic following endocytosis, the helical hairpin is thought to assemble, and simultaneously the fusion peptide to insert into the bilayer of the target membrane (Stegmann et al., 1991; Tsurudome et al., 1992; Binley and Moore, 1997; Chan et al., 1997; Dimitrov, 1997; Weissenhorn et al., 1997) analogous to a SNAREpin.

SNAREpins form an extremely versatile and general fusion machinery, since there are many cognate v-SNAREs and t-SNAREs in different cellular membranes. Viral fusion proteins can now be understood as a special case of SNAREpins (Figure 5) in which the equivalent of a v-SNARE (inserted into the viral membrane) is covalently and permanently attached to the equivalent of a t-SNARE. From this perspective, the hydrophobic fusion peptide of the viral protein would be the equivalent of the membrane anchor of the t-SNARE, except that in this special case membrane insertion of the fusion peptide would be prevented until the helical hairpin of the viral fusion protein assembles when the fusion protein is activated. The fact that many viral fusion proteins are activated by receptor-dependent removal of a blocking protein enables target membrane-specific fusion in spite of the covalent attachment of v- and t- portions of this viral analog of a SNAREpin. Where there is no blocking protein (as in influenza), cell type specificity is achieved by receptor-selective endocytosis followed by nonspecific activation by the acidic pH in endosomes. The triggered assembly of viral hairpins by receptor-dependent removal of a blocking protein is reminiscent of the triggering of SNARE assembly when a t-SNARE blocking protein (Sec1 family) is removed in a Rab-dependent process (Pevsner et al., 1994; Lupashin and Waters, 1997).

Recent evidence (Patel et al., 1998; Rabouille et al., 1998) suggests that homotypic fusion may rely solely on the t-SNAREs identifying a compartment and does not involve v-SNAREs. Assuming that identical t-SNAREs can indeed specifically pair between bilayers to link compartments for homotypic fusion, one can well imagine how such t-t SNAREpins could closely resemble the v-t SNAREpins illustrated in Figure 5 and how they could fuse linked bilayers by the same basic mechanism.

Following fusion, dissociation of now thermodynamically stable SNARE complexes to enable later rounds of fusion requires investment of energy and therefore additional core machinery. The need for SNAPs and the ATPase NSF (and analogous proteins; Rabouille et al., 1995; Kondo et al., 1997) as core fusion machinery can most readily be understood from this perspective, as the minimal "add-on" needed for repeated use of a bilayer-fusing helical hairpin: by separating v-SNAREs from t-SNAREs at the expense of energy from ATP, SNAPs and NSF can make them available for subsequent inter-bilayer-partnering. Indeed, recent evidence implies that in some cases this may be the only role for NSF and SNAPs (Mayer et al., 1996; Nichols et al., 1997; Ungermann et al., 1998). In light of the recent discovery of a pre-SNARE docking system in the Golgi in which vesicles are tethered by p115/GM130 strings (Nakamura et al., 1997; Orci et al., 1998), a sufficient and now the simplest explanation of the accumulation of docked vesicles at the Golgi in the absence of NSF (Orci et al., 1989; Søgaard et al., 1994) (and a different explanation from the one we originally proposed) is that vesicles are tethered but unable to fuse because Golgi t-SNAREs are tied up in complexes with v-SNAREs residing in the same membranes.

Nonetheless, the possibility remains that SNAPs and NSF may also regulate fusion by acting on SNAREpins bridging two lipid bilayers, along the lines originally envisioned, in view of the 20S docking and fusion particle as a prefusion rather than postfusion intermediate (Söllner et al., 1993a). Preliminary studies do not reveal a major effect of the presence or absence of SNAPs, NSF, and ATP in the minimal fusion system. However, these proteins could well affect a step that is not rate-limiting for the overall fusion process in the minimal system under our present conditions. Only when the overall fusion reaction has been carefully dissected into its constitutive steps can the possibility that SNAPs and NSF have a regulating role at any particular step be tested rigorously.

Fortunately, with the minimal fusion machinery now identified, these and other kinds of mechanistic studies are now possible, and they should reveal underlying biophysical mechanisms. In addition, cognate and non-cognate SNAREs can now be presented to each other in a functional context to probe the limits and determinants of their specificity. The assembly of SNAREpins

in cells must be closely regulated by restraint and by catalysis. This follows from the obvious fact that the many compartments in eukaryotic cells do not simply coalesce, degenerating the cell into a single prokaryote-like sac. Many candidate proteins are already known that could regulate SNAREpin assembly by controlling the proximity of vesicles to targets (Pfeffer, 1994, 1996; TerBush et al., 1996; Orci et al., 1998), by affecting the conformation or availability of free SNARE proteins (Aalto et al., 1992; Hata et al., 1993; Garcia et al., 1994; Pevsner et al., 1994), or by stabilizing or promoting fusion intermediates to provide sensitivity to signals (Brose et al., 1992; Schiavo et al., 1997). The minimal fusion machinery now provides a foundation upon which a scaffold of regulatory proteins can be erected and understood in functional and physiological terms.

Experimental Procedures

Plasmid Constructions

All plasmids were propagated in the *E. coli* strain DH5α, and standard protocols for DNA manipulations were followed (Sambrook et al., 1989). The coding sequence of *VAMP2* was amplified by PCR from a mouse brain cDNA library (Stratagene) with primer 1 (GGAATT CCAT ATGTCGGCTA CCGCTGCCAC C) and primer 2 (CATCGTT TAC TTCAGCACTG GATCCTCTAG AGC). The PCR product was digested with NdeI and BamHI and ligated into the pET3a (Novagen)–based vector pMS/His (Søgaard et al., 1994), which codes for a C-terminal His_6-tag yielding the plasmid pTW2.

The coding sequence for the cytoplasmic domain of *VAMP2* (amino acids 1–94) was amplified by PCR using primer 3 (GCTCTAG ACAT ATGTCGGCTA CCGCTGCCAC) and primer 4 (CCATCGATAG ATCTACCTTC GATCTTGAGG TTTTTCCACC AGT) with pGEXKG-VAMP2 (Calakos et al., 1994) as template. The PCR product was digested with NdeI and BglII and subcloned into the NdeI and BamHI sites of pMS/His (Søgaard et al., 1994), which codes for a C-terminal His_6-tag yielding the plasmid pET-rVAMP2CD.

The coding sequence of syntaxin1A was amplified by PCR from a rat brain λgT11 library (Clontech) with primer 5 (GGAAGATCTG AATTCATGAA GGACCGAACC CAGGAG) and primer 6 (GGGGTAC CAA GCTTTTAGTG ATGGTGATGG TGATGTCCAA AGATGCCCCC GATG). The PCR product was digested with EcoRI and HindIII and subcloned into pFastbac1 (GIBCO). Subsequently, the coding region was excised with RcaI and HindIII and ligated into pET28a (Novagen), which had been cut with NcoI and HindIII yielding the plasmid pTW12.

The cytosolic domain of syntaxin (amino acids 1–265) was subcloned into a vector carrying a kanamycin resistance gene as follows. The coding region, the ribosome binding site, and T7-terminator region were excised from the plasmid pET-Syn1A-CD (kindly provided by Dr. Sidney Whiteheart, University of Kentucky) with XbaI and EcoRI and ligated into pET9a, which has been cut with the same restriction enzymes, resulting in pTW19.

The coding sequence for SNAP25B was amplified by PCR using primer 7 (AGCGGATCCA TGGCCGAGGA CGCAGACAT) and primer 8 (AGCGGATCCT TAACCACTTC CCAGCATCT) from a mouse brain cDNA library (Stratagene). The resulting product was digested with BamHI and ligated into BamHI cut pQE-9 (Qiagen) yielding pQE-mSNAP25B. The GST-fusion construct was obtained by removing the BamHI fragment from pQE-mSNAP25B and ligating it into BamHI cut pGEX-2T (Pharmacia) resulting in the plasmid pGEX-mSNAP25B. All coding sequences were confirmed by DNA sequencing.

Protein Expression and Purification

For coexpression of GST-SNAP25 and syntaxin1A-His_6, the respective plasmids were cotransformed into BL21/DE3 cells. The cells of an 800 ml overnight preculture in superbroth containing 500 μg/ml ampicillin and 50 μg/ml kanamycin were sedimented and used to start an 8 liter culture in superbroth containing 200 μg/ml ampicillin and 50 μg/ml kanamycin. Every hour, an additional 100 μg/ml ampicillin was added. The cells were induced with 1 mM IPTG (isopropyl-β-D-thiogalacto-pyranoside) once they reached an optical density of 0.8 (at 600 nm). After 4 hr, the cells were collected by centrifugation and washed once in 20 mM Tris-HCl (pH 7.5), 150 mM KCl. They were then resuspended in 160 ml of breaking buffer A (50 mM Tris-HCl [pH 8.0], 10% (w/v) glycerol, 300 mM KCl, 10 mM β-mercaptoethanol) followed by the addition of 1/4 vol 20% (w/v) Triton X-100, PMSF to 2 mM, and four complete protease inhibitor tablets (Boehringer Mannheim). The cells were broken by one pass through an Avestin (Ottawa, Canada) cell disrupter at >10,000 psi. After centrifugation of the lysate for 15 min at 12,000 rpm in a SA600 rotor (Sorvall), the resulting supernatant was spun at 35,000 rpm in a Ti45 rotor (Beckman) for 60 min. The supernatant was then incubated overnight at 4°C with 5 ml glutathione agarose beads (Sigma) equilibrated in breaking buffer A containing 1% (w/v) Triton X-100. The beads were washed in breaking buffer A containing 1% (w/v) Triton X-100 to remove unbound proteins, and then the detergent was exchanged for octyl-β-D-glucopyranoside by exhaustive washing (>20 bead vol) with buffer B (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 10% glycerol (w/v), 10 mM β-mercaptoethanol) containing 0.8% octyl-β-D-glucopyranoside. The beads were resuspended in 5 ml of buffer B, and 0.8% (w/v) octyl-β-D-glucopyranoside and 125 μl thrombin (1 U/μl) was added. After 4 hr at room temperature, the thrombin was inactivated by the addition of 100 μl 200 mM PMSF and 100 μl 100 mM AEBSF (Calbiochem) and by incubating an additional 30 min. Finally, the beads were sedimented, and the supernatant was recovered, aliquoted, and frozen in liquid nitrogen. Typical protein yields were 1.25–2.5 mg/liter bacterial culture of the syntaxin1A/SNAP25 complex. The purity of the preparation was assessed by SDS-PAGE and Coomassie blue staining (see Figure 1A). In all preparations, SNAP25 migrated as a double band on SDS-PAGE. The lower band of the SNAP25 doublet is a C-terminal degradation product, as determined by N-terminal Edman degradation. Nonetheless, this band is recognized by an antibody raised against 12 C-terminal amino acids of SNAP25.

The cytoplasmic t-SNARE complex was purified from BL21/DE3 cells carrying the plasmids pTW19 and pGEX-mSNAP25B in an identical manner except that the detergents were ommited from the buffers. The yield was typically around 1 mg/liter culture. The complex could be further concentrated (about 6-fold) using a Microcon concentrator with a 30 kDa cutoff (Millipore).

For expression of full-length VAMP2-His_6, BL21/DE3 cells containing the plasmid pTW2 were grown and harvested as described for GST-SNAP25 and syntaxin1A-His_6, except that kanamycin was omitted from the growth medium. The washed cells were resuspended in 80 ml breaking buffer C (25 mM HEPES-KOH [pH 7.4], 400 mM KCl, 2 mM β-mercaptoethanol) followed by the addition of 1/4 vol 20% (w/v) Triton X-100 and PMSF to 2 mM. The cells were broken by one pass through an Avestin cell disrupter at >10,000 psi. After centrifugation of the lysate for 15 min at 12,000 rpm in a SA600 rotor, the resulting supernatant was spun at 50,000 rpm in a Ti70 rotor for 60 min. This supernatant was then incubated for 2 hr at 4°C with 6 ml of Ni-NTA-agarose (Qiagen) equilibrated in breaking buffer C containing 1% Triton X-100. The beads were washed with breaking buffer C containing 1% Triton X-100, and then Triton X-100 was exchanged with octyl-β-D-glucopyranoside by extensive washes with buffer D (25 mM HEPES-KOH [pH 7.4], 100 mM KCl, 10% glycerol (w/v), 2 mM β-mercaptoethanol, 1% octyl-β-D-glucopyranoside) containing 50 mM imidazole–acetic acid (pH 7.5). The protein was eluted from the Ni-NTA beads with a linear gradient from 50 to 500 mM imidazole in buffer D. Typical yields for mVAMP2-His_6 were 4–6 mg/liter of bacterial culture. The purity of the preparation was assessed by SDS-PAGE and Coomassie blue staining (see Figure 1A). The low molecular weight bands present in all VAMP preparations are N-terminal degradation products of VAMP. These fragments cannot be detected on a Western blot with the monoclonal antibody Cl. 69.1, which is directed against the 16 N-terminal residues of VAMP2 (Edelmann et al., 1995), but can be purified via their C-terminal His_6 tag and reconstituted into vesicles.

In some instances, mVAMP2-His_6 was further purified to remove the N-terminal degradation products of the protein from the preparation (see Figure 1A). The peak fractions of VAMP, eluted from Ni-NTA beads, were pooled (10–15 ml total vol) and diluted 5-fold in

buffer E (25 mM HEPES-KOH [pH 7.0], 10% glycerol (w/v), 1 mM DTT, 1% octyl-β-D-glucopyranoside) containing 50 mM NaCl. Using a FPLC system (Pharmacia), the protein was loaded onto a 1 ml Mono-S Sepharose column (Pharmacia) equilibrated in the same buffer. The column was washed with 5 ml of buffer E containing 100 mM NaCl, after which VAMP was eluted with a 15 ml linear gradient of 100–300 mM NaCl in buffer E. Eluted fractions were analyzed by SDS-PAGE followed by staining with Coomassie blue-R250. Fractions containing VAMP and no detectable degradation product were pooled and aliquoted for reconstitution. These fractions contained approximately 50% of VAMP loaded onto the Mono-S column.

[^{35}S]-labeled VAMP was prepared from metabolically labeled BL21(DE3) expressing pTW2, grown in 1 liter modified Eagle medium in the presence of 6.4 mCi of Easytag (NEN) following a published procedure (Giovane et al., 1997). The purification was essentially as described for the unlabeled protein, with the exception that the bacteria were lysed by treatment with lysozyme in the presence of DNAse I (Sambrook et al., 1989), and the protein was step-eluted (300 mM imidazole) from Ni-NTA agarose. The resulting purified protein typically had a specific activity of 1×10^5 cpm/μg.

BL21/DE3 cells carrying the plasmid pET-rVAMP2CD were grown exactly as described above for the overexpression of full-length VAMP2. From these cells, the cytosolic domain of VAMP (amino acids 1–94) carrying a carboxy-terminal His_6-tag was purified as previously described for [^{35}S]-labeled full-length VAMP. The octyl-β-D-glucopyranoside was then removed by extensive dialysis against 25 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10% glycerol (w/v), 1 mM DTT.

Protein Reconstitution into Vesicles

All lipids were obtained from Avanti Polar Lipids. For each fluorescent donor vesicle preparation, 100 μl of a 3 mM premixed lipid solution in chloroform—POPC (1-palmitoyl, 2-oleoyl phosphatidylcholine):DOPS (1,2-dioleoyl phosphatidylserine):NBD-DPPE (N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine):rhodamine-DPPE (N-(lissamine rhodamine B sulfonyl) 1,2-dipalmitoyl phosphatidylethanolamine) in an 82:15:1.5:1.5 mol ratio—and trace amounts of [^{3}H]-DPPC (1,2-dipalmitoyl phosphatidylcholine) (Amersham) were dried down in 10 × 75 mm glass test tubes by a gentle stream of nitrogen, and any remaining traces of chloroform were then removed under vacuum for 30 min.

A sample to produce v-SNARE-containing donor vesicles was prepared by dissolving the lipid film in 100 μl of a solution containing VAMP2-His_6 (ranging from 3–5 mg/ml) in 1% (w/v) octyl-β-D-glucopyranoside (OG) as eluted from Ni-NTA agarose. Reconstitutions using material dialyzed against reconstitution buffer or VAMP preparations containing only 0.8% (w/v) OG could be reconstituted equally well. A sample to prepare t-SNARE donor vesicles was prepared in the same fashion, but using 100 μl buffer B containing the t-SNARE complex (2–4 mg/ml of total protein) and 0.8% (w/v) OG. A sample to produce protein-free donor vesicles was prepared by dissolving the lipid film with 100 μl of reconstitution buffer (25 mM HEPES-KOH [pH 7.4], 100 mM KCl, 10% glycerol (w/v), 1 mM DTT) containing 0.8% (w/v) OG. In all cases, the lipids were dissolved by gentle agitation for 15 min at room temperature.

Vesicles were then formed from these samples by rapid dilution followed by extensive dialysis as follows. While vortexing vigorously, 200 μl of reconstitution buffer (at room temperature) was added to each of the above 100 μl samples, thereby diluting the detergent OG below its critical micellar concentration promoting vesicle formation. The concentration of OG in protein preparations and reconstitutions was kept to a minimum to avoid unnecessary dilution. Then detergent was removed by dialysis (in Spectrapore 6–8 kDa cutoff dialysis tubing) against 4 liters of room temperature reconstitution buffer containing 4 g Biobeads SM2 beads (Bio-Rad). The dialysis was continued at 4°C overnight with no buffer changes. All reconstitutions done in parallel were dialyzed together. If the total volume of the dialysate exceeded 6 ml, the samples were distributed equally into separate buffer tanks.

Vesicles were recovered and concentrated by flotation in a Nycodenz (Sigma) step gradient. Each ~300 μl dialysate was mixed with 300 μl of 80% (w/v) Nycodenz dissolved in reconstitution buffer and then divided equally into two 5 × 41 mm ultraclear centrifuge tubes (Beckman). Then, each was overlaid with 250 μl 30% (w/v) Nycodenz in reconstitution buffer followed by 50 μl reconstitution buffer lacking glycerol. The samples were then centrifuged in a SW55 rotor (Beckman) with the appropriate adaptors at 48,000 rpm for 4 hr at 4°C. The vesicles were harvested from the 0/30% Nycodenz interface in 75 μl per tube and then combined.

For certain control experiments, (such as Figure 2C) donor vesicles containing t-SNAREs were needed. Gradients used to prepare donor t-SNARE vesicles (or, for that matter, any t-SNARE vesicles) resulted in two closely spaced vesicle bands (v-SNARE vesicle preparation fractionate as a single band). These bands were harvested separately, each in a vol of ~40 μl. The protein concentration of each of these fractions was very similar (approximately 0.25 mg/ml). The lipid concentration in the lower band was ~0.4 mM compared with ~1 mM in the less dense band. The experiment performed in Figure 2C utilized only the denser band of the doublet because the lighter band was noticeably less efficient as a donor (containing fewer t-SNAREs per vesicle).

Unlabeled nonfluorescent acceptor vesicles were prepared as follows: For each acceptor vesicle preparation, 100 μl of a 15 mM lipid mixture in chloroform (POPC:DOPS in an 85:15 mol ratio with trace amounts of [^{3}H]-DPPC) was evaporated into a film as described for the donor vesicles. Samples to yield v-SNARE-containing acceptor vesicles were prepared by dissolving the lipid film in 500 μl of a solution containing 3–5 mg/ml VAMP in 1.0% (w/v) OG as eluted from Ni-NTA agarose. For the preparation of t-SNARE acceptor vesicles, 500 μl of the syntaxin1A/SNAP25 t-SNARE complex (2–4 mg/ml total protein) in buffer B containing 0.8% (w/v) OG were used to dissolve the lipid film. Protein-free acceptor vesicles were prepared by dissolving the lipid film in 500 μl of reconstitution buffer containing 0.8% (w/v) OG. The dissolved lipid-protein mixtures were then diluted with 1 ml of reconstitution buffer and dialyzed as described above. Each ~1.5 ml dialysate was then mixed with 1.5 ml 80% (w/v) Nycodenz in reconstitution buffer and overlaid with 750 μl 30% Nycodenz in reconstitution buffer and 250 μl reconstitution buffer without glycerol in a single 11 × 60 mm ultraclear centrifuge tube (Beckman). After centrifugation for 3 hr and 40 min at 55,000 rpm at 4°C in a SW60 rotor (Beckman), the vesicles were harvested in a vol of 400 μl per tube (in the case of t-SNARE vesicle acceptors, the upper and lower bands of the doublet described above were pooled). These nonfluorescent vesicles could be seen as an opaque band when viewed against a dark background. Lipid concentrations and yields were determined from tracer [^{3}H]-DPPC and protein concentrations and yields by amido black dye binding (Schaffner and Weissmann, 1973). The vesicles were generally used fresh but could also typically be stored at 4°C without apparent loss of activity for up to 5 days. Representative protein and lipid concentrations were as follows: For donor vesicles, lipid concentrations were typically 1 mM and for acceptor vesicles typically 2 mM. VAMP concentrations in donor vesicles were approximately 0.7 mg/ml and t-SNARE concentrations in acceptor vesicles approximately 0.5 mg/ml.

SNARE Complex Formation Monitored by Resistance to Cleavage by Botulinum Neurotoxin D

Acceptor t-SNARE vesicles (10 μl, containing ~12 μg protein and [^{35}S]-labeled v-SNARE vesicles (1 μl, containing ~1 μg protein) were combined in 10 μl of reconstitution buffer and incubated at 4°C for 12 hr with or without a subsequent incubation at 37°C for 2 hr. The molar ratio of t-SNAREs to v-SNAREs in the reaction was approximately 3 to 1. Following the incubation, 20 μl of nonradioactive cytoplasmic domain of VAMP (~15 μg of protein), representing a 20-fold molar excess over [^{35}S]-labeled VAMP and a 6-fold molar excess over the t-SNAREs in acceptor vesicles, was added to the reaction. The reaction was further incubated at 4°C for 1 hr, and then Triton X-100 was added from a 20% stock solution to a final concentration of 0.5% (v/v). The addition of excess nonradioactive cytoplasmic domain of VAMP blocks all free t-SNAREs while vesicles are still intact, preventing the formation of additional SNARE complexes containing [^{35}S]-labeled VAMP following the addition of detergent. Lysis of vesicles by detergent is necessary to allow the toxin to cleave the approximately 25% of [^{35}S]-labeled VAMP facing the vesicle lumen, which would otherwise result in unacceptably

high background. The amount of [^{35}S]-labeled VAMP facing the vesicle lumen was determined by digesting donor vesicles with toxin in the absence of detergent and measuring [^{35}S]-labeled VAMP inaccessible to the toxin. Following vesicle solubilization, 4 μg of recombinant botulinum neurotoxin D light chain (BoNT D) (Glenn and Burgoyne, 1996) was added, and the reaction was incubated for 1 hr at 37°C. An additional 4 μg of BoNT D was then added, and the incubation was continued for 1 hr at 37°C. Neurotoxin digestion was terminated by the addition of 5× Laemmli SDS-PAGE buffer and heating of the reaction at 98°C for 4 min. One half of each sample was resolved by SDS-PAGE on an 18% acrylamide/0.47% bisacrylamide gel. Following electrophoresis, the gel was fixed in 40% methanol/10% acetic acid, dried, and analyzed using a phosphorimager (Molecular Dynamics). Unlike nonradioactive VAMP, [^{35}S]-labeled VAMP migrated as a double band on the 18% acrylamide gel.

To show that cytoplasmic domain of VAMP could block the available t-SNAREs in the acceptor vesicles, donor v-SNARE vesicles were mixed with cytoplasmic domain of VAMP in the amounts described and added to acceptor t-SNARE vesicles. Following an incubation at 4°C for 1 hr, vesicle solubilization with Triton X-100 and BoNT D digestion, protection of [^{35}S]-labeled VAMP was at background levels. To establish that BoNT D was able to completely digest all the VAMP in the reaction, v-SNARE vesicles were mixed with cytoplasmic domain of VAMP in the presence of 0.5% (v/v) Triton X-100 and digested with toxin as described. The resulting protection of [^{35}S]-labeled VAMP remained at background levels.

To determine the maximum fraction of [^{35}S]-labeled VAMP that could partner with t-SNAREs, Triton X-100 (0.5%, v/v) was used to dissolve a mixture of [^{35}S]-labeled VAMP donor vesicles (1 μl, containing ~1 μg protein) and t-SNARE acceptor vesicles (10 μl, containing 12 μg protein). After 1 hr at 4°C, this sample was further processed as described above. Under the conditions used, 30 ± 2% of [^{35}S]-labeled VAMP present in the reaction was toxin-protected, representing the maximum amount of VAMP available for binding. Longer incubations did not increase the amount of toxin-resistant VAMP. When identical quantities of v- and t- SNAREs were allowed to form complexes in the presence of detergent prior to reconstitution into vesicles, the fraction of [^{35}S]-labeled VAMP protected from toxin was the same (30%) as following reconstitution and then Triton extraction. This result shows that reconstitution did not affect the competency of proteins to form SNARE complexes. To show that [^{35}S]-labeled VAMP vesicles behaved identically to nonradioactive VAMP vesicles, the two preparations were tested in parallel in the fusion assay.

Fusion Assays

Standard assays were performed in white 96-well FluoroNunc plates (Nunc). Typically (as in Figure 2B) 5 μl labeled v-SNARE donor vesicles (5.5 nmol phospholipid, 3.5 μg VAMP2 corresponding to 0.25 nmol VAMP2) were mixed with 45 μl unlabeled t-SNARE acceptor vesicles (90 nmol phospholipid, 22.5 μg t-SNARE complex corresponding to 0.39 nmol). The nonstandard experiments described in Figures 2C and 2D use the following additional vesicle preparations: t-SNARE donor vesicles (5 μl per reaction, ~1.25 μg, ~0.022 nmol t-SNARE complex, and 2.2 nmol phospholipid), protein-free donor vesicles (5 μl, 5 nmol phospholipid), v-SNARE acceptor vesicles (45 μl, ~63 μg, ~4.5 nmol VAMP2, and 90 nmol phospholipid), and protein-free acceptor vesicles (45 μl, 90 nmol phospholipid). If the reactions were preincubated, the wells were sealed with Scotch tape, and the plates gently agitated in the dark at 4°C overnight. The plates were then placed in the Fluorimeter (Fluoroskan II, Labsystems) equilibrated to 37°C. NBD fluorescence was followed with filters set at 460 nm (excitation, half band width 25 nm) and 538 nm (emission, half bandwidth, 25 nm). NBD fluorescence was monitored at either 2 or 5 min intervals. In some cases, samples were overlaid with 50 μl mineral oil with identical results. Donor and acceptor vesicles can also be prepared using sucrose rather than Nycodenz gradients for flotation, with no effect on fusion. After 2 hr at 37°C, 10 μl of a 2.5% (w/v) Triton X-100 solution was added to terminate the reaction and allow normalization as described in the text and the legend to Figure 2. Fusion could also be followed on a larger scale in quartz cuvettes in a Perkin Elmer LS 50B fluorimeter (excitation 460 nm, slit width 10 nm; emission 538 nm, slit width 10 nm; cutoff filter 515 nm with or without crossed polarizers) with indistinguishable results from those in Figure 2.

To measure the mixing of the inner leaflets of donor vesicles with the inner leaflets of acceptor vesicles in our fusion assays, we monitored the NBD fluorescence resistant to external dithionite treatment and therefore contributed by the inner leaflet before and after fusion. For this purpose, v-SNARE donor vesicles were prepared as described above except that NBD-DOPS (N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dioleoyl phosphatidyl-serine) was substituted for NBD-DPPE. These v-SNARE donor vesicles were incubated in standard fusion reactions overnight at 4°C with acceptor vesicles containing t-SNARES or (as a control) protein-free acceptor vesicles. NBD fluorescence was then monitored in a FluoroskanII plate reader as a function of time at 37°C. Duplicate samples were incubated in parallel in 500 μl Eppendorf tubes overnight at 4°C and then for 2 hr at 37°C. These samples were then chilled on ice, and 550 μl ice-cold reconstitution buffer was added to each. NBD fluorescence was then followed in a Perkin Elmer LS50B fluorimeter in quartz cuvettes at 4°C. Excitation was set at 460 nm (slit width 10 nm), and emission was recorded at 538 nm (slit width 10 nm) with a cutoff filter at 515 nm. After 10 min, 20 μl 1 M sodium dithionite was added to abolish the signal resulting from NBD-PS located in the outer leaflet. After an additional 20 min, the vesicles were lysed by adding 15 μl 20% (w/v) Triton X-100, allowing the dithionite access to the NBD that had been in the inner leaflet. The small background (1.3 fluorescence units) due to autofluorescence in control samples without any NBD-PS was subtracted in all cases.

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