Calcium-independent stimulation of membrane fusion and SNAREpin formation by synaptotagmin I

Lara K. Mahal, Sonia M. Sequeira, Jodi M. Gureasko, and Thomas H. Söllner

Cellular Biochemistry and Biophysics Program, Memorial Sloan Kettering Cancer Center, New York, NY 10021

Neurotransmitter release requires the direct coupling of the calcium sensor with the machinery for membrane fusion. SNARE proteins comprise the minimal fusion machinery, and synaptotagmin I, a synaptic vesicle protein, is the primary candidate for the main neuronal calcium sensor. To test the effect of synaptotagmin I on membrane fusion, we incorporated it into a SNARE-mediated liposome fusion assay. Synaptotagmin I dramatically stimulated membrane fusion by facilitating SNAREpin zippering. This stimulatory effect was topologically restricted to v-SNARE vesicles (containing VAMP 2) and only occurred in trans to t-SNARE vesicles (containing syntaxin 1A and SNAP-25). Interestingly, calcium did not affect the overall fusion reaction. These results indicate that synaptotagmin I can directly accelerate SNARE-mediated membrane fusion and raise the possibility that additional components might be required to ensure tight calcium coupling.

Introduction

Interneuronal communication and information processing in the brain is mediated by the quantal release of neurotransmitters triggered by the influx of calcium into the nerve terminal. These calcium-induced events occur on the submillisecond timescale, thus suggesting that the calcium sensor is tightly coupled to the machinery that fuses neurotransmitter-containing synaptic vesicles with the plasma membrane. SNAREs, a family of compartment-specific transmembrane proteins, initiate membrane fusion when a v-SNARE on a transport vesicle pairs with its cognate t-SNARE on the target membrane, forming SNAREpins (trans SNARE complexes) (Söllner et al., 1993; Weber et al., 1998). In the case of neurotransmitter release, the synaptic vesicle-bound v-SNARE VAMP 2 interacts with its cognate t-SNARE, consisting of syntaxin 1 and SNAP-25, on the presynaptic plasma membrane. Subsequent stepwise SNARE assembly into a four helix bundle brings the two membranes in close apposition and drives lipid bilayer mixing (Sutton et al., 1998; Weber et al., 1998; Nickel et al., 1999; Chen et al., 2001). Regulatory components, such as the calcium sensor, may alter the kinetics of SNARE complex assembly at distinct stages by trapping or accelerating reaction intermediates.

The prime candidate for a calcium sensor in the central nervous system is synaptotagmin I, a type I transmembrane protein localized on synaptic vesicles (Brose et al., 1992; Südhof and Rizo, 1996). Gene deletions of synaptotagmin I in Mus musculus, Caenorhabditis elegans, and Drosophila melanaster all demonstrate a marked loss of calcium-evoked fusion (Littleton et al., 1993; Nonet et al., 1993; Broadie et al., 1994; Geppert et al., 1994). In addition, replacement of endogenous synaptotagmin I in mice with a mutant version that has a twofold lower affinity for calcium in the presence of phospholipid membranes causes a concomitant decrease in the calcium sensitivity of neurotransmitter release (Fernández-Chacon et al., 2001). These results provide compelling in vivo evidence that synaptotagmin I is the major calcium sensor for fast synaptic exocytosis. At the molecular level, synaptotagmin I is characterized by two conserved cytoplasmic calcium-binding domains, C2A and C2B, which interact in a calcium-dependent manner with the synaptic t-SNAREs, acidic lipids, and phosphoinositides (Bennett et al., 1992; Brose et al., 1992; Schiavo et al., 1996, 1997; Davis et al., 1999; Gerona et al., 2000; Earles et al., 2001). In addition, the C2B domain of synaptotagmin I is responsible for the homo- and heterooligomerization of synaptotagmins (Osborne et al., 1999; Desai et al., 2000) of which there are 13 known mammalian isoforms (Kelly, 1995; Augustine, 2001; Südhof, 2002). Recent electrophysiological studies of dense core vesicle fusion in PC12 cells have shown that overexpression of synaptotagmins with different calcium affinities differentially modulate fusion pore kinetics (Wang et al., 2001). These experiments further link synaptotagmin I to the actual fusion process.
Despite strong evidence that synaptotagmin I couples calcium sensitivity to vesicle fusion, the molecular mechanisms by which it acts remain elusive. Two main hypotheses exist about how synaptotagmin I might regulate membrane fusion: (1) synaptotagmin I acts as a clamp, preventing fusion of docked vesicles until an influx of calcium releases the clamp, and (2) synaptotagmin I stimulates fusion upon the influx of calcium. In addition, there are variations of these models that require additional components to control calcium-triggered exocytosis (Kelly, 1995).

Deconvolution of the mechanism of action of synaptotagmin I in vivo has been hindered by the degeneracy of synaptotagmin isoforms and the difficulty of separating the molecular functions of synaptotagmin I from those of other accessory proteins in the complex cellular environment. Thus, we used a simplified in vitro model of membrane fusion that allowed us to examine the role of synaptotagmin I in a defined and accessible system (Weber et al., 1998). This approach has been used previously to establish that SNAREs are the minimal machinery for membrane fusion (Weber et al., 1998) and to show that SNARE-mediated fusion is specific and topologically restricted (Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000). In addition, this assay has demonstrated that the NH$_2$-terminal regulatory domain (NRD)* of syntaxin I not only regulates t-SNARE assembly but also effects SNAREpin formation (Parlati et al., 2000). However, the relatively slow fusion kinetics of the in vitro system, in comparison with those of regulated exocytosis in vivo, indicate that additional components may be required to accelerate the overall reaction. In this article, we test the effect of synaptotagmin I on SNARE-mediated fusion, and we examine its putative regulatory roles using the in vitro fusion assay.

Results

**Sytg I reconstituted into v-SNARE vesicles accelerates SNARE-dependent fusion**

We monitored membrane fusion using a fluorescent resonance energy transfer–based lipid mixing assay (Struck et al., 1981). Briefly, when two fluorescent lipids, nitro-2,1,3-benzoazadiazole 1,2-dipalmitoyl-SN-glycero-3 (NBD)–phosphatidylethanolamine (PE) and lissamine rhodamine B 1,2-dipalmitoyl-SN-glycero-3–PE (rhodamine-PE) are incorporated at appropriate concentrations (0.8 mol%) into donor vesicles, the fluorescence signal emitted by NBD-PE is quenched by rhodamine-PE. Fusion of the labeled donor vesicles with unlabeled acceptor vesicles dilutes the fluorescent lipids causing a distance-dependent increase in NBD-PE fluorescence over time. Finally, the NBD signal is normalized to the maximum fluorescence signal obtained by infinite dilution in the presence of detergent and plotted as the percentage of the total fluorescence versus time. In the following experiments, VAMP 2 (v-SNARE) was reconstituted into labeled donor liposomes, and coexpressed syntaxin 1 and SNAP-25 (t-SNAREs) were incorporated into unlabeled acceptor liposomes (Weber et al., 1998).

To facilitate synaptotagmin I expression and reconstitution into liposomes, we used a modified NH$_2$-terminal truncated synaptotagmin I (Sytg I), which begins at aa 57 (see Material and methods for details). Donor liposomes containing reconstituted VAMP 2, VAMP 2 and Sytg I (Sytg I–VAMP 2), and Sytg I alone (Fig. 1 A) were prepared using standard protocols (Weber et al., 2000). These liposomes were mixed with acceptor t-SNARE liposomes in the presence of buffer or the cytosolic domain of VAMP 2 (cd–VAMP 2) without any calcium addition, and the fusion activity was monitored for 2 h at 37°C. Sytg I–VAMP 2 liposomes showed a remarkable increase in fusion activity when compared with liposomes containing only VAMP 2 (Fig. 1 B). Sytg I enhanced the fusion kinetics of VAMP 2 liposomes in a dose-dependent manner as demonstrated by the increased initial fusion rates (Fig. 1 C). At the highest concentration of Sytg I there is an approximate twofold excess of Sytg I over t-SNARE on a liposome to lipidome basis. However, there is twofold more VAMP 2 than Sytg I in the v-SNARE liposomes, and the total amount of t-SNARE in the fusion assay is sixfold higher than Sytg I. For technical reasons, we could not incorporate more Sytg I without lowering the amount of VAMP 2 molecules, thus the observed fivelfold stimulation of the initial fusion rate by synaptotagmin I must be considered as a minimal number. Soluble cd–VAMP 2 inhibited the stimulatory effect of Sytg I, confirming that the observed membrane fusion is SNARE dependent (Fig. 1 B). Furthermore, liposomes containing Sytg I but lacking VAMP 2 did not fuse with t-SNARE liposomes, although Sytg I interacts with t-SNAREs (Figs. 1 B and see Fig. 6 B) (Gerona et al., 2000). These results clearly show that synaptotagmin I by itself does not have any fusogenic activity and that the cross-linking of liposomes via Sytg I–t-SNARE binding interactions is insufficient to induce fusion.

**Soluble cd–Sytg I stimulates SNARE-dependent fusion**

One possible explanation for the effect of Sytg I on fusion is that the t-SNARE binding activity of Sytg I potentiates vesicle binding, and therefore, the stimulatory effect on fusion is simply a consequence of the additional docking sites provided by Sytg I. To determine if this is the case, we constructed a cytosolic domain of synaptotagmin I (cd–Sytg I) that started immediately after the transmembrane region at aa 82. Notably, this construct differs from previously published soluble constructs, which start at aa 95 or 96 (Davis et al., 1999; Osborne et al., 1999). We then studied the ability of cd–Sytg I to accelerate fusion in an assay that is more sensitive to stimulatory components. For this purpose, we incorporated t-SNAREs into labeled donor liposomes and VAMP 2 into unlabeled acceptor liposomes. In addition, we reduced the amount of VAMP 2 incorporated into the acceptor liposomes ~15-fold compared with the standard assay. This reduces the number of copies of VAMP 2 per lipidosome from ~375 in the standard assay to ~25 in the modified assay, a number comparable to the VAMP 2 concentration in synaptic vesicles (Jahn and Südhof, 1994; Weber et al., 1998).

The low concentration of v-SNAREs incorporated into the acceptor liposomes resulted in no observable fusion in the absence of cd–Sytg I within the 2-h incubation period (Fig. 2). However, the addition of 9 µM cd–Sytg I signifi-
Synaptotagmin I accelerates SNARE-mediated fusion | Mahal et al. 275

...cantly stimulated fusion. This stimulation was SNARE dependent, since it was inhibited by the presence of cd–VAMP 2. It should be noted that the overall concentration of synaptotagmin I in this assay is ~10-fold higher than the ratio of synaptotagmin to t-SNARE in the presence and absence of the cytosolic domain of Sytg I (cd–Sytg I, ~10 μM final concentration) and cd–VAMP 2 (see Fig. 1B legend). Note that in assays treated with cd–VAMP 2, the donor liposomes were pretreated with cd–VAMP 2 for 15 min before their mixing with acceptor liposomes. The increase in NBD fluorescence was monitored, and the results were normalized as before.

The stimulatory effect of Sytg I on fusion is topologically restricted

Given that synaptotagmin I, which is localized on synaptic vesicles, cycles through the plasma membrane after exocytosis, Sytg I might also stimulate fusion when localized at the plasma membrane. To test this possibility, we reconstituted Sytg I into t-SNARE–containing unlabeled acceptor liposomes, which represent the plasma membrane in our assay...
Sytg I accelerates fusion-committed docking

To further understand how Sytg I regulates fusion, we analyzed the effect of Sytg I on different stages of the overall reaction. In the in vitro fusion assay, the following steps can be resolved (Fig. 4 A). First, t-SNARE complex activation involves the functional removal of the NH$_2$-terminal domain of syntaxin 1A (NRD; Fig. 4 A) (Parlati et al., 1999). Subsequently, SNAREpins are formed between liposomes and “zip up” into a partially assembled helical bundle. This reaction is initially reversible and then becomes resistant to treatment with cd–VAMP 2 and neurotoxins (Weber et al., 1998; Melia et al., 2002). Low temperature (4°C) blocks membrane fusion allowing us to accumulate these SNAREpin intermediates (Weber et al., 1998). Finally, lipid bilayers fuse, and SNAREpins are fully assembled into cis-SNARE complexes in which membrane-spanning regions reside in the same membrane and their cytoplasmic domains form a “fully zipped up” four-helical bundle.

To test whether the stimulatory effect of synaptotagmin I is comparable to that caused by the removal of NH$_2$-terminal domain of syntaxin 1A, we used a t-SNARE construct containing syntaxin 1A with a thrombin-cleavable NH$_2$-terminal domain (N–t-SNARE) (Parlati et al., 1999). Acceptor liposomes containing N–t-SNARE were prepared and treated with either thrombin (tc–N–t-SNARE) or AEBSF-inactivated thrombin (N–t-SNARE) immediately before addition into the fusion assay. As expected, cleavage of the NH$_2$-terminal domain of syntaxin 1 increased the fusion efficiency in presence of VAMP 2 liposomes (Fig. 4 B) (Parlati et al., 1999). However, when the fusion kinetics of VAMP 2, tc–N–t-SNARE liposomes were compared with those of Sytg I–VAMP 2, N–t-SNARE liposomes, we observed that the NRD removal has a much less prominent effect than that caused by Sytg I (Fig. 4 B). Furthermore, cleavage of the inhibitory NH$_2$-terminal domain of syntaxin 1A did not further enhance the initial kinetics of Sytg I–VAMP 2 liposome fusion, although it modestly increased the final fusion signal (Fig. 4 B). These results indicate that synaptotagmin I has a function that exceeds the mere removal of the NRD of syntaxin 1A and suggests a role in facilitating a later stage of SNARE complex assembly.

To examine if Sytg I affects the rate of SNARE assembly between liposomes, we captured docked complexes at 4°C (Weber et al., 1998) in the presence and absence of Sytg I and tested at which time they become resistant to the addition of cd–VAMP 2. This assay measures how far SNAREpin assembly has proceeded and indicates the time at which docked liposomes become fusion committed. Donor liposomes containing either VAMP 2 alone or Sytg I–VAMP 2 liposomes were incubated with acceptor t-SNARE liposomes at 4°C for varying amounts of time before cd–VAMP 2 was added (Fig. 5 A). All reactions were kept on ice until the final time point when the reactions were warmed to 37°C and fusion was allowed to take place. Liposomes containing Sytg I attained a fusion-committed docked state much faster than liposomes without Sytg I (Fig. 5, C and D, respectively). In fact, after only 1 h at 4°C Sytg I–VAMP 2 liposomes display the same signal that VAMP 2 liposomes attain after an overnight incubation. In contrast to VAMP 2 liposomes, which have a basal fusion activity of 5% of the total fusion signal at 4°C overnight, Sytg I–VAMP 2 liposomes showed an increased fusion potential at this low temperature (16% of the total fusion signal [unpublished data]). Thus, we conclude that Sytg I promotes the formation of fusion-committed SNAREpins. It may also catalyze SNARE-mediated membrane mixing, but that distinction is hard to make without the ability to examine individual fusion events.
The stimulatory effect of Sytg I is calcium independent
So far, our experiments indicate that Sytg I stimulates fusion in absence of external calcium. However, since synaptotagmin I is the prime candidate for a neuronal calcium sensor, we analyzed the effect of calcium on the fusion reaction in the presence of Sytg I. To our surprise, neither the addition of EGTA nor an effective calcium concentration of ~900 μM altered our basic results (Fig. 6 A). Compared with EGTA, calcium reproducibly caused a minor reduction of the initial fusion kinetics and a small increase in the extent of final fusion of the Sytg I–containing liposomes; however, the significance of these minor changes remains to be determined. We confirmed that our Sytg I construct retains the basic calcium-dependent properties, such as calcium-dependent liposome binding and oligomerization (unpublished data). We also reexamined the binding of Sytg I to t-SNARE. Due to the calcium-dependent oligomerization of the synaptotagmin construct, we prebound Sytg I to protein A beads using the anti-Sytg monoclonal antibody M48 and then added t-SNARE in the presence or absence of calcium. At saturating concentrations of t-SNARE (1 μM), significant binding between Sytg I and t-SNARE was observed in the absence and presence of calcium; however, calcium still increased the overall interactions (Fig. 6 B). We obtained similar results with the cd–Sytg I construct starting at aa 82 and with another anti-Sytg monoclonal antibody CI. 41.1 (unpublished data). These results are in agreement with data previously obtained with full-length native synaptotagmin I (Chapman et al., 1995b; Gerona et al., 2000). This largely calcium-independent synaptotagmin I–t-SNARE interaction may provide the basis for the calcium-independent stimulatory effect on fusion that we observed.

Discussion
Synaptotagmin I plays a critical role in calcium-evoked neurotransmitter release, and accumulating evidence indicates that it functions as the major calcium sensor in neurons. SNARE complex formation, the process underlying exocytosis, is highly regulated in neuronal systems with synap-
totagmin I forming part of the regulatory machinery. Herein we demonstrate that synaptotagmin I profoundly accelerates SNARE mediated membrane fusion in a topologically restricted manner, even in the absence of calcium.

SNARE complex formation in neurons begins with the controlled formation of the t-SNARE, a process which is negatively regulated by the NRD and other syntaxin binding proteins such as Munc 18-1 (Dulabova et al., 1999; Misura et al., 2000; Fisher et al., 2001). The NRD block must be released to allow t-SNARE formation, a process most likely mediated by a component such as Munc 13 (Betz et al., 1997). In the process of t-SNARE formation, the NRD block on SNAREpin formation seen in our fusion assay is most likely relieved. After t-SNARE formation on the plasma membrane, SNARE complexes assemble into a four-helix bundle in a zippering reaction that may involve multiple steps, a theory supported by several in vivo studies (Hua and Charlton, 1999; Xu et al., 1999). SNARE complex formation starts at the membrane-distal end of the SNARE motif and may be slowed down by the inherent instability of the membrane-proximal domains of the t-SNARE (Fiebig et al., 1999; Melia et al., 2002) and the increasingly repulsive forces between the lipid bilayers. Although membrane fusion will eventually proceed, the rate may be too slow to ensure fast regulated exocytosis, and additional components accelerating the reaction may be required (Weber et al., 1998; Fasshauer et al., 2002).

Our data indicate that synaptotagmin I could be such an accelerating component. Synaptotagmin I accelerates the initial rate of fusion at least by a factor of five (Fig. 1 C). These results are consistent with the recent observation that synaptotagmin I promotes the assembly of cytoplasmic SNARE domains in vitro (Littleton et al., 2001). Synaptotagmin I overcomes the block caused by the NRD (Fig. 4 B) and, more significantly, accelerates SNAREpin formation (Fig. 5). Accordingly, synaptotagmin I converts the initially reversible SNAREpin into a fusion-committed state that can no longer be inhibited by the addition of cd–VAMP 2. In the absence of synaptotagmin I and at low temperatures, the initial v-liposome–t-liposome interaction occurs within minutes, but it takes several hours or an overnight incubation for the reversible SNAREpin interaction to become resistant to neurotoxins or to the inhibitory cytoplasmic domain of VAMP 2 (Weber et al., 1998). The latter reaction, which is greatly enhanced by synaptotagmin I, may involve the membrane-proximal domain of the t-SNARE. Indeed, it has been shown that synaptotagmin I interacts with the membrane-proximal part of the syntaxin 1 SNARE motif containing VAMP 2 with and without Sytg I at 4°C. After time equals x, cd–VAMP 2 (equimolar to t-SNARE) was added, and the reaction was allowed to incubate at 4°C until the final time point. The mixture was then warmed to 37°C, and the reaction is monitored as described in Fig. 1 legend. (B) Kinetic profiles of fusion from a time course of functionally docked VAMP 2 liposomes. The assay was performed as outlined in A. Note that the normalized data ignores the preassay fusion and an initial drop in fluorescence, which is due to temperature effects on the fluorescent probes (Chapman et al., 1995a), is typically observed. In addition, please note that the time course for SNAREpin formation is concentration dependent. (C) Kinetic profiles of fusion from a time course of functionally docked VAMP 2–Sytg I liposomes. The assay was performed as outlined in A.

Figure 5. Sytg I accelerates fusion-committed docking. (A) Acceptor t-SNARE liposomes (45 μl) were mixed with donor liposomes (5 μl)
plasma membrane would not affect the fusion of vesicles lacking synaptotagmin I (e.g., constitutive transport vesicles), thus adding an additional level of control over which vesicles are primed for calcium-dependent fusion.

However, surprisingly, the stimulatory effect of synaptotagmin I in its calcium-dependent form was calcium independent. At first glance, this result seems to directly contradict the concept that synaptotagmin I is either an inhibitory clamp (hypothesis 1) or a calcium-dependent promoter of fusion (hypothesis 2). Indeed, the stimulatory effect of synaptotagmin I is incompatible with the clamp hypothesis. However, we cannot completely exclude that calcium might further stimulate the reaction. After calcium influx into the nerve terminal, the first synaptic vesicle fuses within less than 1 ms, indicating that SNAREpins are already preassembled. If synaptotagmin I–accelerated SNAREpin assembly is the rate-limiting step in our fusion assay, we would not be able to detect any faster reactions that may follow. We attempted to overcome this potentially rate-limiting step by accumulating prefusion intermediates in the presence of synaptotagmin at 4°C and then adding calcium during the warm-up phase. Even under these conditions, we could not detect a significant stimulatory effect of calcium (unpublished data). This raises the possibility that synaptotagmin I and SNAREs alone are not sufficient to mediate calcium-regulated exocytosis, and additional components, such as lipids or proteins (perhaps even a different synaptotagmin isoform), may be required. Indeed, genetic evidence has indicated that additional components are necessary (Kelly, 1995).

In summary, our data demonstrates that synaptotagmin I plays an important accelerating role in SNAREpin assembly and membrane fusion. Future experiments will reveal whether the addition of further components to the reconstructed assay will confer calcium sensitivity.

Materials and methods

All lipids were from Avanti with the exception of [125I]-1,2-dipalmitoyl phosphatidylcholine ([125I]-DPPC), which is from Amersham Pharmacia Biotech. The anti–synaptotagmin I monoclonal antibody CI.41.1 was from Synaptic Systems.

Plasmid constructions

Construction of membrane-anchored synaptotagmin I. The cDNA encoding full-length synaptotagmin I was amplified by PCR using a rat brain k-GT11 cDNA library (CLONTECH Laboratories, Inc.) and the following oligonucleotides: (a) GGGGGATCCATGGTGAGTGCCAGTCATCC and (b) GGGGAGCTCTTACTTCTGACAGCCAGCATGGC and cloned into PCR-Script (Stratagene) according to the manufacturer’s instructions. This construct was digested with PstI and EcoRI, and a double-stranded oligonucleotide of phosphorylated oligonucleotides, (c) GGTAGGAGGAGGAGGTTGATGCCTGCTGGCTGTCAAGAAGGAGCTCCTGAGG and (d) AATTCCTCGAGTCACCCCATGCGCTGGCTGTCAAGAAGGAGCTCCTGAGG and (e) AGATCTCCATGGTGAGTGCCAGTCATCC and (f) CTAATCCGAGTTGAGCTGTTC and template pTW27 was digested with the same enzymes and then ligated into the cut pTW27 yields pTW25, thus removing the stop codon. The coding sequence for synaptotagmin I was excised by digestion of pTW25 with NcoI and XhoI. This fragment was ligated into pET-28b (Novagen) digested with the same enzymes and cloned into pET-28b encoding full-length synaptotagmin I with a COOH-terminal His tag. To remove the luminal domain, pTW27 was digested with NcoI and KpnI. The PCR product of oligonucleotides (a) AGATCTCCATGGTGAGTGCCAGTCATCC and (b) CTAATCCGAGTTGAGCTGTTC and (c) GGTAGGAGGAGGAGGTTGATGCCTGCTGGCTGTCAAGAAGGAGCTCCTGAGG and (d) AATTCCTCGAGTCACCCCATGCGCTGGCTGTCAAGAAGGAGCTCCTGAGG and (e) AGATCTCCATGGTGAGTGCCAGTCATCC and (f) CTAATCCGAGTTGAGCTGTTC and template pTW27 was digested with the same enzymes and then ligated into the cut pTW27 yielding pTW70. This construct encodes synaptotagmin I under the control of a T7 promoter without its luminal domain (aa 1–56) but with a COOH-terminal His tag. In addition, the protein encoded is an isoform in which calcium-dependent oligomerization is abolished (Desai et al., 2000) and contains an amino acid sequence that is compatible with calcium-dependent fusion.
Published July 15, 2002

Lys 95, was made in an identical manner to pLM7 but with the following ammonium, 5
1.5 h at 4°C (25 mM Hepes · KOH, pH 7.4, 100 mM KCl, 5 mM
added a protease inhibitor cocktail (final concentrations: 1.2
(Desai et al., 2000) using site-directed mutagenesis (QuikChange kit;
shiba, 2000). However, this does not correspond to the condition in vivo,
teines have been shown to form disulfide bonds in lipid bilayers in vitro
a synaptotagmin I capable of calcium-dependent oligomerization with no
GAAAAGGCGGAGGTTACGAC, to yield the final plasmid pLM6 encoding
GACTAAAAGGAC, (2) Cys 79 to Ile using primers (a) CCTGCGCCTT-
TCTTTTTGAACAATACTTAATGACACAAAAGG, (4) Cys 74 to Ser
TGTTCTTGTCTATCAAGAATTGTTGCTCAAGAAGG, (2) CTTAATGACAAAGGCGGAT
gAG, and (b) CAACTAATTGTCAGAACGAGAGGTCAAG, to yield the final plasmid pLM6 encoding a
synchrony I (110.6 mg) or buffer (control beads) with monoclonal anti-
binding assay.
Fusion assays
For each vesicle preparation (POPC only or POPC/DOPS), 100 µl of lipid
solution (see POPC only and POPC/DOPS lipid mixtures above) was
ried down in a 10 × 75 glass test tube by a stream of nitrogen, and trace
amounts of chlorofluro were removed under vacuum for 1 h. The
dried liposomes were resuspended in 500 µl reconstitution buffer A (R buffer,
25 mM Hepes · KOH, pH 7.4, 100 mM KC1, 5 mM Mg·mercaptoethanol, 1
mM MgCl₂, 0.01 mM CaCl₂, 10% glycerol). To this cell suspension was
added a protease inhibitor cocktail (final concentrations: 1.2 mg/ml
leupeptin, 2 µg/ml antipain, 20 µg/ml turkey trypsin inhibitor, 10 µg/ml benz-
amidine, 5 µg/ml phenylamine, 2.1 µg/ml aprotinin, 5 µg/ml chymostatin,
2.5 µg/ml pepstatin) and 1/4 vol 20% (w/v) Triton X-100. The suspen-
sion was then incubated three times through a syringe containing a 0.22 µm
filter (Millipore Ultracel) and dialyzed against 100 mM imidazole to 500 mM imidazole. Expression and purification of protein from construct plM8 was performed in an identical manner to cd-Syt1.
Full-length t-SNARE complex (mouse his-SNAP 25 and t-calcium 1A) was
expressed and purified from vector pTW34 as described previously (Webber et al., 1998). Thrombin-cleavable (tc) t-SNARE complex (mouse his-SNAP 25 and t-calcium 1A) was expressed and purified from vector pTW69 as described previously (Parlati et al., 1999). Full-length mouse VAMP-2 was expressed and purified from vector pTW2 as described previ-
ously (Webber et al., 1998).
Lipid mixtures
Donor lipid mix. Donor lipid mix contains 83.3 mol% 1-palmitoyl-2-ole-
yl-SN-glycero-3-phosphatidylcholine (POPC). 15.1 mol% 1,2-dioleoyl-
SN-glycero-3-phosphatidylserine (DOPS), 0.8 mol% R-PE, 0.8 mol%
NBD-PE and trace amounts of [3H]-DPPC, and 3 mM total lipid.
Acceptor lipid mix. Acceptor lipid mix contains mol% POPC, 15 mol%
DOPS and trace [3H]-DPPC, and 15 mM total lipid.
POPC/DOPS lipid mix (for lipid binding assay). POPC/DOPS lipid mix contains
75 mol% POPC, 25 mol% DOPS and trace amounts of [3H]-DPPC, and 15 mM total lipid.
POPC/DOPS lipid mix (for lipid binding assay).

Liposome formation and thrombin cleavage of t-SNARE liposomes
Liposomes were formed in the presence of VAMP 2 (0.7–1 mg/ml), Sytg I
(1 mg/ml), and t-SNARE (1.5–3 mg/ml) in various combinations using the previous
previously described technique of dilution and dialysis followed by a Ny-
codenz gradient (Webber et al., 1998) with the donor and acceptor lipid mix
ates defined above. Note that for unlabeled v-SNARE liposomes, the quantity of VAMP2 used was much lower (0.09 mg/ml). Protein amounts
in these detergent liposomes were determined using Coomassie blue–stained
SDS-PAGE with protein standards and Quantity One Quantitation Soft-
ware (Bio-Rad Laboratories). The NH₂-terminal domain of syntaxin was
moved by thrombin cleavage as described previously (Parlati et al., 1999).
Fusion assays
Fusion reactions and data analysis were performed as described previously
(Weber et al., 1998) with the following exceptions: (a) In all cases, 45 µl of acceptor (unlabeled) and 5 µl of donor (labeled) liposomes were used, (b)
unless otherwise noted, both acceptor and donor liposomes were pre-
warmed to 37°C before mixing, and (c) to minimize quenching, 10 µl of 25% (w/v) dodecyl maltoside instead of Triton X-100 was added at the
end of the fusion reaction.

Lipid binding assay
For each vesicle preparation (POPC only or POPC/DOPS), 100 µl of lipid
solution (see POPC only and POPC/DOPS lipid mixtures above) was

Downloaded from jcp.rupress.org on April 26, 2016
ml R buffer (containing either 100 μM EGTA-buffered calcium or 2 mM EGTA as appropriate), and solubilized in 100 μl 10% SDS solution. The mixture was transferred to scintillation vials containing Scintiverse (10 ml; Fischer), and the ‘H radioactivity was counted (5 min per vial; Beckman Coulter LS6001C).

**t-SNARE binding assay**

Syg1 (107 µg) was immobilized on protein A beads (100 µl beads; Amer sham Pharmacia Biotech) by immunoprecipitation with monoclonal antibody CI:41.1 (10 µl) in buffer T (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100) with 1 mg/ml BSA. The beads were diluted to give a final concentration of 20%, split into two pools, and washed with buffer T containing 1 mg/ml BSA and either 1 mM EGTA or 1 mM CaCl2 (Ca). Beads were then pelleted, supernatant was removed, and 1 μl of 1× SDS PAGE sample buffer (50 mM Tris·HCl, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol, 100 mM DTT) was added. Samples were heated at 95°C for 10 min and analyzed by SDS-PAGE. The experiment was also done using cd-Syq1 and monoclonal antibody M48 in an identical manner.

We thank Dr. Thomas Weber (Mount Sinai School of Medicine, New York, NY) for his generous gift of pIv70. In addition, we thank Stéphanie Bourdelie for help with the completion of the article and Dr. Oleg Varlamov for critical reading of the article.

Research was supported by postdoctoral fellowships from the Jane Coffin Childs Memorial Fund to L.K Mahal and the Portuguese Foundation for Science and Technology (to S.M. Sequeira).

Submitted: 27 March 2002

Revised: 5 June 2002

Accepted: 7 June 2002

**References**


