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Reconstituting SNARE-mediated membrane fusion at the single liposome level

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Abstract

Successful reconstitutions of SNARE-mediated intracellular membrane fusion have been achieved in bulk fusion assays since 1998 and in single liposome fusion assays since 2004. Especially in neuronal presynaptic SNARE-mediated exocytosis, fusion is controlled by numerous accessory proteins, of which some functions have also been reconstituted in vitro. The development of and results obtained with two fundamentally different single liposome fusion assays, namely liposome-to-supported membrane and liposome-to-liposome, are reviewed. Both assays distinguish between liposome docking and fusion steps of the overall fusion reaction and both assays are capable of resolving hemi- and full-fusion intermediates and end states. They have opened new windows for elucidating the mechanisms of these fundamentally important cellular reactions with unprecedented time and molecular resolution. Although many of the molecular actors in this process have been discovered, we have only scratched the surface of looking at their fascinating plays, interactions, and choreographies that lead to vesicle traffic as well as neurotransmitter and hormone release in the cell.

INTRODUCTION

Improving knowledge of cellular functions by reconstituting them in vitro has a very long history. Classical examples among many others are the reconstitution of DNA replication by Arthur Kornberg in the 1950s and the reconstitution of mitochondrial oxidative phosphorylation by Efraim Racker in the 1960s. Although membrane fusion in intracellular membrane traffic, fertilization, and cell entry of enveloped viruses has been known to be important in cell biology for decades, its functional reconstitution in vitro has been achieved much later. Fusion of pure lipid liposomes has been studied since the late 1970s (Duzgunes et al., 1984; Lentz, 1994; Papahadjopoulos, Vail, Pangborn, & Poste, 1976; Wilschut & Hoekstra, 1986; Wilschut & Papahadjopoulos, 1979). However, liposomes with typical biological lipid compositions do not fuse naturally. Rather unusual fusogens like relatively high concentrations of calcium or highly cationic peptides in combination with liposomes composed of anionic lipids or high concentrations of water-activity-reducing polymers like polyethylene glycol have to be used to force pure lipid liposomes to fuse with one another. This is physiologically sensible because lipid bilayers with physiological lipid compositions

should not spontaneously fuse with or within cells. To the contrary, such fusion must be controlled by the cell to preserve the identity and integrity of cellular membranes when not carrying out functions that require membrane fusion. Cells have evolved numerous proteins and protein machines to work on their membranes to fuse them in a highly regulated fashion.

Fusion proteins of enveloped viruses, i.e., spike glycoproteins that reside in the viral envelope have been studied in great detail in reconstituted systems. For example, the hemagglutinin of influenza virus has been reconstituted into liposomes, often also called virosomes, and their fusion properties were investigated mostly by fluorescent lipid-mixing assays (Bonnafeous & Stegmann, 2003; Hinterdorfer, Baber, & Tamm, 1994; Markgraf et al., 2001; Stegmann et al., 1987). A fully functional reconstitution of intracellular membrane fusion was achieved only in 1998 (Weber et al., 1998), although it had been known before that soluble *N*-ethylmaleimide-sensitive factor activating protein receptors (SNAREs) are the main proteins that are responsible for intracellular membrane fusion (Söllner et al., 1993). However, the ultimate proof that SNAREs alone are capable of fusing two lipid bilayers came only from reconstitution experiments. Several other proteins have been discovered that regulate the function of SNAREs and their roles in intracellular membrane fusion will be surveyed in the Section 1 of this review.

The most frequently used fusion assays are bulk fusion assays that measure the fusion of many reconstituted protein-containing liposomes (proteoliposomes) in test tubes or spectrofluorimeter measuring cells. Since such samples typically contain millions of proteoliposomes, averages over all particles in the system are recorded in this class of experiments. Any fusion reaction consists of at least two steps: (1) a docking step, in which the two membranes bind to each other and (2) the actual fusion reaction that results in a single merged membrane. Since these two steps are not synchronized in ensemble measurements and since it is desirable to measure the fusion reaction per se without the preceding docking step to better understand its molecular mechanism, single liposome fusion experiments have been developed, which permit separate measurements of the docking and fusion reactions. In Sections 2 and 3 we will review a couple of different single liposome fusion approaches that have been developed to investigate the mechanism and regulation of SNARE-mediated membrane fusion. These new approaches to the reconstitution of membrane fusion have led to important new biological insights in how these systems work.

1. THE ROLE OF SNAREs AND ACCESSORY PROTEINS IN MEMBRANE FUSION

SNAREs represent a conserved protein family involved in many different intracellular fusion reactions. All SNAREs contain a conserved stretch of 60–70 amino acids arranged in heptad repeats, referred to as a SNARE motif. SNAREs represent the central catalysts of intracellular membrane fusion. The SNAREs mediating neuronal exocytosis include synaptobrevin (also called VAMP) residing on synaptic vesicles, and the plasma membrane-resident SNAREs syntaxin and SNAP-25 (Jahn & Fasshauer, 2012; Jahn & Scheller, 2006; Rothman, 2014).

SNAREs form a parallel four-helix bundle, one helix each contributed from syntaxin and synaptobrevin and two helices from SNAP-25 (Stein, Weber, Wahl, & Jahn, 2009; Sutton, Fasshauer, Jahn, & Brunger, 1998). The SNARE assembly–disassembly cycle is the engine that ultimately drives the irreversible fusion reaction. According to the “zippering model,” assembly of SNAREs in a “trans”-configuration (i.e., cross-bridging two membranes) is the key step in membrane fusion. Assembly is initiated at the N-terminal ends of the SNARE motifs and then progresses toward the C-terminal membrane anchors down a steep energy gradient, forming a tight connection between the membranes. This is followed by fusion, perhaps involving a hemifusion intermediate. After fusion, SNAREs convert from the “trans” to “cis” configuration. To reset the system after fusion and before subsequent clathrin-mediated endocytosis, SNAREs are disassembled by the *N*-ethylmaleimide-sensitive factor (NSF), which requires magnesium-ATP as an energy source and α -SNAP as a co-factor (Söllner et al., 1993).

Initiation and completion of assembly appear to be tightly controlled by accessory proteins including Munc18, Munc13, complexin, and synaptotagmin, all of which are capable of directly interacting with individual SNAREs and SNARE complexes. Some of these proteins not only bind to SNAREs but also interact with the lipid bilayer of the membrane, thus exerting a dual and synergistic control on membrane fusion (Jahn & Fasshauer, 2012; Rizo & Rosenmund, 2008; Sudhof & Rothman, 2009).

Nucleation of synaptobrevin binding critically depends on the presence of an acceptor complex consisting of SNAP-25 and syntaxin. While synaptobrevin is free to engage in SNARE complexes, syntaxin and SNAP-25 undergo complex homo- and heterooligomerizations that are well characterized in solution and that critically affect the ability to nucleate synaptobrevin binding. For instance, a SNAP-25/syntaxin binary complex readily recruits a second syntaxin molecule that occupies the synaptobrevin binding site, thus effectively blocking nucleation. These associations between syntaxin and SNAP-25 also occur in intact membranes (Halemani, Bethani, Rizzoli, & Lang, 2010). Moreover, syntaxin forms homooligomeric clusters in membranes that are cholesterol-dependent and that appear to be crucial for function (van den Bogaart et al., 2011; Lang et al., 2001; Murray & Tamm, 2009, 2011). The equilibria and thus the concentrations of different SNARE acceptor complexes are a critical determinant of fusion kinetics since only the binary 1:1 complex forms an acceptor site for synaptobrevin binding. Importantly, these equilibria are regulated by accessory proteins. Indeed, a stabilizing effect on the formation of a binary acceptor complex was reported for Munc13, Munc18, complexin, and synaptotagmin (Weninger, Bowen, Choi, Chu, & Brunger, 2008), and for the priming factor CAPS (James, Kowalchuk, Daily, Petrie, & Martin, 2009). A binary SNARE complex can be stabilized by a peptide corresponding to the C-terminal SNARE motif of synaptobrevin (Pobbati, Stein, & Fasshauer, 2006). This complex is not only homogeneous but allows for rapid synaptobrevin binding and fusion of liposomes that is at least an order of magnitude faster than without such stabilization.

Munc18 belongs to the family of SM-proteins that are essential for fusion and that exert their effects by directly binding to syntaxin. At least two different binding modes have been described: one involving binding to a closed conformation of syntaxin, in which the N-

terminal portion of the SNARE motif is bound to the N-terminal Habc three-helix regulatory domain of syntaxin, thus preventing syntaxin from entering SNARE complexes and a second in which a short peptide at the N-terminal end of the syntaxin is bound to the Munc18 protein (Burgoyne & Morgan, 2007; Carr & Rizo, 2010; Toonen & Verhage, 2007). It has been shown that in some cases Munc18 interacts with syntaxin simultaneously in both binding modes (Khvotchev et al., 2007; Shen, Tareste, Paumet, Rothman, & Melia, 2007). While earlier reports suggested that binding of the N-terminal peptide is needed for syntaxin to interact with SNAP-25, it appears that the N-terminal peptide must at least transiently be released for the binary complex to form (Burkhardt, Hattendorf, Weis, & Fasshauer, 2008). This lends support to the view that Munc18 is needed for the formation of the syntaxin/SNAP-25 acceptor complex (Rodkey, Liu, Barry, & McNew, 2008; Weninger et al., 2008; Zilly, Sorensen, Jahn, & Lang, 2006), perhaps in cooperation with Munc13 (Guan, Dai, & Rizo, 2008). It is controversial whether Munc18 remains bound to the SNARE complex after nucleation as proposed by most current models or whether it is displaced. Some suggested that Munc18 is displaced by synaptobrevin binding (Zilly et al., 2006), but others favor the view that Munc18 functionally interacts with the partially assembled *trans*-SNARE complex to drive fusion (Deak et al., 2009). Munc13 has been proposed to open the closed and Munc18-bound form of syntaxin allowing integration of other SNAREs into the trans-SNARE complex in preparation for full SNARE complex assembly and fusion (Ma et al., 2013).

Numerous studies involving mutated Munc18 in intact cells and animals have shown that the protein is absolutely essential for exocytosis (Toonen & Verhage, 2003). Munc18 has been observed to stimulate liposome fusion (Diao, Su, Lu, et al., 2010; Shen et al., 2007; Tareste, Shen, Melia, & Rothman, 2008), but it is unclear whether it exerts its effect during docking, fusion, or both, with support for the latter being provided by a study in which the two binding modes were selectively disrupted by mutagenesis (Deak et al., 2009). The original idea that Munc18 is needed for syntaxin to assume an open conformation as required for SNARE-binding (Misura, Scheller, & Weis, 2000) was challenged by the observation that a constitutively “open” syntaxin mutant does not bypass the need for Munc18 in exocytosis (Weimer et al., 2003).

Complexins are small proteins that bind with high affinity to the surface of the neuronal SNARE complex. While deletion of complexins generally results in an inhibition of calcium-dependent neurotransmitter release, not only facilitatory but also inhibitory effects of complexin have been observed, and thus no clear picture of their role has yet emerged (Brose, 2008; Rizo & Rosenmund, 2008; Sudhof & Rothman, 2009). SNARE-binding is mediated by a central helix that aligns in an antiparallel fashion in a groove formed by syntaxin and synaptobrevin (Bracher, Kadlec, Betz, & Weissenhorn, 2002; Chen et al., 2002; Pabst et al., 2000) and SNARE-binding by this helix is essential for function (Cai et al., 2008; Xue et al., 2007). Furthermore, mutations in the complexin binding site of synaptobrevin result in a phenotype similar to complexin deletion mutants, supporting the view that complexin exerts its function downstream of SNARE nucleation but upstream of calcium/synaptotagmin-mediated triggering of fusion (Brose, 2008; Maximov, Tang, Yang, Pang, & Sudhof, 2009). Recent results suggest that the N-terminal portion of complexin

competes with the C-terminal part of synaptobrevin in the final zippering step (Giraudo et al., 2009; Lu, Song, & Shin, 2010; Xue et al., 2007), exerting a block that is relieved by synaptotagmin which, according to this model, displaces complexin upon arrival of the calcium stimulus (Giraudo, Eng, Melia, & Rothman, 2006; Schaub, Lu, Doneske, Shin, & McNew, 2006; Tang et al., 2006). The results of the effects of complexin on liposome fusion are confusing: both inhibitory (Schaub et al., 2006) and stimulatory (Malsam et al., 2009; Yoon et al., 2008) effects were observed, with the latter apparently requiring the N-terminal domain and a putative phosphorylation site (Malsam et al., 2009). The accessory helix of a superclamp mutant of complexin was found in a crystal structure to cross over a neighboring partially assembled SNARE complex, giving rise to a zig-zag cross-linked arrangement of multiple SNARE complexes prior to a displacement of the complexin clamp and to fusion (Kümmel et al., 2011). However, this model has been challenged by structural and binding studies in solution that were complemented with neurotransmitter release experiments in neurons (Trimbuch et al., 2014). A model in which the accessory helix of complexin interacts electrostatically with the membrane accounts better for these latter results.

Synaptotagmin-1 functions as the calcium sensor for synchronous neuronal exocytosis. Synchronous events account for the largest fraction of calcium-triggered neurotransmitter release, and synaptotagmin-1 acts to synchronize and accelerate calcium-triggered membrane fusion. Overexpression of synaptotagmin-1 stabilizes fusion pores as measured by amperometry (Chapman, 2008), and genetically modifying synaptotagmin to alter its calcium affinity alters the calcium-sensitivity of fusion in mice (Sudhof & Rothman, 2009). Synaptotagmin-1 is a synaptic vesicle membrane protein, which contains two C2 domains (termed C2A and C2B) and is anchored to the membrane by a single N-terminal TM domain. Point mutations within the C2A or C2B domains of synaptotagmin impair fusion to varying degrees and also reduce the membrane affinity of the soluble C2 domains (Fernandez-Chacon et al., 2001, 2002; Mackler, Drummond, Loewen, Robinson, & Reist, 2002; Robinson, Ranjan, & Schwarz, 2002). In general, mutations in C2B appear to be more severe than mutations in C2A (Chapman, 2008). For example, some mutations that impair calcium binding in C2A do not alter fusion (Fernandez-Chacon et al., 2002; Robinson et al., 2002), while mutations that alter the calcium-binding residues of C2B severely impair calcium-dependent exocytosis (Mackler et al., 2002). However, tryptophan residues placed within the calcium-binding loops of either C2A or C2B shift the calcium requirements for exocytosis, presumably because they shift the membrane affinity of either domain (Rhee et al., 2005). Thus both domains are required for synaptotagmin function and appear to function cooperatively.

Synaptotagmin binds to membranes and interacts with SNAREs. The C2 domains of synaptotagmin penetrate into bilayer interfaces and interact via their first and third calcium-binding loops in a calcium-dependent manner (Bai, Tucker, & Chapman, 2004; Herrick, Sterbling, Rasch, Hinderliter, & Cafiso, 2006). The insertion of the C2 domains into lipid bilayers has been observed to induce positive curvature in bilayers (Martens, Kozlov, & McMahon, 2007), an event that may promote lipid mixing and the formation of a fusion stalk. While intriguing, these observations do not provide a molecular mechanism for this curvature induction and the role of curvature in fusion has not been tested. Synaptotagmin

also interacts with bilayers in a calcium-independent manner through the polybasic face of C2B (Bai et al., 2004; Kuo, Herrick, Ellena, & Cafiso, 2009). Although weaker than calcium-dependent binding, the affinity is sufficiently strong so that C2B will never be free in the cytosol. A soluble fragment of synaptotagmin comprising C2A and C2B has the capacity to aggregate pure lipid vesicles in a calcium-dependent manner. This appears to be mediated by the two (i.e., the calcium-dependent and calcium-independent) membrane-interacting surfaces of C2B (Xue, Ma, Craig, Rosenmund, & Rizo, 2008), and by a configuration of membrane-bound C2AB, which orients the membrane-binding surfaces of the two C2 domains in opposite directions (Herrick et al., 2009). The soluble C2AB domains of synaptotagmin were also found in a recent study to form rings of variable sizes on membranes in the absence, but not in the presence of calcium (Wang et al., 2014). If physiologically relevant, these rings could sterically prevent SNARE assembly in the regions they occupy, but promote SNARE assembly and fusion when the rings are disassembled by calcium.

Numerous studies have been directed at investigating protein–protein interactions between synaptotagmin and SNAREs (Chapman, 2008; Rizo, Chen, & Arac, 2006). Synaptotagmin is reported to interact with syntaxin and SNAP-25, but not synaptobrevin (Sudhof, 2002; Tucker & Chapman, 2002; Zhang, Kim-Miller, Fukuda, Kowalchuk, & Martin, 2002). Solution NMR experiments indicate that the binding of synaptotagmin to the SNARE complex is mediated primarily by the C2B domain; however, the data suggest that synaptotagmin–SNARE interactions may not be highly specific. Recent work using pulse EPR (Herrick et al., 2009) and single molecule FRET (Choi et al., 2010; Vrljic et al., 2010) are consistent with this view, although interesting differences are apparent when the results of these two approaches are compared. Some of these can be attributed to different salt concentrations used in the experiments. For example, EPR yields a different average configuration for C2AB when bound to SNAREs, and C2AB is observed to make multiple contacts with SNAREs, only one of which is reported by FRET. These differences are important, because they lead to different interpretations of the role of synaptotagmin at the site of fusion. In general, both membrane and SNARE interactions appear to play a role in synaptotagmin-mediated fusion. However, it is not yet clear at what step during SNARE assembly synaptotagmin might act.

The molecular mechanisms by which synaptotagmin synchronizes and promotes neuronal exocytosis are not understood. As indicated above, synaptotagmin might compete with complexin, or it might promote the close approach of bilayers, perhaps through charged membrane-binding sites on either side of C2B (Rizo & Rosenmund, 2008). Synaptotagmin may control fusion by directly interacting with the SNAREs (Chapman, 2008), perhaps transiently at some intermediate state. Synaptotagmin might also act by controlling SNARE assembly, perhaps by promoting the assembly of SNAP-25 onto membrane-associated syntaxin (Bhalla, Chicka, Tucker, & Chapman, 2006). The fact that well-documented aggregation effects of synaptotagmin on liposomes are often not controlled in fusion assays raises questions about some of the reported effects of synaptotagmin on fusion (Arac et al., 2006; Stein, Radhakrishnan, Riedel, Fasshauer, & Jahn, 2007). Finally, as mentioned above, synaptotagmin may assist SNARE-mediated fusion by promoting positive membrane curvature, thereby lowering the energy barrier to form the fusion pore (Hui, Johnson, Yao,

Dunning, & Chapman, 2009; Martens et al., 2007). However, both degree and kinetics of the effects of synaptotagmin vary widely depending on the experimental conditions (Arac et al., 2006; Tucker, Weber, & Chapman, 2004). Furthermore, no calcium-dependent acceleration was observed when membrane-anchored synaptotagmin (Stein et al., 2007) or synaptic vesicles that contain endogenous synaptotagmin are used (Holt, Riedel, Stein, Schuette, & Jahn, 2008). However, an interesting recent report indicates that a calcium-dependent acceleration of docking can be achieved using full-length synaptotagmin provided that calcium levels are kept in a low micromolar concentration range, and appropriate lipid compositions are used in vesicle and target membranes (Lee et al., 2010).

2. SNARE-MEDIATED FUSION OF SINGLE LIPOSOMES TO SUPPORTED MEMBRANES

Supported lipid bilayers (SLBs) have been popular to study membrane related processes for over 30 years. The planar geometry in combination with advanced fluorescence microscopy techniques like total internal reflection fluorescence microscopy (TIRFM) permits the design of experiments to test complex functions of membrane proteins reconstituted in vitro in near-natural environments with high signal-to-noise ratios. It is therefore not surprising that different laboratories have utilized SLBs to study SNARE-mediated membrane fusion of single liposomes with SLBs. In all these works, the SLB mimics the postsynaptic plasma membrane and contains the SNAREs syntaxin and SNAP-25. Liposomes containing synaptobrevin and in some cases also synaptotagmin mimic the synaptic vesicle. The lipid compositions used and the state of the proteins within the membrane differ substantially between the approaches used in different laboratories and often depend on the methods that were employed for their preparation. It is important to distinguish between two fundamentally different techniques for SLB formation and thus SNARE protein reconstitution: direct vesicle fusion (VF) to a clean hydrophilic substrate and a combined Langmuir–Blodgett transfer/vesicle fusion (LB/VF) method (Figure 1).

2.1 SNARE RECONSTITUTION INTO SLBs BY THE VF TECHNIQUE

Owing to the simplicity of preparation the direct VF method (Brian & McConnell, 1984; Kalb, Frey, & Tamm, 1992) has been preferred by most laboratories to reconstitute SNARE-mediated liposome fusion to SLBs. Here, an acceptor complex containing syntaxin and SNAP-25 (t-SNAREs) or syntaxin by itself are reconstituted into small unilamellar vesicles by detergent removal and added to a carefully cleaned glass substrate. After an incubation time of 1–3 h unfused liposomes are washed away (Figure 1(A)). The technique to observe single liposomes fusing with the SBL is similar between all laboratories and was first described by Fix et al. (2004) who were also the first to implement a single liposome/SLB SNARE fusion assay. Synaptobrevin-containing liposomes are labeled with one or two fluorescent markers and added to the t-SNARE-containing SLB on a TIRF microscope. Fluorophores in liposomes that enter the evanescent field close (within ~100 nm) to the membrane get excited and the fluorescence is recorded by a sensitive camera. Docking, i.e., the immobilization of liposomes at the membrane surface, is recognized in these assays by a fluorescence signal that after its appearance does not change its location or intensity unless fusion or undocking occurs (Figure 2(A)). Fusion of membrane-labeled liposomes with the

SLB is characterized by a sudden increase or decrease due to the orientation change of the fluorophores followed by a slower decrease of fluorescence due to the diffusion of fluorophores into the plane of the SLB (Fix et al., 2004) (Figure 2(C)). Although the time resolution of this first study did not allow a detailed kinetic analysis, it already showed that SNAREs without any additional factors are capable to fuse membranes much faster than the previously observed kinetics of bulk liposome fusion assays that are dominated by the slower docking step (Hernandez, Kreutzberger, Kiessling, Tamm, & Jahn, 2014). However, the fusion efficiency, i.e., the ratio of fused to all docked liposomes, was very low (0.35%) when full-length syntaxin was used and was increased by the addition of divalent cations like calcium (15%) or magnesium (4%) and/or by using syntaxin that had its regulatory N-terminal Habc domain removed (11%). Observing not only the fluorescence from labeled lipids, but also from calcein, which was encapsulated inside the liposomes, Bowen, Weninger, Brunger, and Chu (2004) reported similar fusion efficiencies (10–15%) independent of calcium in their single liposome/SLB fusion assay. Systematic experiments showed, however, that while the amount of docking was proportional to the amount of syntaxin reconstituted into the SLB, SNAP-25 was not needed for docking or fusion. Moreover, fusion, while depending on the presence of syntaxin in the SLB and synaptobrevin in the liposomes, had to be stimulated by either heating from the exciting laser light or an external heat source. The dependence on divalent ions or heat in these first studies and the unusual lack of a requirement on SNAP-25 in the second study indicated that something was wrong with these early “restitutions” because well-established physiological properties of SNARE-mediated fusion could not be reproduced even though the basic methodology of the single liposome/SLB assay appeared to have worked. Moreover, measurements of the lateral mobility of the reconstituted SNAREs in the SLBs revealed that in contrast to lipids only a small fraction of the proteins (3–7%) were mobile (Bowen et al., 2004).

Weisshaar and coworkers used a similar approach and characterized their SNARE-containing SLBs by atomic force microscopy (AFM). They found that the incorporation of larger amounts of protein (protein:lipid (p/l) 1:300) led to aggregated lipid/protein material on the substrate that was not capable of docking of synaptobrevin proteoliposomes (Liu, Tucker, Bhalla, Chapman, & Weisshaar, 2005). However, when they used 100 times lower protein concentrations, fast (25 ms) fusion of the proteoliposomes to the SLBs was observed. But, as in the study of Bowen et al., SNAP-25 was not required and syntaxin in the SLB and synaptobrevin in the liposomes were sufficient to promote docking and fusion. Calcium and magnesium had no effect on the efficiency or kinetics of fusion in this study. The full potential of the single liposome/SLB assay was shown in two follow-up publications by the Weisshaar group, in which hemi- and full-fusion events were distinguished and release of soluble contents from the liposomes upon full fusion was recorded (Liu, Wang, Chapman, & Weisshaar, 2008; Wang, Smith, Chapman, & Weisshaar, 2009). While the fluorescence signal from the membrane label was sufficient to discriminate between the merger of the outer and inner leaflets of the SLBs and liposomes (Liu et al., 2008), two-color measurements showed that the content dye calcein stayed enclosed in the liposome until the inner leaflets of the two membranes fused (Wang et al., 2009) (Figure 2(B)). However, movies of fusion events with 1 ms time resolution showed that the content

was not released into the narrow cleft between the membrane and substrate as would be expected for a controlled fusion reaction between the two membranes, but escaped into the bulk solution above the membrane. A systematic study showed that more hemifusion was observed with increasing concentrations of phosphoethanolamine (PE) lipids in the liposomes and the SLB (11 et al., 2008).

Rothman and coworkers modified the above assays by using microfluidic flow channels that do not depend on TIRFM to detect single liposome fusion and by doping the SLB with polymer-conjugated lipids (Karatekin et al., 2010). The polymer most likely increases the space between the substrate and bilayer and reduces unspecific docking of the liposomes. The fusion efficiency in this assay was increased to ~50% and all three neuronal SNAREs were required for fusion. The fusion efficiency dropped dramatically when the number of synaptobrevins per liposome decreased from approximately 100 to 10–20. Based on this result and the assumption that half of the reconstituted synaptobrevins were oriented with their SNARE domains pointing into the lumen of the liposomes, the authors concluded that approximately 5–10 SNARE complexes are necessary to promote fast membrane fusion.

The requirement for low protein concentrations in the SLB (protein/lipid <1: 5000) of the assays described above is likely caused by the random orientation of the proteins in the proteoliposomes and SLBs and the preparation technique. SNAREs with their SNARE domains facing outward in the liposome interact with the substrate when the SLB is formed by the direct VF technique (Bowen et al., 2004), where they become immobilized and unavailable for SNARE complex formation. Although the reported mobility of properly oriented syntaxins is low even at these low protein concentrations, this might not interfere with the protein's ability to promote docking and fusion. At higher protein concentrations aggregation caused by the incorrectly oriented SNAREs might render all proteins in the SLB inactive.

2.2 SNARE RECONSTITUTION INTO SLBs BY THE LB/VF TECHNIQUE

Preparation of SLBs by the combined LB/VF method is a two-step process: First, a lipid monolayer is transferred from the air–water interface of a Langmuir trough to a carefully cleaned substrate. In a second step, proteoliposomes are added to the dry supported monolayer to form the second leaflet of the SLB (Kalb et al., 1992) (Figure 1(B)). The lipid compositions of the two leaflets can be different and this compositional asymmetry is maintained for several hours (Crane, Kiessling, & Tamm, 2005; Kiessling, Crane, & Tamm, 2006). The first monolayer can be doped with polymer-conjugated lipids to improve protein mobility and bilayer stability (Wagner & Tamm, 2000). SNARE proteins reconstituted into the SLB are preferentially oriented with their large cytosolic domains facing away from the substrate and 83% of the protein stay mobile even at higher protein densities (p/l 1:500) (Liang, Kiessling, & Tamm, 2013; Wagner & Tamm, 2001). The first functional SLB reconstitution of a syntaxin/SNAP-25 acceptor complex showed that it readily forms the ternary SNARE complex in the membrane and that the acceptor complex interacts strongly with negatively charged lipids like phosphatidylserine (PS) and phosphatidylinositol-4,5-bisphosphate (PIP2) (Wagner & Tamm, 2001). Later, it was revealed that the ternary SNARE complex stays upright in a POPC bilayer, indicating a rigid linker region between

the *trans*-membrane domain and the SNARE domain of syntaxin under these conditions (Kiessling & Tamm, 2003).

Fast 20 ms single liposome fusion in this system was observed when an acceptor complex consisting of syntaxin, SNAP-25, and a peptide representing the C-terminal half of the SNARE motif of synaptobrevin were reconstituted into a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) SLB containing 20% cholesterol (Domanska, Kiessling, Stein, Fasshauer, & Tamm, 2009). The synaptobrevin peptide ensures a 1:1 stoichiometry between syntaxin and SNAP-25 and prevents the formation of a 2:1 syntaxin/SNAP-25 complex that in vitro prevents fast docking and fusion (Pobbati et al., 2006). Docking of synaptobrevin-containing liposomes is proportional to the density of SNARE acceptor complexes in the membrane up to protein/lipid ratios of ~1:2000. Almost no docking was observed when syntaxin without SNAP-25 was reconstituted or when the full 96-residue SNARE motif of synaptobrevin was added to the acceptor complex containing SLB. Fusion in this assay was independent of calcium or heat and thus represented the first reliable functional reconstitution of SNARE-mediated fusion in the supported membrane format. From an evaluation of more than 1000 fusion events a kinetic model was derived that revealed that eight independent reactions occur between docking and the onset of fusion (Domanska et al., 2009). This model is consistent with the interpretation that eight SNARE complexes are necessary to promote fast fusion under these conditions. Later it was found with the same experimental setup that the number of parallel reactions is modulated by the lipid environment. Increasing the amount of phosphatidylethanolamines in the SLB or the synaptobrevin-containing liposomes reduced the number of required reactions and thus potentially the number of formed SNARE complexes to three (Domanska, Kiessling, & Tamm, 2010). The fact that the number of SNAREs catalyzing fusion is variable and that it can be adjusted to the requirements needed under certain conditions was later shown by comparing the fusion efficiencies of large and small liposomes (Hernandez et al., 2014).

During synaptic transmission, synaptic vesicles fuse with the plasma membrane in order to release neurotransmitter into the synaptic cleft. To prove their physiological relevance, it is desirable to demonstrate that in vitro fusion assays with reconstituted liposomes mimic the fusion behavior of synaptic vesicles and that not only lipids are mixed, but also contents are released through the fusion pore. As described above, membrane labels in the liposomes are already well suited to distinguish between full- and hemifusion in an SLB assay (see Figure 2). In fact, using a polarized TIRFM assay it can be shown that the complete labeled lipid content of fusing spherical liposomes collapse into the plane of the membrane within 10 ms (Kiessling, Domanska, & Tamm, 2010). Encapsulating rhodamine as content marker into the liposomes shows that the observed full-fusion events always coincide with content release into the narrow cleft between substrate and SLB (Kiessling et al., 2013). The specificity and relevance of the SNARE-SLB fusion assay was further tested by using membrane-labeled purified synaptic vesicles from rat brain, showing that synaptic vesicles fuse with SLBs as efficiently and fast as their counterparts that were reconstituted from purified components (Kiessling et al., 2013). Fusion efficiencies were 21% in the absence and 43% in the presence of calcium, similar to the efficiencies observed with the reconstituted synaptobrevin liposomes with and without added calcium. When synaptotagmin was co-

reconstituted into synaptobrevin liposomes, the fusion efficiencies were 39% in the absence of calcium and 82% in the presence of calcium. The relatively modest calcium dependence observed in these experiments, even in the presence of phosphatidylserine and PIP2 in the SLB, is not yet sufficient to mimic the high calcium sensitivity of synaptic VF *in vivo*. Thus, the molecular players that efficiently suppress fusion in the absence of calcium and render the neuronal fusion machinery strictly calcium-dependent have yet to be found in this system. Because the observed fusion kinetics were very fast (4–200 ms) and the efficiencies were high (20–80%), it will be very interesting to discover the factors that block fusion before calcium is added and couple the action of calcium-synaptotagmin to the release of this block. These factors may not just be proteins, but the activity of SNAREs may also be strongly modulated by conformational equilibria of the SNAREs in membranes with changing lipid compositions (Liang et al., 2013). Therefore, functional reconstitution strategies must not only consider the minimal sets of required proteins, but also their contexts in the lipid bilayer. Functional reconstitutions of SNARE and other proteins in SLBs combined with single liposome assays and structural studies offer unprecedented opportunities to further unravel the inner workings of the calcium and SNARE-controlled neuronal fusion machine.

3. SNARE-MEDIATED FUSION OF INDIVIDUAL SURFACEIMMOBILIZED LIPOSOMES

The principle of SNARE-mediated fusion of single surface-immobilized liposomes is similar to bulk fusion assays. Two sets of SNAREs, t-SNAREs and v-SNAREs, are reconstituted into separate sets of liposomes, and the encounter of liposomes with opposing SNAREs then leads to docking and fusion of liposomes. The main difference between bulk and this class of single liposome fusion assays is that one set of vesicles is immobilized on the surface of a substrate with a biotin–neutravidin–biotin system and, as in the single liposome-to-SPB fusion assays, the spatial resolution and high signal-to-noise ratio of TIRFM is utilized to detect the binding (docking) and fusion of single SNARE liposomes to immobilized liposomes with the complementary SNAREs (Figure 3).

To immobilize liposomes, quartz or glass surfaces are first coated with polyethyleneglycol (PEG)/biotin-PEG. The PEG/biotin-PEG surface is treated with neutravidin and then with SNARE-liposomes containing a small amount of biotinylated lipids. The presence of the PEG cushion eliminates the nonspecific binding of liposomes to the substrate and the biotin/neutravidin system controls the specific binding of desired liposomes. Subsequently liposomes containing the other type of SNAREs are flowed into the measuring chamber and allowed to dock and fuse with the immobilized liposomes. Since there are two different sets of SNARE proteins, either the t- or v-SNARE liposomes may be immobilized on the surface. Both configurations have been successfully employed to recapitulate different aspects of SNARE-mediated membrane fusion (Kyoung et al., 2011; Yoon, Okumus, Zhang, Shin, & Ha, 2006). As in all other assays, fusion may be monitored by lipid mixing and/or content mixing. To measure lipid mixing, fluorescent lipid labels are incorporated into the liposomes and the change of the TIRFM signal upon lipid mixing is recorded by measuring FRET to an acceptor fluorophore on an opposing liposome (Figure 3(A)–(C)) (Yoon et al.,

2006) or by measuring fluorescence dequenching due to dilution (Figure 3(D)–(F)) (Kyoung, Zhang, Diao, Chu, & Brunger, 2013). To monitor content mixing soluble labels are encapsulated in one type of proteoliposomes (Diao, Su, Ishitsuka, et al., 2010; Kyoung et al., 2011), and analogous dequenching or FRET techniques are employed to detect fusion. It is highly desirable to combine measurements of lipid and content mixing in the same experiments because such experiments readily distinguish between hemifusion and full-fusion events (Diao, Zhao, Zhang, Kyoung, & Brunger, 2013) and also detect spurious fusion events involving content release into the bulk solution that may happen when proteoliposomes burst rather than fuse (Wang et al., 2009).

Yoon et al. (2006) observed multiple lipid mixing intermediates in a FRET-based assay, in which v-SNARE-containing proteoliposomes were immobilized. Using yeast SNAREs, this study proved for the first time that the immobilized proteoliposome system can be used to dissect individual intermediate states of the SNARE-mediated fusion pathway. With neuronal SNAREs, the same group showed that complexin suppresses SNARE complex formation and liposome docking but, once docked, promotes SNARE complex stabilization and fusion. It also showed that the presence of 1 mM calcium can increase lipid mixing in this system (Yoon et al., 2008). The single liposome assay was critical for observing the opposing effects of complexin because the different effects on docking and fusion could be differentiated, which is not possible in ensemble fusion assays. Munc18 was also shown to stimulate SNARE-mediated lipid mixing with both full-length syntaxin and truncated-syntaxin that had its regulatory Habc and N-terminal peptide Munc18 binding domains removed (Diao, Su, Lu, et al., 2010). The authors concluded that this stimulation effect of Munc18 originates from its binding to the SNARE complex. In a system with reconstituted synaptobrevin and full-length synaptotagmin in the immobilized liposomes and the acceptor t-SNARE complex in the opposing liposomes, Lee et al. (2010) showed that the presence of 10 μ M calcium accelerates liposome docking, whereas increasing the calcium concentration to 100 μ M aborted this acceleration. Although this study showed that synaptotagmin can stimulate vesicle docking under a physiological calcium concentration, the stimulating effect on lipid mixing is not clearly demonstrated and moreover the stimulated docking effect is greatly attenuated when 3 mol% PS lipid content was increased to the more physiological 15 mol% PS content in the synaptobrevin/synaptotagmin liposomes (Takamori et al., 2006). The stimulating effects of calcium and synaptotagmin were revisited by lipid- and content-mixing assays (Lai, Diao, et al., 2013). Interestingly, a monotonic increase of the fusion efficiency was observed upon increasing the calcium concentration (up to 1 mM). Since the content mixing signal showed a marked delay when a larger content marker was used (Diao, Su, Ishitsuka, et al., 2010), the authors hypothesized that this delayed opening provided evidence for a pathway for SNARE-mediated liposome fusion to undergo either kiss-and-run or full fusion (Lai, Diao, et al., 2013). It is also of interest to note that in this system SNAREs alone can promote lipid mixing (hemifusion); however, they are not effective in promoting full fusion (Lai, Diao, et al., 2013), in contrast with what has been observed in bulk ensemble fusion experiments (Weber et al., 1998) and in the single liposome to supported membrane fusion studies described in the previous section (Domanska et al., 2009). Mutations in the synaptotagmin linker region that connects the transmembrane helix and tandem C2 domains indicated that charge segregation in this region is important for a

fast opening of the fusion pore, but it has no effect on liposome docking (Lai, Lou, Jho, Yoon, & Shin, 2013).

Brunger and collaborators introduced a single surface-immobilized liposome assay with the distinct characteristic that it could be triggered by calcium. In this setup, the t-SNARE-containing liposomes were immobilized through a biotin/ neutravidin/biotin linkage on the substrate, and lipid and content double-labeled liposomes containing v-SNAREs and synaptotagmin were flowed into the chamber. After a period of incubation and buffer wash, docked but not-yet fused liposome complexes were triggered by calcium influx to initiate fusion, as observed by fluorescence dequenching of the lipid and content labels (Diao, Zhao, et al., 2013; Kyoung et al., 2011, 2013). Soluble regulatory proteins, such as complexin may be added through buffer washes in this experimental setup. Employing this approach, Kyoung et al. (2011) showed that docked liposomes underwent either immediate full fusion, delayed full fusion, hemifusion only, or just remained docked. The fusion efficiency in this system is relatively high, with a majority (61%) of liposomes undergoing complete fusion when full-length synaptotagmin and complexin were co-reconstituted. An analysis of the time delays showed that more than 20% of all full fusion events occurred within the first 200 ms after the calcium trigger, which itself was monitored separately with a calcium-sensitive dye. A double exponential fit of all full-fusion events revealed a fast fusion component with a time constant of 250 ms. In contrast, in a system with neuronal SNAREs alone, only 20% of interacting vesicles exhibited full fusion with a slow time constant of 3 s. It should be noted that the calcium-triggered fusion required calcium concentrations as high as 5 mM in this system. At this calcium concentration, rapid fusion and docking efficiencies were enhanced by complexin, whereas at a lower calcium concentration, complexin had little effect. With a modified experimental protocol and a reportedly improved quality of the proteins, the calcium sensitivity could be improved to 250–500 μ M (which is still $\sim 10\times$ physiological) and all fast fusion events were found to bypass hemifusion and proceed directly to full fusion upon calcium triggering (Diao, Zhao, et al., 2013). In contrast, initially hemifused states were slow to fuse. At this lower calcium concentration, the inclusion of complexin was found to shift fusion toward the immediate fusion pathway, effectively synchronizing fusion (Diao et al., 2012). The presence of complexin increased the fusion efficiency at 500 μ M calcium 85-fold from the very low spontaneous fusion efficiency of 0.02% to 1.7%, whereas in the absence of complexin the respective fusion efficiencies increased sixfold from 0.1% to 0.6% (Lai et al., 2014). The authors took the apparent more than 10-fold efficiency increase as evidence for the ability of complexin to synchronize SNARE-mediated fusion. The application of various mutants of complexin revealed that the N-terminal domain of complexin is more important for fusion synchronization by complexin, whereas membrane binding of the C-terminal domain suppressed spontaneous fusion (Diao, Cipriano, et al., 2013; Lai et al., 2014).

These studies of SNARE-mediated fusion with surface-immobilized liposomes demonstrated a clear separation of the docking and fusion steps and revealed fusion kinetics on the order of hundreds of millisecond, i.e., much faster than the overall fusion rates measured in bulk fusion assays, which are on the order of tens of minutes. The fastest kinetics were observed to be 250 ms in the 5 mM calcium-triggered fusion experiments when SNARE-mediated fusion was further boosted with synaptotagmin and complexin.

Perhaps surprisingly, these kinetics are still an order of magnitude slower than the 20 and 4 ms kinetics revealed by the SNARE-mediated fusion of single liposomes to SLBs described above (Domanska et al., 2009; Kiessling et al., 2013). The difference is even more striking considering that in the calcium-triggered immobilized liposome experiments the SNARE complexes start from a tethered conformation, whereas in the supported bilayer studies time zero is defined as the time when liposomes initiate docking onto the bilayer. It is unlikely that the different geometrical constraints of the two systems are sufficient to explain the sizable difference in the fusion kinetics. The immobilized liposomes may decrease the energy barrier and thus increase the rates of membrane fusion relative to those measured with planar membranes, but the relatively large liposomes used in these studies are not likely to have large curvature effects on the rates of fusion. It is possible that surface-immobilized liposomes are entangled in fashions that limit their rates and efficiencies of fusion. In summary, it is clear that multiple factors that include details of protein preparation, the stoichiometric compositions of prepared acceptor t-SNARE complexes, the orientation preferences of SNAREs on the liposome and supported bilayer surfaces, as well as lipid compositions and curvatures of the reconstituted proteoliposomes all contribute to the measured fusion kinetics in immobilized liposome and supported bilayer systems.

OUTLOOK

The reconstitution of intracellular membrane fusion has made dramatic progress over the past 10–15 years. After the introduction of successful reconstitutions of intracellular membrane fusion in bulk fusion assays, single liposome methods have been developed that have lead to significant new insights into the molecular mechanism of intracellular SNARE-mediated membrane fusion. Despite this progress, major questions remain. For example, no consensus has been reached yet about the speed of SNARE-mediated fusion reactions in the reconstituted in vitro assays. While the fastest single liposome assays reach fusion rates on the order of 10 ms, i.e., only about an order of magnitude slower than the speed of fusion in synaptic exocytosis, others have reached much slower fusion rates. Some of the discrepancies may be attributed to the definition of the starting point of fusion in the different assays, but others may very well arise from different properties and states of the reconstituted proteins. In our opinion, the characterization of the reconstituted SNAREs in the initial liposomes has not received the full attention it deserves. SNAREs undergo multiple conformational equilibria that depend to a great deal on the lipids and physical states of model membranes in which they are embedded, on accessory proteins like Munc18, and on their respective concentrations in the reconstituted membranes. These factors should all be better controlled and standardized in future experiments. Another question that has occupied the field for quite some time is the question of how many SNARE complexes need to form to open a productive fusion pore. While numbers have ranged from 1 to about 14, some consensus now appears to have been reached. This stoichiometry is not fixed but depends, among other factors, on the lipid composition and size, i.e., curvature, of the reconstituted liposomes (Hernandez et al., 2014). Unless the liposomes are unusually small or relatively inert lipids are used, we believe that on the order of three or four SNARE complexes are needed to open a fusion pore (Domanska et al., 2010). A very important question for the future will be to resolve how calcium and synaptotagmin control the

opening of the fusion pore by presynaptic neuronal SNAREs. Wildly different calcium sensitivities of reconstituted synaptotagmin-controlled fusions have been reported in the literature. Most if not all of these assays have used relatively slow and somehow suppressed rather than optimized fusion assays. In our opinion, the factor that physiologically suppresses neuronal SNARE-mediated membrane fusion under optimized fusion conditions has not yet been found. Neuronal SNAREs promote fast fusion in optimized assays without the action of calcium and synaptotagmin, which clearly differs from what happens in the cell. Therefore, a very important future goal in this field must be the functional reconstitution of the molecular break that arrests neuronal SNARE-mediated fusion in the absence of calcium, but releases the break at full (millisecond) speed upon the addition of physiological (a few tens of micromolar) concentrations of calcium. Reconstitutions of intracellular membrane fusion have clearly opened many new windows to look at these important cellular functions, but despite the appearance that much is understood in this field, it is very clear that many very fundamental questions regarding the pertinent molecular mechanisms still await answers. We are convinced that the tools described in this review will continue to provide these answers in the not too distant future.

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FIGURE 1. Reconstitution of SNARE proteins into supported lipid bilayers
(A) Direct vesicle fusion (VF). (B) Langmuir–Blodgett/vesicle fusion (LB/VF).

FIGURE 2. Signal readout from single liposome-supported lipid bilayer fusion assays
 (A) Liposomes are labeled with a membrane lipid label (red (dark gray in print versions)) and a soluble content label at self-quenching concentrations (green (very dark gray in print versions)). Upon docking the membrane label gets excited by the evanescent field of a total internally reflected laser beam (not shown), resulting in a sharp increase of the observed (membrane-) fluorescence. (B) Upon hemifusion the outer leaflets of the liposome and the supported lipid bilayer merge allowing labeled lipids to diffuse into the plane of the supported lipid bilayer. The orientation change of the fluorophores relative to the evanescent field increases or decreases the observed fluorescence depending on the polarization of the exciting light. The subsequent diffusion of the fluorophores away from the fusing liposome decreases the observed fluorescence to about half of its original value. (C) Upon full fusion both leaflets of the liposome and the supported lipid bilayer merge allowing labeled lipids to diffuse into the plane of the supported lipid bilayer. The orientation change of the fluorophores relative to the evanescent field increases or decreases the observed fluorescence depending on the polarization of the exciting light. Opening of the fusion pore allows content dye to diffuse into the cleft between substrate and membrane thereby dequenching the (content-) fluorescence. The subsequent diffusion of membrane- and content-fluorophores away from the fusing liposome decreases the observed fluorescence to zero.

FIGURE 3. Signal readout from single liposome–liposome fusion assays

(A–C) FRET assay. Liposomes labeled with donor lipid fluorophores (green (very dark gray in print versions)) are added to surface-immobilized acceptor liposomes labeled with acceptor lipid fluorophores (red (dark gray in print versions)). (A) Upon docking the donor fluorophore gets excited by the evanescent field of a total internally reflected laser beam (not shown), resulting in a sharp increase of the observed donor fluorescence. (B) Upon hemifusion the outer leaflets of the two liposomes merge allowing donor and acceptor fluorophores to diffuse within the connected leaflet. FRET between about half of all donor

and acceptor fluorophores decreases the donor fluorescence and increases the acceptor fluorescence. (C) Upon full fusion both leaflets of the two liposomes merge allowing donor and acceptor lipid fluorophores to diffuse within the connected bilayer. FRET between all donor and acceptor fluorophores decreases the donor fluorescence and increases the acceptor fluorescence. (D–F) Dequenching assay. (D) Observation starts in the docked state. Membrane (red (dark gray in print versions)) and content (green (very dark gray in print versions)) label fluorescence are quenched due to the high dye concentrations in the respective compartments. (E) Upon hemifusion the outer leaflets of the two liposomes merge allowing labeled lipids to diffuse into the immobilized unlabeled liposome. The dilution of about half of the labeled lipids dequenches the fluorescence signal. (F) Upon full fusion both leaflets of the two liposomes merge allowing labeled lipids to diffuse into the immobilized unlabeled liposome. The dilution of all labeled lipids dequenches the fluorescence signal. The merging of the two liposome lumina dequenches fluorescence signal from the content label.