Close Is Not Enough: SNARE-dependent Membrane Fusion Requires an Active Mechanism that Transduces Force to Membrane Anchors

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Abstract. Is membrane fusion an essentially passive or an active process? It could be that fusion proteins simply need to pin two bilayers together long enough, and the bilayers could do the rest spontaneously. Or, it could be that the fusion proteins play an active role after pinning two bilayers, exerting force in the bilayer in one or another way to direct the fusion process. To distinguish these alternatives, we replaced one or both of the peptidic membrane anchors of exocytic vesicle (v)- and target membrane (t)-SNAREs (soluble N-ethylmaleimide-sensitive fusion protein [NSF] attachment protein [SNAP] receptor) with covalently attached lipids. Replacing either anchor with a phospholipid prevented fusion of liposomes by the isolated SNAREs, but still allowed assembly of trans-SNARE complexes docking vesicles. This result implies an active mechanism; if fusion occurred passively, simply holding the bilayers together long enough would have been sufficient. Studies using polyisoprenoid anchors ranging from 15–55 carbons and multiple phospholipid-containing anchors reveal distinct requirements for anchors of v- and t-SNAREs to function: v-SNAREs require anchors capable of spanning both leaflets, whereas t-SNAREs do not, so long as the anchor is sufficiently hydrophobic. These data, together with previous results showing fusion is inhibited as the length of the linker connecting the helical bundle-containing rod of the SNARE complex to the anchors is increased (McNew, J.A., T. Weber, D.M. Engelman, T.H. Sollner, and J.E. Rothman. 1999. *Mol. Cell.* 4:415–421), suggests a model in which one activity of the SNARE complex promoting fusion is to exert force on the anchors by pulling on the linkers. This motion would lead to the simultaneous inward movement of lipids from both bilayers, and in the case of the v-SNARE, from both leaflets.

Key words: lipid mixing • isoprene • liposome • lipid anchor • vesicular transport

Introduction

Membrane fusion, in which two distinct lipid bilayer membranes are merged into one, is the common final step in the transport of proteins among intracellular compartments, the controlled release of hormones and neurotransmitters by exocytosis, and the penetration of the genomes of enveloped viruses into the cytoplasm. Remarkably, common physical principles appear to underlie these diverse biological processes that involve fusion on both sides of cellular membranes.

Intracellular membrane fusion is triggered by the pairing of cognate vesicle (v)[1]-SNAREs (soluble N-ethylmaleimide-sensitive fusion protein [NSF] attachment protein [SNAP] receptor) and target membrane (t)-SNAREs between opposite bilayers (Sollner et al., 1993; Nichols et al., 1997; Weber et al., 1998; Chen and Whiteheart, 1999; McNew et al., 1999; Nickel et al., 1999; Parlati et al., 1999). These proteins assemble into a helical bundle (Poirier et al., 1998; Sutton et al., 1998) in which the hydrophobic anchors of both v- and t-SNAREs emerge at the same end (Hanson et al., 1997b; Hohl et al., 1998; Poirier et al., 1998; Sutton et al., 1998). As a result, the involved bilayers are held into close apposition by these SNAREpins (Weber et al., 1998). Fusion from this structure occurs with high ef-

ficiency and at a reasonable rate in the absence of any other proteins, demonstrating that the SNAREpins themselves are the underlying principle of intracellular membrane fusion (Weber et al., 1998). In fact, fusion requires only the helical bundle portion of the SNARE complex (Parlati et al., 1999).

Viral membrane fusion is mediated by extracellular viral-encoded proteins embedded in the viral envelope that are activated by receptor binding at the cell surface or endocytosis (Dalgleish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1986; Feng et al., 1996). Upon activation, they can undergo a dramatic conformational switch to form a helical bundle; like the SNAREpin, this viral hairpin is simultaneously inserted into both membrane partners (reviewed by Skehel and Wiley, 1998). Before activation, viral fusion proteins are anchored solely in the viral envelope. Therefore, during this switch, the viral protein must insert into the cellular membrane. This is typically mediated by a specialized fusion peptide that is buried in the protein structure before activation (Wilson et al., 1981).

These models raise the fundamental question of whether membrane fusion is an essentially passive or an active process. It could be that simply pinning two bilayers together long enough is sufficient for fusion; the bilayers could do the rest spontaneously. Or, it could be that the fusion proteins play an active role after pinning, in one or another way perturbing the lipids by exerting force in the bilayer. The assembly of the helical bundles that comprise pins would seem to afford an ample source of energy to do work on the bilayer, since isolated SNARE complexes and viral helical bundles only denature at temperatures above 80–90°C (Hayashi et al., 1994; Lu et al., 1995; Lamb et al., 1999).

In principle, it is possible to distinguish an active from a passive mechanism by examining the effect upon fusion activity of replacing one or both of the peptidic membrane anchors of fusogenic pins with covalently attached lipids. If fusion occurs passively, it should not be adversely affected by lipid anchors since simply holding the bilayers together long enough is by definition sufficient. If, however, fusion is found not to occur with some or all species of lipid anchors, then it necessarily involves an active mechanism, since in at least some cases simply holding the bilayers together long enough did not suffice. Of course, for these conclusions to hold, the separation of the two bilayers must remain constant independent of the nature of the anchor, or equivalently, fusion must be shown to be insensitive to variations in separation in the range resulting from substituting lipid anchors. Also, the assembly of the pin must be unaffected by lipid substitution.

Elegant and pioneering studies have shown that replacing the natural peptidic anchor that holds viral fusion proteins into the envelope with an encodable phospholipid-based glycerophosphoinositide (GPI) unit prevents fusion and results in hemifusion, a state where outer leaflets merge, but the inner leaflets do not (Kemble et al., 1994; Melikyan et al., 1995). However, a recent study suggests that GPI-linked HA can produce nonexpanding fusion pores under certain experimental conditions (Markosyan et al., 2000). These results establish the importance of the viral membrane anchor and are consistent with the possibility that fusion involves an active mechanism. However, it is also possible that hemifusion and subsequent effects are a unique consequence of a GPI-anchor and the viral fusion peptide. Insertion of the fusion peptide may destabilize bilayer structure and possibly facilitate the bilayer-to-nonbilayer transition that is likely required for hemifusion. Unfortunately, it is not presently possible to lipid-anchor viral fusion proteins in both the viral and target membrane to eliminate viral fusion peptide-specific phenomena.

By contrast, SNARE proteins are well suited for such studies because the two membrane anchors can be manipulated in separate polypeptide chains and tested later when reconstituted into liposomes. Here, we make precisely such changes in cognate SNAREs mediating exocytosis, vesicle-associated membrane protein 2 (VAMP2, the v-SNARE), a heterodimer of syntaxin1A, and a synaptosomal-associated protein of 25 kD (SNAP-25B, the t-SNARE; Trimble et al., 1988; Baumert et al., 1989; Oyler et al., 1989; Bennett et al., 1992; Sollner et al., 1993). We find that close proximity of two membranes is not sufficient for fusion, though there are certain lipid anchors that appear to permit lipid mixing. Additionally, we describe the synthesis and utilization of four novel isoprenoid lipid anchors.

Materials and Methods

Chemicals

The isoprenyl alcohols used for lipid synthesis were obtained from the following vendors. Undecaprenol, solanesol, and farnesol were from Sigma-Aldrich and geranylgeraniol was from Metrya. N, N′-dicyclohexyl-carbodiimide and N-maleoly-β-alanine were from Fluka, and 4–dimethylaminopyridine (DMAP) was from Sigma-Aldrich. All other phospholipids and 1,2-dioleoyl-sn-glycero-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide] were from Avanti Polar Lipids as chloroform solutions.

DNA Manipulations and Plasmid Constructs

All PCR was done with *Pwo* polymerase (BM), except QuickChange mutagenesis, which was done with *Pfu* polymerase according to the manufacturer's instructions (Stratagene). All other DNA modifying enzymes were from New England Biolabs. The *E. coli* strains were DH5a (GIBCO BRL) for standard cloning or XL1-Blue for QuickChange mutagenesis.

Generation of Constructs for Lipid Anchor Attachment

The cytoplasmic domain of syntaxin1A was generated by PCR using pJM26 (McNew et al., 1999) as a template and the oligo NcoI–rSyn1A (CGCCATGGCC ATGAAGGACC GAACCCAGG) and rSyn1A-Cys (GGCTCGAGTT AGCAAAGCTT CTTCCTGCGT GCC). This NcoI–XhoI fragment was ligated into pET28a (Novagen), cut with the same enzymes. This plasmid (pJM52) codes for syntaxin1A without a transmembrane domain (TMD), but an ultimate cysteine residue. A naturally occurring cysteine residue at amino acid 145 was removed from pJM52 by site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer's instructions generating the plasmid pJM57-2. The oligos rSyn1A-C145Sf (GACTACCGAG AACGaTcgAA AGGGCGCATC CAGAGGC) and rSyn1A-C145Sr (GCCTCTG-GAT GCGCCCTTTc gAtCGTTCTC GGTAGTC) were used for this manipulation.

In a similar manner, the cytoplasmic domain of VAMP was obtained by PCR with pJM27 (McNew et al., 1999) as a template and the oligos mVAMP2-His$_6$ (Weber et al., 1998) and mVAMP2-Cys (CCGGATCCTT AGCAAAGCTT GAGGTTTTTC CACC). A NdeI–BamHI fragment was ligated into pJM50 (McNew et al., 1999) generating pJM51. pJM50 is a modified pET15b (Novagen) vector that has the HindIII site at bp 29–34 removed by digestion with HindIII, filling in with Klenow, and religated. The final plasmid (pJM51) codes for VAMP2 without a TMD, but an ulti-

mate cysteine residue. This protein has no additional cysteine residues. A VAMP protein with two COOH-terminal cysteines was also generated in the same manner as above (pJM60). In this case, the PCR reaction contained a new 3′ oligo, mVAMP2-C2 (CCGGATCCTT AGCAACCACC GCAAAGCTTG AGGTTTTTCC ACC) that codes for the amino acids Cys Gly Gly Cys. The protein resulting from this expression of pJM60 has two cysteine residues spaced by two glycine residues (see Fig. 1).

Three SNAP-25b constructs were used in this study. pFP247 (Parlati et al., 1999) expresses His$_6$-SNAP-25b from pET-15b. A mutant SNAP-25b that does not contain cysteine residues was constructed in the following manner. A 40-bp AvrII–HindIII fragment was removed from pGEX-mSNAP-25b (Weber et al., 1998) and replaced with a synthetic double-stranded oligo (SN25C-Sf, CTAGGAAAAT TCTCCGGACT TTCCGT-GTCC CCCTCCAACA, SN25C-Sr, AGCTTGTTGG AGGGGGACAC GGAAAGTCCG GAGAATTTTC), generating pJM58. This dsOligo mutates all four cysteine residues in the specified region of DNA to serine. A His$_6$-tagged version of this protein was constructed by removing a 382-bp BamHI–AvrII fragment from pJM58 and ligating it into pFP247 cut with the same enzymes. The resulting plasmid (pJM72) expresses His$_6$-SNAP-25b [C85S, C88S, C90S, C92S].

Synthesis of Novel Maleimido Lipids

Maleimidopropionic Acid Undecaprenylester (C55). 4 mg of undecaprenol in carbon tetrachloride (Sigma-Aldrich) was dried down in a 1-ml reacti-vial by a gentle stream of nitrogen and then dried under vacuum for 30 min. 2.25 mg maleoyl-β-alanine and 3.2 µg dicyclohexyl carbodiimde in 50 µl dry ethylacetate each were added and the solution was stirred at room temperature for 10 min. At this point, 5.2 µl of a 10-mM solution of DMAP in dry ethylacetate was added, and stirring continued overnight at room temperature. The slightly pinkish solution was applied to silica gel TLC plate that was developed in methylene chloride/methanol (50:1). The UV absorbing band with the highest R$_f$ value (R$_f$ = 0.79) was scratched out and eluted with ether. Evaporation of the eluent yielded 3.6 mg (75%) of a colorless oil.

^{1}H-NMR spectra were recorded on a Bruker AMX-400 Mhz instrument and referenced to CHCl$_3$ (^{1}H-NMR) δ = 7.24. Mass spectra were recorded on a PE-Sciex Model API 100 instrument. IR spectra were recorded on Perkin Elmer 1600 series FT-IR spectrometer.

^{1}H-NMR (CDCl$_3$): δ = 1.6–1.75 (m, 36 H), 1.95–2.1 (m, 40 H), 2.63 (t, 2 H, J = 7 Hz), 3.83 (t, 2 H, J = 7 Hz), 4.56 (d, 2 H, J = 7 Hz), 5.12 (br d, 10 H, J = 4 Hz), 5.32 (t, 1 H, J = 7 Hz), 6.69 (s, 2 H).

MS (electrospray): 940.9 (M$^+$ + Na$^+$), 952.8 (M$^+$ + Cl$^-$).

IR (CHCl$_3$): 1712 (C = O), 1730 (C = O) cm^{-1}.

Maleimidopropionic Acid Solanesylester (C45). C45 was synthesized from solanesol (Sigma-Aldrich) as described for C55, yield 75%.

^{1}H-NMR (CDCl$_3$): δ = 1.45–1.75 (m, 15 H), 1.95–2.10 (m, 12 H), 2.63 (t, 2 H, J = 7 Hz), 3.83 (t, 2 H, J = 7 Hz), 4.59 (d, 2 H, J = 7 Hz), 5.11(br t, 3 H, J = 6 Hz), 5.32 (t, 1 H, J = 7 Hz), 6.70 (s, 2 H).

MS (electrospray): 804.5 (M$^+$ + Na$^+$), 816.6 (M$^+$ + Cl$^-$).

IR (CDCl$_3$): 1712 (C = O), 1730 (C = O) cm^{-1}.

Maleimidopropionic Acid Geranylgeranylester (C20). C20 was synthesized from geranylgeraniol (Metrya) as described for C55, yield 70%.

^{1}H-NMR (CDCl$_3$): δ = 1.5–1.75 (m, 30 H), 1.95–2.10 (m, 32 H), 2.64 (t, 2 H, J = 7 Hz), 3.83 (t, 2 H, J = 7 Hz), 4.59 (d, 2 H, J = 7 Hz), 5.10 (br m, 3 H), 5.32 (t, 1 H, J = 7 Hz), 6.70 (s, 2 H).

MS (electrospray): 442 (M$^+$ + H$^+$), 464.0 (M$^+$ + Na$^+$), 476.7 (M$^+$ + Cl$^-$).

IR (CDCl$_3$): 1712 (C = O), 1730 (C = O) cm^{-1}.

Maleimidopropionic Acid Farnesylester (C15). C15 was synthesized from farnesol (Sigma-Aldrich) as described for C55, yield 61%.

^{1}H-NMR (CDCl$_3$): δ = 1.6 (s, 6 H), 1.68 (s, 6 H), 1.69, (s, 3 H), 1.85–2.18 (m, 8 H), 2.64 (t, 2 H, J = 7 Hz), 3.83 (t, 2 H, J = 7 Hz), 4.59 (d, 2 HJ = 7 Hz), 5.07–5.11 (br m, 2 H), 5.31 (dt, 1 H, J = 6 Hz, 1 Hz), 6.70 (s, 2 H).

MS (electrospray): 391.4 (M$^+$ + NH$_4^+$) 396.1 (M$^+$ + Na$^+$), 412.3 (M$^+$ + K$^+$), 407.7 (M$^+$ + Cl$^-$).

IR (CHCl$_3$): 1,712 (C = O), 1,730 (C = O) cm^{-1}.

Expression and Purification of t-SNARE Complex

A 100 ml preculture of BL21(DE3) cells transformed with plasmid pTW34 (Parlati et al., 1999) was grown overnight at 37°C in Luria-Broth (LB) medium containing 0.5% (wt/vol) glucose and 50 µg/ml kanamycin. This preculture was used to inoculate a 12 l containing the same medium. After overnight growth at 37°C, this culture was used to seed 300 liters of LB medium containing 50 µg/ml kanamycin. The cells were grown until they reached a density of 0.8 A$_{260}$ and were then induced with IPTG (0.2 mM final concentration). After 1 h at 37°C, an additional 15 g of kanamycin sulfate was added and the incubation continued for an additional 3.5 h. After centrifugation, the cell paste was frozen in liquid nitrogen in three aliquots.

One aliquot of this cell paste (∼900 g) was resuspended in 2 liters of breaking buffer (25 mM Hepes-KOH, pH 7.40, 100 mM KCl, 10% [wt/vol] glycerol) and 10 mM β-mercaptoethanol). After addition of 30 ml 200 mM PMSF in ethanol and 500 ml 20% (wt/vol) Triton X-100, the cells were disrupted by one passage through an Emulsiflex C5 cell disrupter (Avestin) at >10,000 psi. Cell debris was then removed by centrifugation in a GS3 rotor for 30 min at 8,000 rpm. The supernatant was additionally clarified by centrifugation in a Ti45 rotor for 45 min at 35,000 rpm. To this lysate 30 ml packed Ni-NTA agarose (Qiagen) that have been washed first in breaking buffer containing 1% (wt/vol) Triton X-100 and 500 mM imidazole (pH adjusted to pH 7.5 with acetic acid) and then equilibrated with breaking buffer containing 1% (wt/vol) Triton X-100. After mixing gently overnight at 4°C on an orbital shaker, the slurry was poured into a column and successively washed with 300 ml of breaking buffer containing 1% (wt/vol) Triton X-100; breaking buffer containing 1% (wt/vol) n-octyl-β-D-glucopyranoside (OG); and breaking buffer containing 50 mM imidazole and 1% (wt/vol) OG. Finally, the t-SNARE complex was eluted with a linear gradient (∼250 ml) to breaking buffer containing 500 mM imidazole and 1% (wt/vol) OG. All fractions containing significant amounts of t-SNARE complex were pooled. In an effort to remove heat-shock proteins, 1 mM in MgCl$_2$ and 100 mg ATP-agarose (Sigma-Aldrich) was added to the pooled fractions. After overnight incubation at 4°C on a turning wheel, the beads were removed by filtration and the t-SNARE complex frozen in 550-µl aliquots that were stored at –80°C. The protein concentration was determined according to Schaffner and Weissmann (1973) using a bovine IgG as a standard, and was found to be 1.19 mg/ml.

Full-length mVAMP2 (pTW2) was purified as previously described (Weber et al., 1998).

The soluble t-SNARE complex containing one modifiable cysteine (SN25C-S/SΔTMD-C) was produced by coexpression of pJM72 and pJM57-2 in the BL21(DE3) *Escherichia coli* strain. 4 ml of preprecuture of Luria-Broth (LB) containing 100 µg/ml ampicillin and 50 µl/ml kanamycin was inoculated with the coexpressing strain and grown at 37°C for ∼3 h. This 4-ml culture was used as the inoculum for a 1,200-ml preculture of Super Broth (SB; Bio 101) containing 500 µg/ml ampicillin, 50 µg/ml kanamycin, and 0.5% glucose. This preculture was grown overnight (12–15 h) at 37°C. The 1,200-ml preculture was spun at 500 *g* and the pelleted cells resuspended in 24 liters of SB containing 200 µg/ml ampicillin and 50 µg/ml kanamycin (4 l media in each of six baffled flasks). The 24-liter culture was grown to an OD$_{600}$ of ∼0.7, then induced with 1 mM IPTG for 4 h at 37°C. The cells were harvested by centrifugation and resuspended in 100 ml of breaking buffer (25 mM Hepes-KOH, pH 7.7, 100 mM KCl, 10% (wt/vol) glycerol, 0.2 mM Tris(2-carboxyethyl)phosphine hydrochloride [TCEP; Fluka], and 2 EDTA-free protease inhibitor tablets [BM]). The reducing agent TCEP was used to allow alkylation of the cysteine to maleimide. The cells were disrupted by two passages though an Emulsiflex C5 cell disrupter. Cell debris was removed by centrifugation at ∼25,000 *g* for 15 min. The supernatant was further clarified by a 60-min centrifugation at 50,000 *g* in a Ti70 rotor (Beckman). The supernatant was added to 15 ml of packed Ni-NTA beads (Qiagen) equilibrated in breaking buffer. Binding proceeded for 3 h at 4°C on a rotating wheel. The slurry was spun at ∼1,000 *g* for 5 min and the majority of the unbound material was removed. The beads were transferred to a 1.5 × 15-cm chromatography column (BioRad). The beads were washed with wash buffer (25 mM Hepes-KOH, pH 7.7, 200 mM KCl, 10% (wt/vol) glycerol, 0.2 mM TCEP, 50 mM Imidazole-OAc, pH 7.5) until the A$_{280}$ reached a stable background (∼60 ml). The protein was eluted with a linear, 7-column volume imidazole (∼100 ml) gradient from 50–500 mM. 3-ml fractions were collected and analyzed by SDS-PAGE. Peak fractions were pooled and frozen at −80°C in 300–500-µl aliquots. The protein concentration was 11.3 mg/ml as determined by a Bradford protein assay (Biorad) with IgG as the standard.

The soluble t-SNARE complex containing up to five modifiable cysteines (SN25/SΔTMD-C) was produced by coexpression of pFP247 and pJM57-2 in the BL21(DE3) *E. coli* strain. The protein was purified as described above.

The cytoplasmic domain of VAMP containing a COOH-terminal ultimate cysteine was produced by expression of pJM51 in the BL21(DE3) *E. coli* strain. A 200-ml preculture of SB containing 500 µg/ml ampicillin was grown overnight at 37°C. The cells were harvested by centrifugation and used to inoculate 4 liters of 2XYT media containing 200 µg/ml ampicillin.

The culture was grown at 37°C until an OD$_{600}$ of ~0.7 was reached. Ampicillin (100 μg/ml) was added to the growing culture every hour before induction to prevent plasmid loss. The culture was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C. The cells were harvested by centrifugation and resuspended in 50 ml breaking buffer (25 mM Hepes-KOH, pH 7.4, 400 mM KCl, 2 mM β-mercaptoethanol, 0.5 mM 4-(2-aminoethyl) benzenesulfonylfluoride, 1 EDTA-free protease inhibitor tablet). The cells were disrupted by two passages though an Emulsiflex C5 cell disrupter. Cell debris was removed by centrifugation at ~25,000 g for 15 min. The supernatant was further clarified by a 60 min centrifugation at 50,000 in an Ti70 rotor (Beckman). The supernatant was added to 3 ml of packed Ni-NTA beads (Qiagen) equilibrated in breaking buffer. Binding proceeded for 1 h at 4°C on a rotating wheel. The slurry was spun at ~1,000 g for 5 min and the majority of the unbound material was removed. The beads were transferred to a 1.5 × 10-cm chromatography column (BioRad). The beads were washed with wash buffer (25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 10% [wt/vol] glycerol, 1 mM TCEP, 50 mM Imidazole-OAc, pH 7.5) until the A$_{280}$ reached a stable background (~15 ml). The protein was eluted with a linear, 7-column volume imidazole (~20 ml) gradient from 50–500 mM. 1.5-ml fractions were collected and analyzed by SDS-PAGE. Peak fractions were pooled and dialyzed against 4 l of 25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 10% (wt/vol) glycerol, 1 mM TCEP. Significant precipitation occurred during dialysis. The dialyzed material was spun at 60,000 for 15 min in a TLA100.3 rotor in a tabletop ultracentrifuge (Beckman) to remove precipitates. The supernatant was frozen at −80°C in 100-μl aliquots. The protein concentration was 9.9 mg/ml, as determined by an Amido Black protein assay (Schaffner and Weissmann, 1973) with IgG as the standard.

The cytoplasmic domain of VAMP containing two COOH-terminal cysteine residues and the cytoplasmic domain of rat VAMP2 was produced by the expression of pJM60 and pET-rVAMP2CD (Weber et al., 1998), respectively, in the BL21(DE3) *E. coli* strain. The protein was purified as described for His$_6$-mVΔTMD-C, except the dialysis step was omitted for His$_6$-mVΔTMD-C2.

All SDS-PAGE gels are standard 12% Laemmli gels (Laemmli, 1970), except for Fig. 8, which is a 10% Bis-Tris NuPAGE gel (Novex).

SNARE Reconstitutions

The wild-type TMD-containing SNAREs were reconstituted essentially as described (Weber et al., 1998). The primary exception was the t-SNARE complex that was produced by coexpression of untagged syntaxin 1A and His$_6$-SNAP-25B (pTW34; Parlati et al., 1999). This t-SNARE complex reconstitutes substantially better than the combination used previously, likely because the COOH-terminal His$_6$ tag has been removed from syntaxin1A.

Lipid anchored t-SNARE complexes were also reconstituted by detergent dilution and dialysis. 75 nmol (~5 mole percent) of the particular maleimide lipid was dried in a 10 × 75-mm glass tube under a stream of nitrogen from a chloroform stock, and was then dried under vacuum for 30 min. The lipid film was resuspended by the addition of 25 μl of 20% (wt/wt) OG, followed by 200–300 μl of protein solution (SN25C-S/SΔTMD-C or SN25/SΔTMD-C), depending on the protein concentration. To this, 30 μl of 4 M KCl and the remaining volume up to 500 μl of reconstitution buffer (without reducing agent) was added. The mixture was shaken on a vortexer for 30 min at room temperature and then the incubation was continued for another 30 min standing. Unreacted maleimide lipid was quenched by the addition of 5 μl of 100 mM β-mercaptoethanol for 10 min at room temperature. The 500 μl solution of lipid-coupled protein was transferred to another 10 × 75-mm glass tube containing 1,500 nmol of lipid mix 5 g (85 mole% phosphatidylcholine [POPC], 15 mole% phosphatidylserine [DOPS]; 100 μl of 15 mM lipid in chloroform, dried as before). The lipid film was resuspended by vortexing the lipid/protein/detergent solution for 15 min at room temperature. Proteoliposomes were formed by diluting the detergent below its CMC with the addition of 1.0 ml of reconstitution buffer (25 mM Hepes-KOH, pH 7.7, 100 mM KCl, 10% [wt/vol] glycerol) containing 1 mM DTT. Detergent was removed by dialysis in a GIBCO BRL microdialysis chamber against reconstitution buffer containing 1 mM DTT at a flow rate of ~1.5 ml/min for ~15 h. Liposomes were separated from unincorporated protein by flotation in a discontinuous Nycodenz gradient as described in Weber et al. (1998).

Lipid-anchored VAMP was prepared by directly coupling VAMP-ΔTMD-C to preformed, extruded liposomes containing the maleimide lipid at 5 mole%. Liposomes were prepared by drying 300 nmol of particular maleimide lipid mix (77 mole% POPC, 15 mole% DOPS, 1.5 mole% lissamine rhodamine B [rhodamine]-DPPE, 1.5 mole% nitro-2,1,3-benzoxadiazole [NBD]-DPPE, 5 mole% of the maleimide lipid) in 10 × 75-mm glass tubes under a stream of nitrogen from a chloroform stock, and then dried under vacuum for 30 min. 300 μl of reconstitution buffer (without reducing agent) was added to the dried lipid and the film was resuspended by vortexing for 30 min at room temperature. The multilamellar liposomes were frozen in liquid nitrogen for 30 s, and were then thawed in a 37°C water bath for 30 s. This cycle was repeated eight times. Small unilamellar liposomes of ~50 nm were produced by extrusion through 50-nm polycarbonate filters using a Liposofast mini-extruder (Avestin). VAMP-ΔTMD-C was coupled to the extruded liposome in a reaction containing 100 μl liposomes (~100 nmol lipid), 100 μl VAMPΔTMD-C protein, and 100 μl Buffer B (25 mM Hepes-KOH, pH 7.7, 400 mM KCl, 10% [wt/vol] glycerol, 2 mM EDTA). The reaction was mixed briefly by gentle vortexing, and was then incubated at room temperature for 1 h with intermittent gentle mixing. Continual mixing during the coupling reaction is detrimental to the final liposome preparation. Unreacted maleimide lipid was quenched by the addition of 3 μl of 100 mM β-mercaptoethanol for 10 min at room temperature. Liposomes were separated from unincorporated protein by flotation in a discontinuous Nycodenz gradient as described (Weber et al., 1998).

Fusion Assays and Data Analysis

Standard fusion assays were performed as described (Weber et al., 1998) with modification (McNew et al., 1999). 45 μl (~100 nmol lipid, ~660 pmol t-SNARE complex) of unlabeled t-SNARE liposomes were mixed on ice with 5 μl (~ 6.25 nmol lipid, ~220 pmol VAMP) of fluorescently labeled v-SNARE liposomes in a 96-well Fluoronunc polysorp plate (Nunc). The VAMP liposomes are essentially the same as described in Weber et al. (1998), but the t-SNARE liposomes contain about two to three times more protein, due to the increased reconstitution efficiency of the improved His$_6$-tagged SNAP-25/untagged syntaxin t-SNARE complex. These reconstitution yielded ~150 copies of the t-SNARE complex per liposome. The plate was immediately placed in a 37°C fluorescent plate reader (Floroskan II; Labsystems) without any prior preincubation, and NDB fluorescence was read (excitation 460 nm, emission 538 nm) at 2-min intervals. At the end of 120 min, 10 μl of 2.5%(wt/vol) n-docecyl-maltoside (Boehringer) was added to determine NBD fluorescence at infinite dilution. The kinetic fusion data was first converted to percent detergent signal as described (Weber et al., 1998; McNew et al., 1999), and was then converted to rounds of fusion using the equation: $Y = 0.49666*e^{(0.036031X)} - 0.50597*e^{(-0.053946X)}$, where Y is the fold lipid dilution and X is the percent docecylmaltoside signal at a given time interval, as described (McNew et al., 1999; Parlati et al., 1999).

Liposome Docking Assay

A Fluoronunc maxisorp plate was prepared for the docking assay in the following manner. The HPC-1 monoclonal syntaxin antibody (100 μl of 100 μg/ml of purified IgG in PBS) was added to wells of the 96-well microtiter plate and allowed to bind for 1 h at room temperature on a rocking platform. Unbound IgG was washed away with three 300 μl washes with PBS. Nonspecific protein binding was blocked by the addition of 300 μl of 10 mg/ml bovine IgG (Calbiochem) in PBS for 1 h at room temperature on a platform. Residual IgG was removed with five 300 μl washes with PBS. Nonspecific lipid binding was prevented by the addition of 50 μl of 3 mM small unilamellar liposomes (SUV's, 85 mole% POPC, 15 mole% DOPS in reconstitution buffer, prepared by sonication). Bulk lipid binding continued for 1 h at room temperature on a platform. At this time, 10 μl of the blocking liposomes was removed and replaced with a mixture of 1:1 unlabeled t-SNARE liposomes and labeled v-SNARE liposomes that were docked overnight at 4°C in a 500-μl microfuge tube. 30 μl of each v- and t-SNARE liposomes (60 μl total) were allowed to dock overnight. Where indicated, 5 μl (~15 μg) of competitor VAMP-soluble domain was added to inhibit docking. 10 μl of this mixture (five v- and five t-SNARE) was added to wells (five replicates) of a 96-well microtiter plate for each docking pair. Adherence of the docked liposomes was done for 1 h at room temperature on a platform. Undocked v-SNARE–containing liposomes were removed by five 300 μl washes with PBS. The remaining docked, v-SNARE–derived rhodamine fluorescence was determined by solubilizing the remaining lipid in 100 μl of 2% SDS and measuring rhodamine fluorescence (excitation 535 nm, emission 590 nm, emission cutoff at 570 nm) in a fluorescent plate reader (SpectraMax Gemini, Molecular Devices). The data presented in Figs. 2 and 4 are the average of five wells and the error bars show SD from the mean.

Results

### Covalent Attachment of SNARE Cytoplasmic Domains to Lipids and the Fusion Assay

The cytoplasmic (soluble) domains of VAMP2 and syntaxin1A were expressed in *E. coli* and then attached to a maleimide derivative of a lipid moiety via the SH group of a unique, engineered cysteine residue (Fig. 1 A). The sole cysteine residue (position 145) in the native sequence of syntaxin1A was replaced by a serine by in vitro mutagenesis. VAMP2 normally lacks cysteine in the cytosolic domain. For certain experiments, the four (naturally palmitoylated) cysteines in SNAP-25 were also replaced with serines by mutagenesis (Fig. 1 A). The location of a single cysteine at the COOH terminus of VAMP2 and syntaxin1A assures that proteins will be attached to lipids in
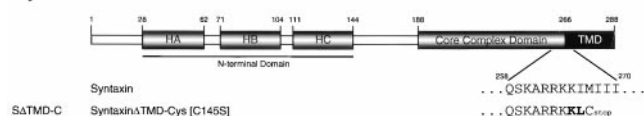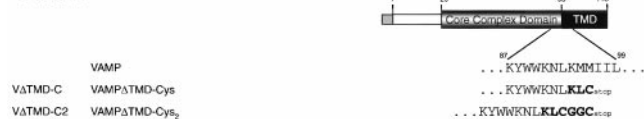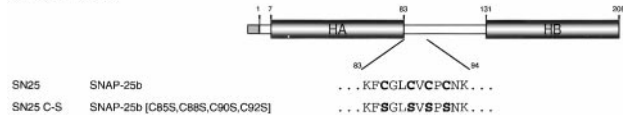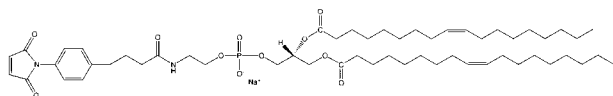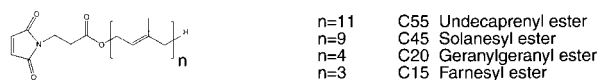

Figure 1. Neuronal SNARE proteins and lipid structures. A, The domain structure of the full-length SNARE proteins are graphically depicted. In modified regions, the amino acid sequence is shown to expose the exact nature of the changes. v- and t-SNAREs were constructed that were lacking the TMDs and terminated in an ultimate cysteine residue. To produce t-SNARE complexes containing only one modifiable cysteine residue, the naturally occurring cysteines in SNAP-25 were removed by site directed mutagenesis. B, The structure of a commercially available PE and four novel synthetic isoprenyl lipids are shown.

bilayers with the same orientation as when they are linked to their natural peptidic anchors. Cytoplasmic domains of syntaxin and SNAP-25 (or related constructs) were coexpressed in *E. coli* and the resulting soluble t-SNARE complex was purified from these extracts (Weber et al., 1998).

As one lipid moiety to anchor soluble VAMP and/or syntaxin/SNAP-25, we employed a commercially available maleimide derivative of phosphatidylethanolamine (Fig. 1 B). In addition, we synthesized four isoprenoid derivatives of varying chain lengths: 15 carbons (farnesyl); 20 carbons (geranylgeranyl); 45 carbons (solanesyl); and 55 carbons (undecaprenyl). Farnesyl and geranylgeranyl are used biologically to anchor otherwise soluble proteins (Fu and Casey, 1999). Undecaprenol (also termed ficaprenol-11) is used biologically to anchor intermediates in the synthesis of oligosaccharide chains in bacteria (Troy, 1979; Reusch, 1984). A maleimide group attached to a linker was added to the terminal OH of the isoprenoids as described in Materials and Methods.

The conjugate that results from the attachment of the lipid anchor to the SNARE can then be use in the formation of proteoliposomes. In all cases, the location of the modifiable cysteine (the point of attachment of lipid anchor; Fig. 1 A) is immediately following the membrane-delimiting lysine residue. This placement puts the SNARE in a very similar position relative to the membrane as its native TMD counterpart. The short linker that bridges the final cysteine residue in the protein to the lipid anchor (~18 Å in the maleimide phosphatidylethanolamine derivative [PE] lipid anchor and ~9 Å in the isoprenoid lipids) is well within the tolerance of normal membrane fusion identified by proteinaceous linkers (9 amino acids, ~30 Å in an extended conformation; McNew et al., 1999).

The fusion activity of the full-length protein-anchored and the lipid-anchored soluble SNARE proteins was monitored by the well-characterized lipid-mixing assay described in detail previously (Struck et al., 1981; Weber et al., 1998). Two populations of vesicles containing VAMP (v-SNARE or donor vesicles) and the syntaxin-SNAP-25 complex (t-SNARE or acceptor vesicles) are reconstituted by detergent dialysis in the presence of excess phospholipids (85 mole% POPC and 15 mole% DOPS). The vesicles containing the v-SNARE were labeled by including 1.5 mole% of each of the fluorescent lipids NBD-PE and rhodamine-PE at the expense of POPC in the reconstitution. The fluorescence of NBD is quenched by rhodamine in an energy transfer process that is strongly dependent on distance. Therefore, when fluorescent v-SNARE vesicles fuse with unlabeled t-SNARE vesicles, fluorescent lipids are diluted, the average distance between the fluorophores increases, and NBD fluorescence increases as quenching is relieved. This effect is monitored at the NBD emission maximum at 538 nm and normalized to infinite lipid dilution by detergent addition. The normalized fluorescent signal is converted to rounds of donor vesicle fusion after the calibration procedure previously described (McNew et al., 1999; Parlati et al., 1999).

SNAREs Anchored by a Single Phospholipid neither Fuse nor Hemifuse

v-SNARE liposomes were produced that contained VAMP

that was attached to the bilayer either by its native TMD or by the PE derivative (Fig. 1 B). Donor liposomes (POPC, DOPS, NDB-PE, and rhodamine-PE, ~50 nm) that contained 5 mole% of the maleimide-PE derivative were prepared by extrusion through polycarbonate filters. VAMP was coupled directly to the preformed liposomes in solution. The coupling reaction was terminated and excess maleimide lipid was quenched by the addition of 1 mM β-mercaptoethanol to the reaction. The amount of VAMP added to the coupling reaction was empirically determined to parallel the yield of full-length VAMP reconstituted by detergent dilution and dialysis (Fig. 2 C). Unmodified VAMP did not significantly associate with the resulting liposomes. The extent of coupling was monitored by a shift in molecular weight associated with lipid coupling.

Similarly, liposomes containing the t-SNARE complex syntaxin1A/SNAP-25 were prepared where syntaxin was attached to the membrane via its native TMD, or with the single PE lipid anchor. This was accomplished by coexpressing syntaxin1A without its TMD and with the sole cysteine at its COOH terminus (SΔTMD-C) together with a mutant version of SNAP-25 without any cysteines (SN25 C-S, Fig. 1 A). This soluble t-SNARE complex was attached to maleimide-PE in detergent solution and used in normal reconstitution by detergent dilution and dialysis. We confirmed that the SN25 C-S was completely functional for fusion when coexpressed with the TMD-containing syntaxin and tested with TMD-anchored VAMP (data not shown). Fusion activity was assayed at 37°C (with no preincubation) using all possible combinations of PE and TMD-anchored v- and t-SNAREs (Fig. 2). Importantly, VAMP attached with maleimide-PE lacked significant fusion activity when tested with the TMD-linked t-SNARE syntaxin/SNAP-25 (Fig. 2; open circles). The same result was obtained when the t-SNARE was anchored by PE and these liposomes were tested with TMD-linked v-SNARE liposomes (filled diamonds). These results show that a phospholipid anchor on either v- or t-SNARE prevents fusion. These results also demonstrate that there is no significant hemifusion (mixing of outer, but not inner leaflets), since partial lipid mixing was not observed.

Phospholipid-anchored SNAREs Dock Vesicles

To confirm that the PE-linked SNAREs could still form SNARE complexes pinning donor and acceptor vesicles together, even though they failed to fuse, we developed a liposome docking assay that measures the amount of fluorescently labeled v-SNARE donor liposomes that are bound to unlabeled t-SNARE acceptor liposomes. For this purpose the v- and t-SNARE liposomes were allowed to dock (Weber et al., 1998), then the t-SNARE liposomes were selectively retrieved by immunoisolation. Fluorescence associated with the isolated t-SNARE vesicles (above controls for nonspecific binding) reflects bound v-SNARE vesicles. Selective recovery was accomplished by coating microtiter plates with an mAb (HPC-1; Inoue et al., 1992) that specifically recognizes the NH2-terminal regulatory domain of syntaxin1A, which is not involved in the core complex (Sutton et al., 1998).

Fig. 2 B shows the results of the liposome docking assay that clearly demonstrate that both PE-linked v-SNAREs
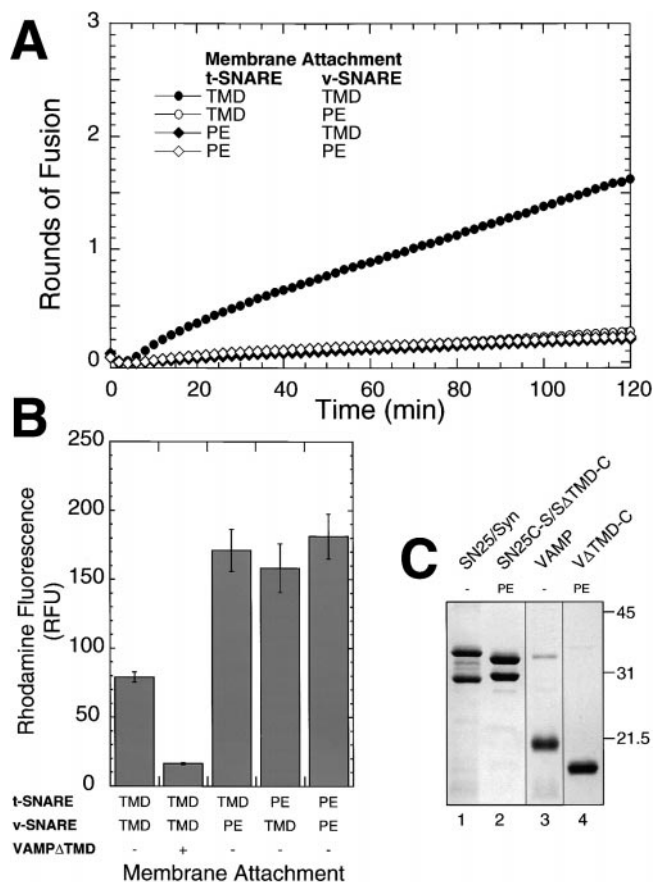
Figure 2. Proximity alone is not sufficient to fuse SNARE-containing vesicles. A, The TMD of either VAMP or syntaxin was replaced with a modifiable cysteine residue (Fig. 1). These proteins were produced in bacteria and coupled in vitro to maleimido-PE (Fig. 1) through a thioether linkage. The t-SNARE complex was produced by coexpression of syntaxin (SΔTMD-C) with SN25 C-S. This t-SNARE complex contains only one modifiable cysteine residue located at the COOH terminus of syntaxin. The lipid-anchored proteins were incorporated into vesicles by detergent dilution and dialysis (t-SNARE complex) or coupled directly to preformed vesicles containing 5 mole% maleimide-PE (VAMP). Vesicles containing the lipid-anchored or normal SNAREs were mixed on ice in various combinations and incubated for 120 min at 37°C in a standard fusion reaction. The extent of fusion is represented as rounds of fusion measured as fold lipid dilution in the reaction. Full-length TMD syntaxin fuses with TMD VAMP to wild-type levels (closed circle). VAMP with a single PE attachment does not lipid mix with wild-type TMD syntaxin (open circles). Similarly, t-SNARE complexes with a single PE attachment do not lipid mix with TMD VAMP (filled diamonds). When both SNARE partners are attached with a single PE lipid anchor, little or no lipid mixing is observed (open diamonds). B. Liposome docking assay. v- and t-SNARE liposomes were incubated in a 1:1 mixture. The t-SNARE–containing liposomes were bound to microtiter plates via an interaction with preciously attached HPC-1 antibodies (see Materials and Methods). Unbound liposomes were removed by washing with PBS and docked v-SNARE liposomes were detected by determining plate-bound rhodamine fluorescence after detergent solubilization. The amount of VAMP liposome-derived rhodamine fluorescence retained on the plate is expressed as the average of five wells for each condition with the error bars representing SD. C, Coomassie blue-stained gel of the vesicles used in the fusion reactions.

and t-SNAREs are capable of mediating vesicle docking. In fact, the lipid-anchored liposomes show an enhanced ability to dock. This enhanced docking efficiency could be explained by an increased conformational freedom of the lipid-attached SNAREs, which in turn would favor trans-SNARE complex formation between liposomes. The specificity of the docking reaction was confirmed by the ability of soluble VAMP to effectively inhibit docking of liposomes containing either VAMP anchored by its transmembrane region (Fig. 2 B) or lipid-anchored VAMP (data not shown). The observation that PE-linked SNAREs dock vesicles also rules out the possibility that sufficient force is generated by SNARE assembly to uproot one lipid anchored SNARE from its bilayer and drive its insertion into the other bilayer housing a TMD-anchored SNARE.

The docking results imply that PE-anchored SNARE complexes can form between vesicles without fusion. Since the small difference in linker length between the PE- and TMD-linked SNAREs outside of the bilayer is well within the tolerance for fusion (McNew et al., 1999), SNARE-dependent fusion follows an active mechanism. Proximity, though a prerequisite, is not sufficient.

Polyisoprenoid Anchors that May Span the Membrane Can Link SNARE Assembly to Bilayer Mixing

Since proximity is not by itself sufficient for bilayer fusion, it follows that in one or another way SNAREpins functional in fusion exert force in the bilayer, pushing it or its constituent lipids along the pathway towards fusion. Evidently, the native TMD was selected in evolution at least in part because it could effectively transduce this force. Is it possible that other lipid anchors, if not PE, might be able to transduce force and thus permit fusion after SNAREpin assembly?

One obvious difference between PE and a TMD is the depth of bilayer penetration. A phospholipid spans only half the bilayer, while a TMD spans all of it. If this difference accounts for the loss of fusion activity, then fusion activity should be observed when SNAREs are linked to lipids that are anchored in both leaflets. With the exception of some rare archaebacterial lipids (Sprott, 1992), phospholipids are not known in nature that have the capacity to span both leaflets. Therefore, to test this possibility we chose the 55-carbon isoprenoid undecaprenol (C55). Though, to our knowledge, it has never been formally shown that undecaprenol spans the bilayer, this is strongly suggested by its length, ~52 Å when fully extended, as compared with the thickness of most bilayers of 35–40 Å. The terminal OH of undecaprenol was esterified with N-malonyl β-alanine to yield the desired lipid, C55 (Fig. 1 B, and Materials and Methods). The linker length of this lipid (including the sulfur of the COOH-terminal cysteine of the attached SNARE) is estimated to be ~8–9 Å. Soluble SNAREs were attached to this maleimide lipid as described for the maleimide-PE derivative. Coupling conditions were chosen that ensured similar reconstitution efficiencies of the C55-coupled and wild-type SNAREs (Fig. 3 C).

Fusion assays (Fig. 3 A) reveal that both C55-anchored v-SNARE and t-SNARE are active with their TMD-linked cognates, and even lipid mix with each other to a
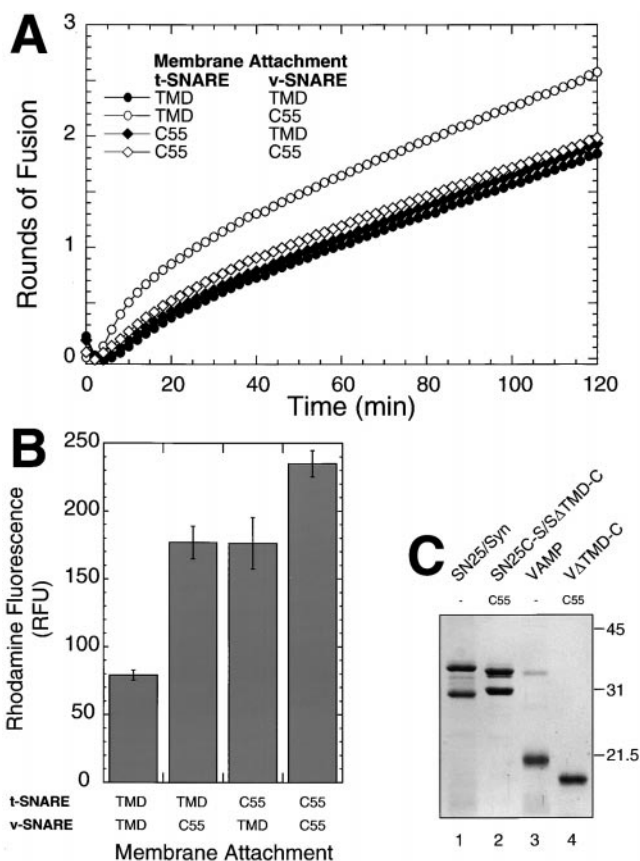
Figure 3. Lipid anchors can functionally substitute for a proteinaceous TMD. A, The TMD of either VAMP or syntaxin was replaced with a modifiable cysteine residue (Fig. 1). These proteins were produced in bacteria and coupled in vitro to C55 (Fig. 1) through a thioether linkage. The t-SNARE complex was produced by coexpression of syntaxin (SΔTMD-C) with SN25 C-S. This t-SNARE contains only one modifiable cysteine residue located at the COOH terminus of syntaxin. The lipid-anchored proteins were incorporated into vesicles by detergent dilution and dialysis (t-SNARE complex) or coupled directly to preformed vesicles containing 5 mole% C55 (VAMP). Vesicles containing the lipid-anchored or normal SNAREs were mixed on ice in various combinations and incubated for 120 min at 37°C in a standard fusion reaction. The extent of fusion is represented as rounds of fusion measured as fold lipid dilution in the reaction. Full-length TMD syntaxin fuses with TMD VAMP to wild-type levels (closed circle). VAMP with a single C55 attachment fuses ~40% better than wild-type with TMD syntaxin (open circles). t-SNARE complexes with a single C55 attachment fuses to near wild-type levels with TMD VAMP (filled diamonds). Most surprisingly, when both vesicle populations contain lipid-anchored SNAREs, wild-type levels of fusion are still observed (open diamonds). B, Liposome docking assay (see Fig. 2). C, Coomassie blue-stained gel of the vesicles used in the fusion reactions.

significant extent. Remarkably, the C55 lipid-anchored v-SNARE fuses with the TMD-linked t-SNARE substantially better than does the native TMD-linked v-SNARE. This is likely the result of increased flexibility in the juxtamembrane region afforded by the lipid anchor. These surprising results show that lipid mixing comparable to native SNARE proteins still occurs, even when both v- and t-SNAREs are attached via lipid anchors, implying
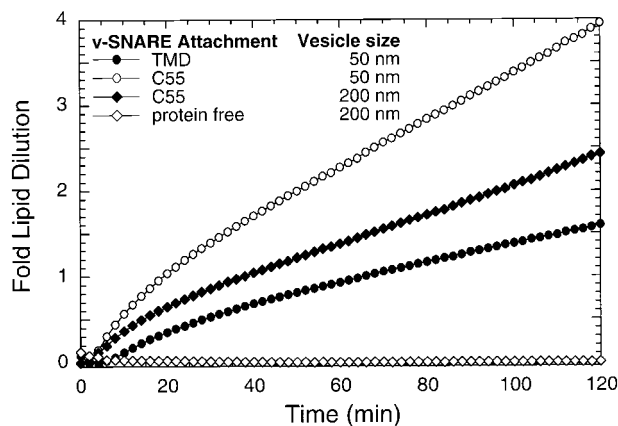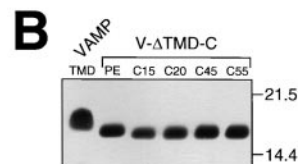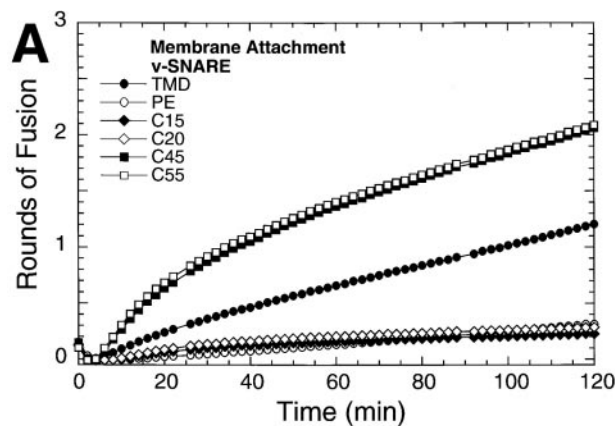
Figure 4. Larger vesicles containing C55-linked VAMP are fusion competent. VAMP (VΔTMD-C) was coupled in vitro to pre-formed liposomes containing 5 mole% C55 extruded through either 50- or 200-nm polycarbonate filter. Protein-free 200-nm liposomes containing 5 mole% C55 were also prepared. Vesicles containing the lipid-anchored v-SNAREs were mixed on ice with wild-type ~50-nm TMD-containing t-SNARE liposomes, and were then incubated for 120 min at 37°C in a standard fusion reaction. The extent of fusion is represented as fold lipid dilution since the size assumption for the rounds of fusion calibration is not valid. Full-length TMD VAMP fuses to wild-type levels (closed circle). VAMP coupled to 50-nm vesicles fused substantially better than wild-type (open circles). VAMP coupled to 200-nm vesicles fused better than wild-type, however, not to the same extent as 50-nm vesicles (filled diamond), likely due to the probe-dilution nature of the assay. Protein-free 200-nm liposomes containing C55 did not fuse with acceptor liposomes. Morphological analysis by cryoelectron microscopy confirmed that the liposome extruded through 200-nm filters were larger than those with 50-nm filters.

Figure 5. Short isoprenoid lipids do not lipid mix. A, VAMP (VΔTMD-C) was coupled in vitro to preformed liposomes containing 5 mole% of either the PE lipid-anchor or one of four different isoprenoids of increasing length. Vesicles containing the lipid-anchored v-SNAREs were mixed on ice with wild-type TMD-containing t-SNARE liposomes, and were then incubated for 120 min at 37°C in a standard fusion reaction. The extent of fusion is represented as rounds of fusion measured as fold lipid dilution in the reaction. Full-length TMD VAMP fuses to wild-type levels (closed circle). All of the short lipid anchors (up to and including C20) show little or no ability to lipid mix. The longer lipid anchors, C45 (closed squares) and C55 (open squares), lipid mix to a similar extent (~75% better than wild-type). B, Coomassie blue-stained gel of the vesicles used in the fusion reactions.

that the undecaprenol anchors can transduce force from the SNARE complex into the bilayer.

Routinely, 50 nm v-SNARE liposomes are prepared to allow a direct comparison with liposomes containing TMD-linked VAMP. When VAMP was coupled to liposomes prepared by extrusion through 200-nm polycarbonate filters, fusion also resulted upon incubation with ~50-nm TMD-linked t-SNARE liposomes (Fig. 4). Unfortunately, it was not possible to test larger t-SNARE liposomes since the t-SNARE complex was not efficiently coupled to preformed liposomes.

To determine if chain length is the critical determinant of the ability of C55 isoprenoid anchors to couple SNARE assembly to fusion, polyisoprenoids of differing chain lengths were tested. Fig. 5 provides these results. Neither C15 nor C20 anchors promote lipid mixing, but they do dock liposomes (data not shown). Neither of these lipid anchors is long enough to penetrate both leaflets. The C45 anchor behaved identically to the C55 anchor, allowing lipid mixing to nearly twice the extent of TMD-linked SNAREs. Since there are no differences among this homologous series in the cytoplasmic or linker regions, or even at the bilayer interface, it is clear that chain length is the sole factor that enables the longer anchors to couple fusion (lipid mixing) to SNARE assembly, and that accounts for the inability of shorter lipid chains to do so.

The fact that C55 and C45 anchors allow lipid mixing to the same or greater extent than TMD-linked SNAREs implies that a complete fusion reaction has occurred with these lipid anchors, as has been independently documented for TMD-linked SNAREs by separately measuring inner leaflet mixing after selective reduction of outer monolayer NBD-PE by dithionite (Weber et al., 1998) and by contents mixing (Nickel et al., 1999). Hemifusion, by definition, would have resulted in a smaller signal. Unfortunately, we have not been able to confirm complete fusion using either of the above assays because the C45 and C55 maleimide derivatives render even unfused liposomes leaky to dithionite, and even to oligonucleotides (data not shown).

Since not all PE or isoprenoid derivatives present in the donor or acceptor liposomes are actually attached to SNAREs (i.e., they are added in excess to promote efficient coupling of protein) it was important to rule out the unlikely possibility that the presence of the uncoupled maleimide lipid derivatives in the lipid bilayer has a significant effect on promoting or inhibiting normal SNARE-mediated fusion. Control experiments confirmed that concentrations up to 5 mole% of either PE (data not shown) or C55 (quenched with 1 mM β-mercaptoethanol) had no
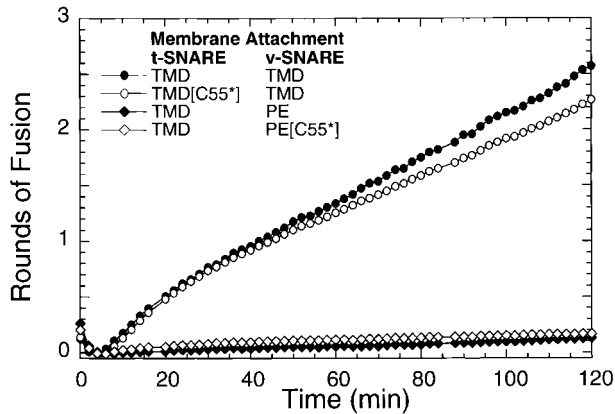
Figure 6. C55 does not stimulate or inhibit SNARE mediated fusion. Vesicles containing the indicated SNAREs were mixed on ice, and were then incubated for 120 min at 37°C in a standard fusion reaction. The extent of fusion is represented as rounds of fusion measured as fold lipid dilution in the reaction. Lipid mixing is seen with vesicles containing TMD-containing SNAREs and the standard lipid composition (filled circles). Similar rates and extents of mixing are seen when the t-SNARE acceptor population contains 5 mole% C55 (quenched with β-mercaptoethanol, open circles, C55*). When donor liposomes containing PE-linked VAMP are used (filled diamonds), the presence of 5 mole% quenched C55 does not stimulate lipid mixing (open diamonds, C55*).

significant effect. Fig. 6 shows that quenched C55 (C55*) has a marginal inhibitory effect when incorporated at 5 mole% in the acceptor t-SNARE liposomes containing the t-SNARE complex. Donor liposomes containing PE-linked VAMP and 5 mole% quenched C55* were still unable to generate a fusion signal, demonstrating that the long-chain C55 isoprenoid is not inherently fusogenic or fusion-promoting on its own.

Asymmetric Lipid Anchor Requirements for v- and t-SNAREs

A careful analysis of the lipid mixing activity of various lipid-anchored SNAREs revealed distinct requirements for v- and t-SNAREs. In the native t-SNARE complex, the SNAP-25 component contains four conserved cysteine residues that are normally fatty-acylated in vivo (Hess et al., 1992; Veit et al., 1996). Unless these residues are purposefully removed (as in the experiments reported above), they too should be modified with lipid during the coupling reaction, resulting in t-SNAREs anchored by multiple lipids: one at the extreme COOH terminus of syntaxin and up to four attachments in the cysteine loop region of SNAP-25. When this t-SNARE complex is coupled to PE, it retains significant capacity to lipid mix with TMD-linked v-SNARE (~50% of the TMD-linked t-SNARE; Fig. 7 A, filled diamonds). Because even five attached PE molecules cannot span the bilayer, the common factor between the ability of C45–55 and multiple PE attachments is not bilayer penetration, but rather appears to be overall hydrophobicity as the critical factor in determining t-SNARE anchor activity.
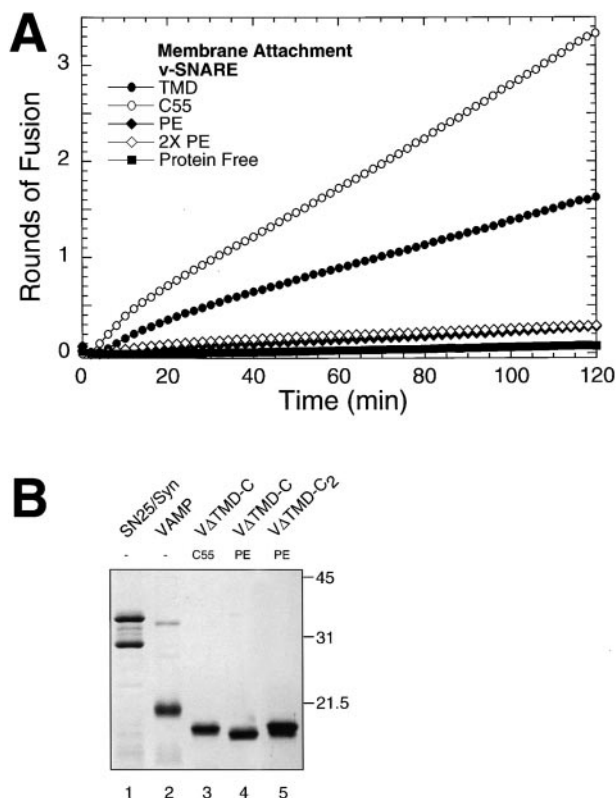
The attachment of multiple PE molecules in the above
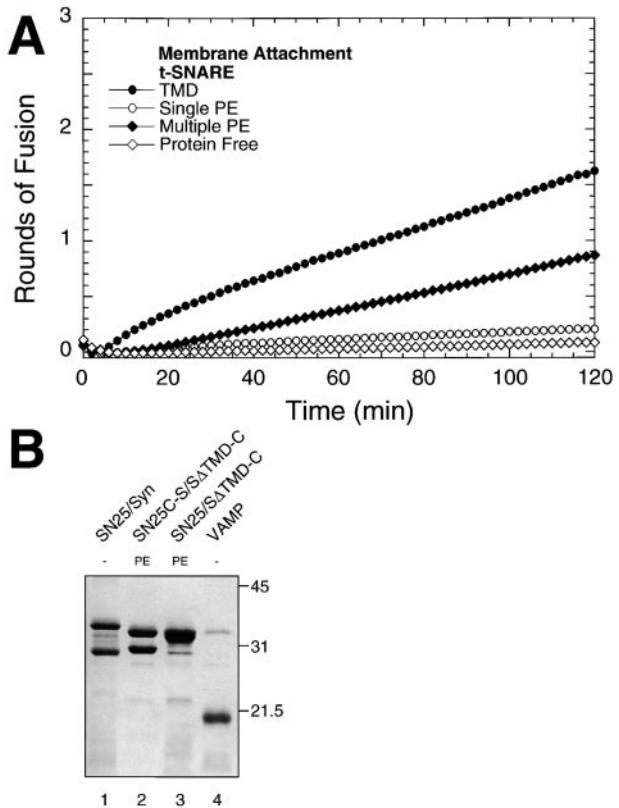
Figure 7. Asymmetric lipid anchor requirements. Overall amount of hydrophobicity is important for the t-SNARE complex. A, Syntaxin (SΔTMD-C) was coexpressed with either SN25 C-S or wild-type SNAP-25, producing t-SNARE complexes with either a single modifiable cysteine residues or a complex with five modifiable residues. These proteins were coupled in vitro to the PE lipid-anchor generating t-SNARE complexes with varying amounts of hydrophobicity. The lipid-anchored proteins were incorporated into vesicles by detergent dilution and dialysis. Vesicles containing the lipid-anchored t-SNAREs were mixed on ice with wild-type TMD-containing VAMP liposomes, and were then incubated for 120 min at 37°C in a standard fusion reaction. The extent of fusion is represented as rounds of fusion measured as fold lipid dilution in the reaction. Full-length TMD syntaxin fuses to wild-type levels (closed circle), whereas t-SNARE complexes with a single PE attachment (open circles) show no fusion above protein-free liposomes (open diamonds). However, the t-SNARE complex with multiple PE attachments (up to five, filled diamonds) fuses to ~50% of wild-type levels. B, Coomassie blue-stained gel of the vesicles used in the fusion reactions.

experiments was confirmed by the significant gel shift of wild-type SNAP-25 relative to the C-S mutant (Fig. 7 B). A t-SNARE complex anchored to membrane only by PE-linked SNAP-25 (presumably involving up to four PE molecules per SNAP-25, made using a syntaxin cytoplasmic domain lacking any cysteine) is also fusogenic (data not shown).

To see if increasing the overall hydrophobicity (with anchors exclusively in the outer leaflet) would also create a v-SNARE competent for lipid mixing, soluble VAMP constructs with several cysteines at the COOH terminus were produced to test the requirements on the v-SNARE side. When a VAMP with two cysteines (Fig. 1 A, VΔTMD-C2)

Discussion

The Role of TMDs in SNARE-dependent Membrane Fusion

It is likely that the TMDs of SNARE and viral fusion proteins have an important role in membrane fusion. All VAMP or syntaxin-type SNARE proteins identified to date, with one notable exception have a COOH-terminal TMD. Even the exception (the yeast SNARE Ykt6p and its homologues (McNew et al., 1997) is membrane-anchored at its COOH terminus by an encoded polyisoprenoid. Viral fusion proteins, and some SNAREs, have certain common and characteristic features in the placement of glycines residues within their transmembrane anchors and point mutations in these and other regions of the TMD have been found to affect fusion (Owens et al., 1994; Cleverley and Lenard, 1998; Melikyan et al., 1999). Also, replacing the TMD with a GPI lipid anchor prevents fusion (Kemble et al., 1994; Melikyan et al., 1995). While these data are certainly consistent with an active role for the natural peptidic anchors in force transduction required for fusion, other interpretations cannot be rigorously excluded due to the inherent complexity of the viral fusion proteins and, in some cases, the assays employed as discussed in the Introduction.

It is more straightforward to address the basic issue of whether anchors play a passive or an active role in fusion in the context of SNARE proteins. This system is inherently more tractable to modification because the SNARE-pin functional in fusion assembles from two separate parts, two half pins, during the fusion process. As a result, the v-SNARE and t-SNARE can be separately modified and all combinations can be directly tested. By contrast, the viral hairpin implicated as fusogenic assembles intramolecularly only in the context of a complex refolding reaction in a much larger protein (Wilson et al., 1981; Carr and Kim, 1993; Bullough et al., 1994; Skehel and Wiley, 1998). Also, since the assay for fusion activity involves isolated recombinant SNARE proteins, a variety of distinct lipidic anchors can be added to already folded and purified cytoplasmic proteins by chemical modification. Additionally, the diversity of the attached lipid anchors is not limited to the few moieties whose addition is encodable on a given side of a membrane in living cells.

The fact that lipid mixing is not observed when either a v-SNARE or a t-SNARE (or both) are anchored to a single phospholipid argues forcefully against a passive mechanism because the same SNARE complex is involved and bilayer proximity is not significantly altered. Lack of lipid mixing means that neither complete fusion (involving both leaflets of both vesicles), nor hemifusion (involving outer leaflets only) takes place. Yet trans-SNARE complexes, in which v-SNAREs anchored in one bilayer assemble into helical bundles with t-SNAREs anchored in another, must still form because vesicles still dock. This means that donor and acceptor vesicles held together by the same SNARE complex fuse when both have their normal peptidic anchors, but fail to fuse when either peptidic anchor is replaced by a phospholipid. Furthermore, the two vesicles are held in functionally indistinguishable and structurally similar proximity, since incidental changes in

Figure 8. Asymmetric lipid anchor requirements. Length of the hydrophobic component is important for v-SNAREs. A, VAMP (VΔTMD-C) was coupled in vitro either to the PE lipid anchor or the longer C55 isoprenoid. Additionally, another VAMP protein was made that contained two cysteine residues at its COOH terminus (Fig. 1). This protein was produced to generate VAMP with multiple attachment points, similar to the t-SNARE complex in Fig. 7. The VAMP proteins were coupled directly to pre-formed vesicles containing 5 mole% C55, or 5 mole% PE as indicated. Vesicles containing the lipid-anchored v-SNAREs were mixed on ice with wild-type TMD-containing t-SNARE liposomes, and were then incubated for 120 min at 37°C in a standard fusion reaction. The extent of fusion is represented as rounds of fusion measured as fold lipid dilution in the reaction. Full-length TMD VAMP fuses to wild-type levels (closed circle). As seen in Fig. 3, VAMP coupled to the C55 lipid anchor fuses better than wild-type (open circles). However, VAMP with a single PE attachment (filled diamonds) shows no fusion above protein-free liposomes (filled squares). Interestingly, VAMP with multiple PE attachments (open diamonds) shows no ability to fuse in contrast to a similarly anchored t-SNARE complex. Additional experiments show that even four PE lipid anchors added to VAMP do not significantly increase its fusion potential (data not shown). B, Coomassie blue-stained gel of the vesicles used in the fusion reactions.

is coupled to PE, little or no lipid mixing is observed with acceptor liposomes containing TMD-linked t-SNAREs (Fig. 8 A, open diamonds). The same was true when the VAMP had four COOH-terminal cysteines (not shown). However, when these same v-SNAREs are anchored by C55, bilayer mixing now occurs (data not shown). These results suggest that while overall hydrophobicity determines t-SNARE anchor activity, chain length determines v-SNARE anchor activity.

linker length associated with changing peptidic to lipid anchors are without significant effect on fusion (McNew et al., 1999). The linkers that attach the cytoplasmic domain of the SNARE proteins to these phosphatidylethanolamine headgroups (including the COOH-terminal cysteine of the VAMP or syntaxin) are ~18 Å long when fully extended. This linker length is well within the variations that have been shown to be well-tolerated with no significant adverse affect on VAMP fusion activity (McNew et al., 1999). Therefore, the dramatic loss of fusion activity when peptidic anchors are replaced by a phospholipid is not due to incidental variations in linker length, but rather to change in anchor unit.

In sum, two bilayers held together by the same device fuse with peptidic anchors, but not with phospholipid anchors. If spontaneous fluctuations in bilayer lipids were to drive fusion in a passive mechanism, the sole role of the SNARE complex would be to create adequate proximity without interacting with (i.e., exerting force to) membrane lipids. This state would be created as well with phospholipid anchors as with peptidic anchors, and would result in fusion after docking; it does not do so. The fact that even intimate bilayer proximity is not sufficient to promote fusion adds further weight to the already considerable evidence that SNAREpins utilize a specific and biologically relevant mechanism to promote fusion (Weber et al., 1998; Chen et al., 1999; McNew et al., 1999; Nickel et al., 1999; Parlati et al., 1999; Weber, T., F. Paralti, J.A. McNew, R.J. Johnston, B. Westermann, T.H. Söllner, and J.E. Rothman, manuscript submitted for publication) and do not simply dock membranes that then fuse by an unknown mechanism (Ungermann et al., 1998).

The finding that replacement of certain viral TMDs with a GPI-based phospholipid anchor abrogates fusion (Kemble et al., 1994; Melikyan et al., 1995) is consistent with our result with SNAREs; however, it is not clear why in the viral case a hemifusion process is created (or persists), whereas with SNAREs no fusion process remains. Conceivably this difference relates to the fact that a fusion peptide is not a traditional TMD since it can reside either folded within a larger protein or associated with a cellular membrane. Even then, the fusion peptide may or may not penetrate both leaflets of the cellular membrane; the geometry of its interaction with the bilayer has been the subject of much debate (Durell et al., 1997; Pecheur et al., 1999; Peuvot et al., 1999). By contrast, v- and t-SNAREs must each have a permanent and traditional membrane anchor since they reside in separate membranes before fusion. If the fusion peptide penetrates the outer leaflet, but not the inner leaflet, it could displace sufficient lipids to allow hemifusion (Chernomordik et al., 1998, 1999; Bentz, 2000); this could point to a difference in force transduction mechanisms between viral and SNARE-dependent fusion.

Possible Mechanisms for Active Coupling of SNARE Assembly to Motion in the Bilayer Promoting Fusion

Our results clearly imply that assembly of the SNAREpin must in one way or another do work on the bilayer to promote fusion, either during or after its assembly. This coupling could occur during assembly, when free energy is made available by folding of the v-SNARE and favorable interactions with the t-SNARE (VAMP is in a random coil before assembly and in an α helix within a helical bundle after assembly; Fasshauer et al., 1997; Poirier et al., 1998; Sutton et al., 1998). Or it could occur after assembly if the SNAREpin is in a high energy state relative to bilayer lipids that can be relieved due to a rearrangement of these lipids. Current genetic, biochemical, and physiological results point to a loose state in which the SNAREs are partially assembled in the membrane-distal portion, and a tight state in which they are completely zipped-up (Hanson et al., 1997a; Katz et al., 1998; Hua and Charlton, 1999; Xu et al., 1999). It seems likely that the transition from the loose to the tight state, which structurally would force attached membranes into close proximity, may be when most of the work on the bilayer is done to trigger fusion.

A priori, there are two general (and nonexclusive) mechanisms by which bilayer lipids can be actively perturbed by assembled (or, assembling) pins: direct conformational coupling, either via lipids associated with the anchor, linker, or surface of the rod of the pin; or pulling on the membrane anchors due to force exerted from the rod via taut linkers. Limits can now be placed on these mechanisms in the case of SNARE-dependent fusion.

It appears that direct conformational coupling involving the anchor, linker, and rod as a unit structure can be ruled out, as can conformation-based coupling between the anchor or the linker and lipids. The fact that essentially free torsional rotation can be introduced into the linker without abolishing fusion (McNew et al., 1999) implies that conformational coupling between rod and anchor is not important. Therefore, a continuous region of secondary or tertiary extending from rod through linker to anchor either does not occur or is not important for fusion if it does. Specific interactions between amino acid side chains of the linker and phospholipids are unlikely to be important for fusion because their nature and precise spacing from the membrane can be altered without effect (McNew et al., 1999). Finally, since we now know that C45 and C55 isoprenoid anchors unrelated in chemical structure to peptidic anchors can support lipid mixing, potentially specific interactions between peptidic anchors (v-v, v-t, or t-t) or between their side chains and lipids are not important for fusion.

The current evidence neither speaks for or against conformational coupling between the bilayer and the rod. For example, the assembly of the rod at the interface between the two bilayers could recruit certain species of lipids via interactions between side chains protruding from the rod and the lipid head groups, creating a local high-energy state of the bilayer that contributes to the activation energy for fusion. However, the fact that fusion is dramatically reduced as either the v- or t-SNARE linkers are significantly extended (McNew et al., 1999) or abolished by PE anchors, implies that if rod–bilayer coupling contributes to fusion, it is not sufficient for fusion.

This same observation (McNew et al., 1999) suggests that force may be transduced, at least in part, by pulling on the membrane anchors. The origin of the strain would lie in the stability of the helical bundle of the rod, which would generate a twisting force to do work on the bilayer as the rod completes its assembly (loose to tight). This process is likely linked to overcoming the steric exclusion of

the attached membranes that becomes prominent when they are forced into intimate proximity as the SNAREs zip-up their membrane proximal ends (Xu et al., 1999). Extending the linker would gradually relieve this strain and accordingly uncouple SNARE assembly from fusion.

Force Transduction by SNAREs during Membrane Fusion

In sum, current evidence suggests that the helical bundle of the SNARE complex in one or another way exerts force on the membrane anchors, and that this provides an essential driving force for fusion. Other interactions, such as possible linkage between the rod and the bilayer, may also be important.

The pattern of activity of various lipid anchors is consistent with this model and adds considerable detail. Presumably, pulling on a membrane anchor serves to displace the anchor from the bilayer, lifting out associated lipids towards the contact point between the two bilayers, a process which has been proposed to be an important trigger for artificial bilayer fusion (Chernomordik and Zimmerberg, 1995; Chernomordik et al., 1995a,b, 1998, 1999). A membrane anchor (whether peptidic or lipidic) that spans both leaflets will perturb lipids on both sides of the membrane as it is displaced; an anchor that samples only the outer monolayer (corresponding to the cytoplasmic leaflet in a cell) will perturb those lipids only in the outer monolayer. Our finding that C55 and C45 isoprenoid v-SNARE anchors (which can in principle sample both monolayers) promote fusion (measured by lipid mixing), but neither C20 nor C15 isoprenoids (which can only reside in the outer monolayer) promote fusion, suggests that the v-SNARE anchor needs to interact with both leaflets in order to promote fusion as it is displaced toward the apposing bilayer when pulled on by the attached linker.

An anchor of up to four phospholipids (involving up to eight fatty acid chains) that is much more hydrophobic than C55, but which is restricted to the outer monolayer, does not permit the v-SNARE to fuse after docking, confirming that bilayer topology, rather than hydrophobicity, is critical for the function of the v-SNARE anchor unit. By contrast, either multiple phospholipids or a single long chain (C45 or C55) isoprenoid served as a functional t-SNARE anchor, implying that overall hydrophobicity and not transmembrane topology is the critical feature of a t-SNARE anchor. The fusogenic activity of a t-SNARE anchor limited to the outer monolayer implies that perturbation of these lipids only is sufficient for fusion on the t-SNARE side.

The dynamic asymmetry implied by our results with lipid anchors should not be surprising since the SNARE-pin is not a symmetrical structure relative to the membrane plane (Sutton et al., 1998). Asymmetric application of force on the two sides of a fusion junction would explain the asymmetric dimpling of membranes engaged in exocytosis, observed by EM (Monck and Fernandez, 1992, 1994, 1996; Monck et al., 1995). It should also be noted that the resistance of SNAREpins engaging in fusion to enzymatic disruption by the ATPase NSF (Weber, T., F. Paralti, J.A. McNew, R.J. Johnston, B. Westermann, T.H. Söllner, and J.E. Rothman, manuscript submitted for publication) is well-explained by the existence of forces during fusion

which would draw the bilayers together and effectively seal them off from NSF or other extrinsic macromolecules.

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