

The Length of the Flexible SNAREpin Juxtamembrane Region Is a Critical Determinant of SNARE-Dependent Fusion

James A. McNew,* Thomas Weber,*
Donald M. Engelman,† Thomas H. Söllner,*
and James E. Rothman*†

* Cellular Biochemistry and Biophysics Program
Memorial Sloan-Kettering Cancer Center
1275 York Avenue
New York, New York 10021

† Department of Molecular Biophysics
and Biochemistry
Yale University
New Haven, Connecticut 06520

Summary

The topology of a SNARE complex bridging two docked vesicles could act as a mechanical couple to do work on the lipid bilayer resulting in fusion. To test this, we prepared a series of modified SNARE proteins and determined their effects on SNARE-dependent membrane fusion. When two helix-breaking proline residues are introduced into the juxtamembrane region of VAMP, there is little or no effect on fusion, and the same change in syntaxin 1A only reduced the extent and rate of fusion by half. The insertion of a flexible linker between the transmembrane domain and the conserved coiled-coil domain only moderately affected fusion; however, fusion efficiency systematically decreased with increasing length of the linker. Together, these results rule out a requirement for helical continuity and suggest that distance is a critical factor for membrane fusion.

Introduction

Members of the SNARE (SNAP receptor) protein family (Söllner et al., 1993a, 1993b) are key components in the process of transport vesicle docking and fusion. Individual SNARE family members are maintained in discrete locations throughout the secretory pathway providing a roadmap of vesicle flow patterns (Hay and Scheller, 1997; Linial, 1997; Advani et al., 1998; Nichols and Pelham, 1998; Steegmaier et al., 1998). The assembly of *trans*-SNARE complexes (SNAREpins) between membranes is likely the underlying principle of lipid bilayer fusion (Weber et al., 1998; Chen et al., 1999), and as such, it must be highly regulated in many cell types. A sequential series of protein interactions, as yet incompletely understood, ensures proper SNARE activation and vesicle tethering and finally culminates in proper SNAREpin formation. The machinery mediating these events includes proteins such as Rab proteins, their associated partners (Lazar et al., 1997; Novick and Zerial, 1997; Martinez and Goud, 1998; Schimmoller et al., 1998), sec1 family members (Aalto et al., 1991; Cowles et al., 1994; Pevsner et al., 1994; Schulze et al., 1994;

Halachmi and Lev, 1996), tethering proteins like p115/Usolp, giantin, GM130 (Nakajima et al., 1991; Waters et al., 1992; Nakamura et al., 1995, 1997; Sapperstein et al., 1995; Orci et al., 1998; Sönnichsen et al., 1998), and the exocyst complex (TerBush et al., 1996; Kee et al., 1997; Sacher et al., 1998). Additional general factors acting on SNAREs are NSF (NEM-sensitive factor; Block et al., 1988) and SNAPs (soluble NSF attachment proteins; Clary et al., 1990), which alter the conformation of SNAREs and disassemble v-t SNARE complexes, thereby regenerating separate v- and t-SNAREs for repeated use.

Most SNARE proteins possess a single transmembrane domain at their extreme carboxy terminus and are predicted to have a high propensity to form coiled-coil structures. Assembled cytosolic domains of SNARE proteins form very stable structures in all cases that have been closely examined (Hayashi et al., 1994; Yang et al., 1999), likely due to their coiled-coil nature. Furthermore, electron microscopic (Hanson et al., 1997b; Hohl et al., 1998) and biophysical (Lin and Scheller, 1997; Poirier et al., 1998) analysis of assembled full-length neuronal SNARE complexes revealed that the transmembrane domains of both the v-SNARE VAMP/syntaxin 1A (Trimble et al., 1988; Baumert et al., 1989; Südhof et al., 1989) and the t-SNARE syntaxin 1A (Bennett et al., 1992, 1993) emerge at the same end of the 7S or 20S particle establishing a parallel orientation of the assembled SNARE proteins. These characteristics suggested that SNARE proteins are also directly responsible for membrane fusion (Hanson et al., 1997a, 1997b; Lin and Scheller, 1997; Hohl et al., 1998), and this has now been directly demonstrated with artificial liposomes and isolated SNAREs (Weber et al., 1998) and confirmed with permeabilized cells (Chen et al., 1999).

The recently obtained three-dimensional structure of the neuronal SNARE complex (Poirier et al., 1998; Sutton et al., 1998) has provided additional information to guide structure-function studies aimed at clarifying the biophysical mechanisms involved in fusion. The crystal structure confirms the previous biophysical predictions of coiled-coil structure and provides a structural framework to explain the intrinsic stability of the assembled SNARE complex. Another striking feature of the core SNARE complex structure is its overall similarity to the proposed fusogenic cores of a variety of virally encoded fusion proteins (Skehel and Wiley, 1998). This similarity lends compelling indirect support to the proposition that SNARE proteins are the cellular fusion machinery and suggests that pin-like structures are a general principle of fusion processes.

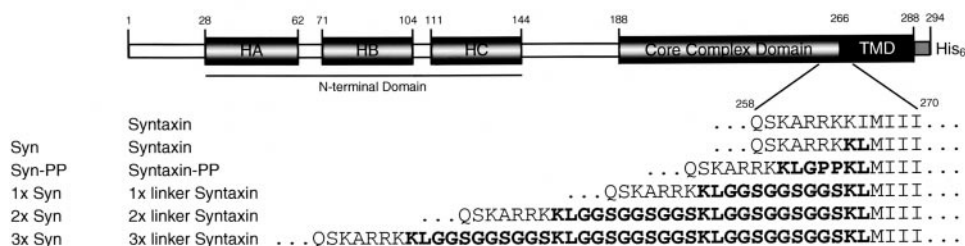
Results

Helical Continuity between the SNARE Core Helix and the Transmembrane Domain Is Not Necessary for Membrane Fusion

One of several models for fusion proposes that a SNAREpin mechanically couples the stable membrane

† To whom correspondence should be addressed (e-mail: j-rothman@ski.mskcc.org).

Syntaxin 1A



VAMP2



Figure 1. Neuronal SNARE Proteins

The amino acid sequence of the full-length SNARE proteins is graphically depicted. Modified regions are magnified to reveal the exact nature of the changes. Flexibility was introduced into the SNARE proteins by engineering a parent DNA construct that contained a unique restriction enzyme site in the juxtamembrane region of the SNAREs into which double-stranded oligonucleotides could be inserted. A repeat unit of nine flexible amino acids was chosen. The linker constructs contain 9, 18, or 27 amino acids intended to introduce flexibility and increase the physical distance form the core SNARE complex and the membrane interface. The proline-proline constructs were designed to prohibit a continuation of the membrane spanning helix into the coiled-coil segment and relieve helical strain.

proximal coiled-coil segment to the v- and t-SNARE transmembrane domains (Weber et al., 1998). This mechanical link could simply result from helical continuity between the juxtamembrane core domain and the transmembrane helix (Harbury, 1998; Sutton et al., 1998). The energy that is generated in the formation of the SNARE complex could then be used to produce a strained conformation in the SNARE proteins making the SNAREpin structure a metastable, high-energy intermediate state (Harbury, 1998; Sutton et al., 1998; Weber et al., 1998). The process of bilayer merger would relieve this stress and return the SNAREs to their lowest energy state.

We began to test these predictions by modifying the SNARE proteins to relieve this potential strain and measure the effect in fusion activity. To this end, we introduced two helix-breaking proline residues (Shultz and Schirmer, 1988) in the juxtamembrane region immediately preceding the transmembrane domain in both syntaxin 1A and VAMP (Figure 1A). The resulting proteins were expressed in *E. coli*, purified by affinity chromatography, and reconstituted into lipid vesicles. The various modified proteins were all well expressed but had dissimilar reconstitution efficiencies. Therefore, differing, empirically derived amounts of the various SNARE proteins had to be added to each reconstitution to maintain a constant protein to lipid ratio in v and t vesicle populations to allow direct comparison between fusion kinetics of vesicles containing modified SNARE proteins (Figure 2C).

The fusion activity of the modified SNARE proteins was monitored by the well characterized lipid-mixing assay (Struck et al., 1981) described in detail previously (Weber et al., 1998). t-SNARE vesicles are derived from a mixture of synthetic lipids containing 85 mole percent

phosphatidylcholine (POPC) and 15 mole percent phosphatidylserine (DOPS). The v-SNARE vesicles contain these lipids in addition to the fluorescent lipids NBD-PE (N-[7-nitro-2,1,3-benzoxadiazole-4-yl]-phosphatidylethanolamine) and rhodamine-PE (N-[lissamine rhodamine B sulfonyl]-phosphatidylethanolamine). The resonance energy transfer between this fluorescent donor and acceptor pair is strongly dependent on the distance between them. Therefore, when fluorescent v-SNARE vesicles fuse with unlabeled t-SNARE vesicles, the average distance between the fluorophores is increased upon lipid dilution producing an increase in the previously transferred NBD fluorescence. This relief of resonance energy transfer is monitored at the NBD emission maximum at 538 nm.

Our standard assay makes use of v vesicles that contain an approximately 10-fold higher concentration of VAMP as compared to the concentration of syntaxin 1A/SNAP-25 in t vesicles; therefore, to maintain an equimolar amount of SNARE proteins in the fusion reaction, we must include an excess of nonfluorescent t vesicles in our fusion assay (~10:1 ratio). Since the concentration of unlabeled lipid is higher than that of the fluorescent lipid, our fusion assay has the potential to yield multiple rounds of fusion of fluorescent v vesicles. When one v-SNARE-containing vesicle fuses with one t-SNARE-containing vesicle, the fluorescent lipid is diluted by half and, likely, all of the t-SNAREs in the new vesicle are complexed with VAMP. However, approximately 90% of the VAMP molecules that were initially present in the original VAMP vesicle are free to participate in another round of fusion. As this process continues, the fluorescent lipids get progressively more diluted as each round of fusion occurs. Both of the ongoing processes, dilution

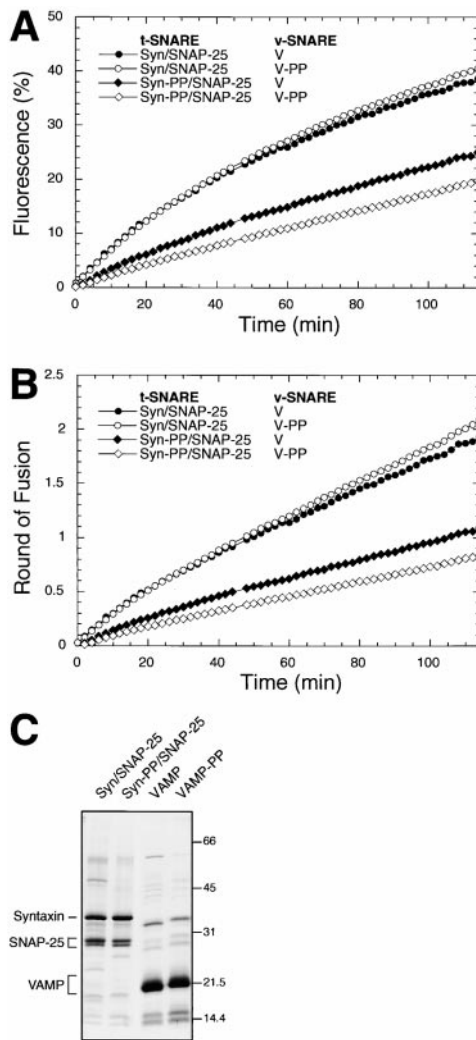


Figure 2. Helical Continuity between the SNARE Core Domains and Their Transmembrane Domain Is Not Required for Membrane Fusion. Vesicles with equivalent amounts of t- or v-SNAREs were preincubated overnight at 4°C and fused for 120 min at 37°C. Fusion curves show a comparison between wild-type SNARE proteins (Syn/SNAP-25 and VAMP, closed circles) and SNAREs that have been modified by the insertion of two helix-breaking proline residues in the juxta-membrane region of VAMP (open circle), syntaxin (closed diamonds), or both (open diamonds). The kinetics of fusion are represented as percent of total NBD fluorescence (A) or rounds of fusion measured as fold dilution of donor lipid in the reaction (B). (C) Coomassie blue-stained gel of the vesicles used in the fusion reaction indicating equivalent amounts of SNARE proteins in the vesicles allowing direct comparison.

of fluorescent lipid and the measured relief of resonance energy transfer, are inherently nonlinear events.

Previously (Weber et al., 1998), the kinetics of the fusion reaction were conventionally (Hoekstra and Duzgunes, 1993) presented as the percent of total NBD fluorescence present at “infinite” separation (Figure 2A, fluorescence), determined when the vesicle phospholipids distribute among many detergent micelles upon addition of detergent. To make it easier to relate this measurement to the efficiency of vesicle membrane fusion, we now calibrate our data in terms of rounds of fusion

of donor vesicles (Figure 2B). To do this, we directly determined the relationship between percent of total NBD fluorescence and fold lipid dilution. This was accomplished by premixing fluorescent lipid stock solutions with nonfluorescent lipids in ratios of 1:0.5, 1:1, 1:2, 1:4, and 1:8. These prediluted fluorescent lipid mixes were used to make VAMP vesicles, and the resulting percent NBD fluorescence was plotted as a function of fold fluorescent lipid dilution. The resulting curve can be fitted with a double exponential function relating these two parameters. The equation of the fitted curve was subsequently used to convert percent NBD fluorescence to fold lipid mixing or rounds of fusion of v vesicles. A detailed description of this calibration will be published elsewhere (Parlati et al., 1999) and is briefly described in the Experimental Procedures.

Figure 2A depicts the result of fusion assays performed with the proline-containing SNARE proteins expressed as percent of total fluorescence. These results were then converted to rounds of fusion by the calibration curve described above (Figure 2B).

The introduction of two proline residues in the juxta-membrane region of VAMP had virtually no effect (Figures 2A and 2B, compare open and closed circles). In fact, a slight enhancement was sometimes observed. However, when the t-SNARE syntaxin contained the helix breakers, both the rate and extent of fusion were diminished. An approximately 50% reduction in the extent of fusion at the 2 hr time point was observed (Figures 2A and 2B, compare closed circles and closed diamonds). A similar effect occurred when both v and t sides were modified (Figures 2A and 2B, open diamonds). These results demonstrate that, while helical continuity is not a prerequisite for membrane fusion, it may enhance the rate 2-fold in the case of the t-SNAREs.

Increasing Distance and Flexibility between the SNARE Core Helix and the Transmembrane Domain Progressively Inhibits Fusion

Since helical continuity between cytoplasmic domains and membrane anchors is not required for fusion, we examined the effect of increasing distance with a flexible linker. The linker sequence is composed of the three-amino acid repeat sequence glycine-glycine-serine repeated three times, (GGS)₃. These amino acids were chosen because glycine has no side chain to restrict rotational freedom and likely adopts a random coil. This modification served two important functions: first, it relieved potential strain by introducing flexibility, and second, it extends the SNARE core helix from its transmembrane domain and presumably distances SNARE complex formation from the lipid bilayer interface.

Figure 3 shows the results of the linker mutations. The introduction of a nine-amino acid linker in the context of the v-SNARE VAMP has only a modest effect on the overall rate and extent of fusion (Figure 3A, open circles). As the length of the linker is increased, a stepwise decrease in fusion activity is observed. While this is most likely the result of increasing the physical distance from the assembled SNAREpin to the membrane interface, we cannot rule out the possibility that the linker protein simply prevents fusion by a steric mechanism.

As seen with the proline mutation, modification of the

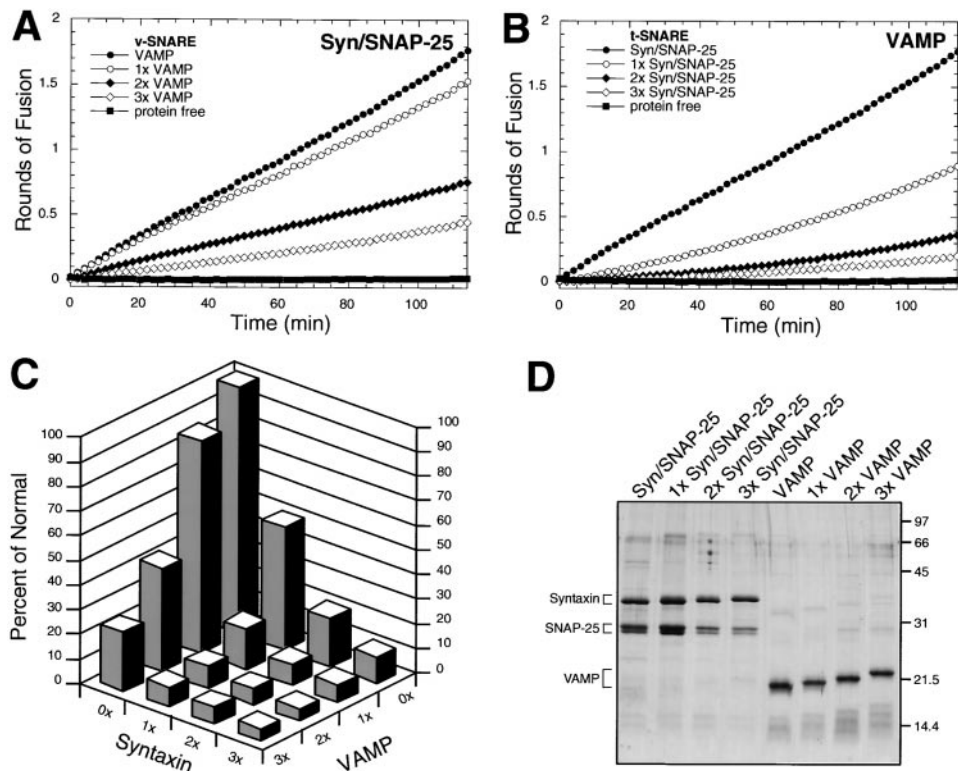


Figure 3. Increasing the Distance between the SNARE Core Domain and the Transmembrane Domain Progressively Inhibits Fusion
Vesicles with equivalent amounts of t- or v-SNAREs were preincubated overnight at 4°C and fused for 120 min at 37°C.
(A) Fusion curves comparing wild-type t-SNARE complex with VAMP proteins containing a linker sequence [(GGS)₃] of increasing length (one, two, or three copies).
(B) Fusion curves comparing various linker syntaxins (isolated in a complex with SNAP-25) relative to normal VAMP. The extent of fusion is represented as rounds of fusion measured as fold dilution of donor lipid in the reaction. Fusion is progressively inhibited by increasing the length of the linker sequence.
(C) Summary of fusion inhibition by linker SNAREs. All possible combinations of linker SNAREs were used in fusion reactions. The extent of fusion at 120 min was normalized to the unmodified SNAREs.
(D) Coomassie blue-stained gel of the vesicles used in the fusion reaction in (A)–(C) indicating equivalent amounts of SNARE proteins in the vesicles allowing direct comparison. The lower band of the SNAP25 doublet is a C-terminal degradation product (Weber et al., 1998).

t-SNARE syntaxin has a more pronounced effect than the modifications of VAMP. With a single linker insertion, fusion is reduced to about 50% of normal SNARE proteins (Figure 3B, open circles). This change is likely due to the relief of helical strain, based on the results seen in Figure 2. Multiples of the linker sequence progressively inhibit fusion as was seen in the case of VAMP. SNARE complex formation appears not to be the limiting factor since the most extreme case (3× linker) forms SNARE complexes as efficiently as the wild-type proteins (data not shown). Figure 3C represents the extent of fusion for all combinations of linker SNAREs as a percent of normal, unmodified SNAREs.

Discussion

The identification of SNARE proteins as basic components of the cellular fusion machinery based on the development of a fusion assay involving isolated SNARE proteins (Weber et al., 1998) now permits a detailed analysis of molecular parameters governing protein-mediated membrane fusion. In this report, we begin

to dissect the fusion mechanism by making selective modifications of the v- and t-SNARE proteins at the cytosol-membrane interface. Surprisingly, we find that changes expected to conformationally uncouple membrane anchors from the body of the SNARE complex only slightly affect the fusion process. The introduction of two tandem proline residues had little effect on fusion (Figure 2), although predictions suggest that the introduction of this pair of helix breakers will lead to the disruption of an α helix (Shultz and Schirmer, 1988). Furthermore, work done on the influenza hemagglutinin viral fusion protein has shown that insertion of two prolines, spaced by as many as 16 residues, does not allow pH-induced helix formation in this region of this well characterized coiled-coil protein (Qiao et al., 1998).

However, increasing the separation between the coiled-coil domains and the transmembrane domains dramatically reduces the efficiency of fusion (Figure 3). In particular, the fact that a single linker insertion or the proline mutations in VAMP have little or no effect on fusion (both of which introduce substantial flexibility) points to distance as the primary determinant of fusion efficiency.

While our results are still compatible with fusion models that rely on mechanical coupling between the coiled-coil domains and the transmembrane-spanning segments, they rule out models in which coupling requires a continuous helix between them. Rather, and surprisingly, our results suggest that transmembrane domains tethered by flexible stems, provided they are the right length, are capable of fusion. Perhaps as SNARE complexes zip up between bilayers, the stems are pulled or twisted to exert force on apposing bilayers.

Experimental Procedures

DNA Manipulations and Plasmid Constructs

Standard genetic manipulation was performed throughout (Sambrook et al., 1989), and all restriction endonucleases were purchased from New England Biolabs. Routine polymerase chain reactions were done with Pwo (Boehringer Mannheim). DNA was routinely propagated in the strain DH5 α (F^- , ϕ 80d*lacZ* Δ M15 Δ [*lacZYA-argF*] U169 *deoR* *recA1* *endA1* *hsdR17*[r_K^- m_K^-] *phoA* *supE44* λ^- *thi-1* *gyrA96* *relA1*; GIBCO-BRL) except for the QuickChange mutagenesis, which used XL1-Blue (*recA1* *endA1* *gyrA96* *thi-1* *hsdR17* *supE44* *relA1* *lac* [F' *proAB* *lac* Δ M15 Tn10 (Tet^r); Stratagene). Protein expression from plasmids requiring the T7 polymerase was performed in the strain BL21(DE3) (F^- *ompT* *hsdS β* [r_B^- m_B^-] *gal* *dcm* [DE3]; Novagen).

Construction of Parental Syntaxin and VAMP Constructs

The HindIII site 3' of the coding region (bp 5955–5960) in pTW12 (Weber et al., 1998) was removed by digesting with HindIII, filling in the overhangs with Klenow, and blunt ligating generating pJM23. This manipulation generated a unique NheI site. The same strategy was employed to remove the HindIII site 5' of the T7 terminator region of the pET3a-derived pTW2 (Weber et al., 1998) generating pJM25. A unique HindIII site was placed in the juxtamembrane region (Figure 1) of both VAMP (pJM27) and syntaxin (pJM26) by site-directed mutagenesis utilizing the QuickChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. The oligonucleotides used for this procedure were rSyn1A-H3f (GCAAGGCAC GCAGGAAGAAGCTTATGATCATCATTTGCTGTGTG), rSyn1A-H3r (CACACAGCAAATGATGATCATAAGCTTCTTCTGCGTGCCTTGC), mVAMP2-H3f (GGTGGAAAACCTCAAGCTTATGATCATCTTGGGA GTG), and mVAMP2-H3r (CACTCCCAAGATGATCATAAGCTTGAGG TTTTCCACC). These vectors (pJM26 and pJM27) served as parent vectors for all subsequent linker insertions.

Generation of Linker and Proline Constructs

The double-stranded oligo GGS-H3f (AGCTCGGTGGTCCGGTG GTTCCGGAGGTCCA) and GGS-H3r (AGCTTGGAACTCCGGAAC CACCGGAACCACCG) codes for the nine-amino acid repeat sequence GSGSGSGS. When this oligo is inserted into the parent vector(s) in the correct orientation, the 5' HindIII site is destroyed and the 3' HindIII site is maintained. Additional amino acids (KL) are added to the ends of the linker from the obligatory HindIII restriction site (Figure 1). This annealed double-stranded oligo was ligated into the VAMP parental plasmid pJM27 generating pJM29-14, pJM32-3, and pJM35-19. These constructs contain one, two, or three copies, respectively, of the double-stranded oligo in the correct orientation. When the double-stranded oligo was ligated into the syntaxin parental plasmid pJM26, the following plasmids were obtained: pJM33-1, pJM28-3, and pJM31-2, respectively. Again, each construct contains one, two, or three copies of the double-stranded oligo in the correct orientation. The same procedure was used to create the proline insertions. The double-stranded oligos, in this case, GPP-H3f (AGCTCGGTCCGCCGA) and GPP-H3r (AGCTCGGCGGACCG), code for the three-amino acid sequence GPP. pJM40-4 is the VAMP plasmid with this insertion, and pJM39-18 is the syntaxin plasmid with the GPP insertion. All mutations were confirmed by DNA sequence analysis.

Recombinant Proteins

All t-SNARE complexes were made by coexpression with pGEX-mSNAP-25 (Weber et al., 1998), which expresses GST-SNAP-25, in

combination with the specific wild-type or linker syntaxin. These proteins were expressed in the BL21(DE3) (Novagen) bacterial strain since the syntaxin constructs are based on pET28a and require the T7 polymerase for expression. Cells expressing t-SNARE complexes were typically grown in 4 liter volumes in SuperBroth (Bio101) containing 200 μ g/ml ampicillin and 50 μ g/ml kanamycin at 37°C. Protein expression was induced by the addition of 1 mM isopropyl- β -D-thiogalacto-pyranoside (IPTG) for 4 hr. The cells were recovered by centrifugation and resuspended in 40 ml breaking buffer (50 mM Tris-HCl [pH 8.0], 300 mM KCl, 10% [w/v] glycerol, 10 mM β -mercaptoethanol, and protease inhibitors [Complete protease inhibitor tablets, Boehringer Mannheim]). After resuspension, 10 ml of 20% (w/v) Triton X-100 was added. The cells were disrupted by a French press, and the extract was clarified by an initial centrifugation at 15,000 \times g_{ave} (GS3, Sorvall) for 15 min followed by 184,000 \times g_{ave} (Ti 70, Beckman) for 1 hr. The high-speed supernatant was bound in batch to 200 mg of glutathione agarose beads (Sigma, equilibrated in breaking buffer containing 1% [w/v] Triton X-100) for 1 hr at 4°C. The GSH beads were washed with approximately 40 ml breaking buffer containing 1% Triton X-100 to remove unbound proteins, then with thrombin cleavage buffer (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 10% glycerol, 10 mM β -mercaptoethanol) containing 0.8% (w/v) n-octyl β -D-glucopyranoside. The detergent exchange was necessary for reconstitution of the t-SNARE complex into vesicles by detergent dilution and dialysis. The t-SNARE complex was eluted from the beads by cleavage with thrombin (80 U; Sigma) for 3 hr at 25°C. The protease was inhibited by the addition of (4-[2-aminoethyl] benzenesulfonyl)fluoride, HCL (AEBSF, Calbiochem) to 4 mM, and the beads were removed by centrifugation.

All VAMP proteins were purified via a carboxy-terminal His₆ tag as previously described (Weber et al., 1998). VAMP was also obtained in n-octyl β -D-glucopyranoside for reconstitution by detergent dilution and dialysis.

SNARE Reconstitutions

All full-length SNARE proteins were purified in 0.8%–1.0% (w/v) n-octyl β -D-glucopyranoside. Proteins were reconstituted into vesicles as previously described (Weber et al., 1998) by detergent dilution and dialysis. In Figure 3, all t-SNARE reconstitutions were normalized to account for differing reconstitution efficiencies among the various linker syntaxins. The following amounts of t-SNARE complex were added to the respective 500 μ l reconstitution: 2.9 mg wild type, 1.8 mg 1 \times linker, 0.3 mg 2 \times linker, and 0.3 mg 3 \times linker. While the various linker VAMP proteins reconstituted similarly, the amount of protein added to the 100 μ l reconstitutions was adjusted to approximately 120 μ g to account for differences in protein concentration. The fluorescently labeled lipid mix used for Figure 3 contained 81 mole percent phosphatidylcholine and 15 mole percent phosphatidylserine, 2.0 mole percent NBD-PE (N-[7-nitro-2,1,3-benzoxadiazole-4-yl]-phosphatidylethanolamine), 2.0 mole percent rhodamine-PE (N-[lissamine rhodamine B sulfonyl]-phosphatidylethanolamine), and trace amounts of ³H-DPPC. Lipid recoveries for the t-SNARE vesicles ranged from 80% to 90%, while v-SNARE vesicles were 50% to 60%.

Fusion Assay and Data Analysis

Because Triton X-100 quenches NDB fluorescence (Struck et al., 1981; data not shown), we replaced Triton X-100 with n-dodecylmaltoside as a nonquenching alternative. In all experiments reported here, n-dodecylmaltoside (Boehringer Mannheim) was used to determine the maximum NDB signal.

Fusion assays were performed as described in Weber et al. (1998). A mixture of 45 μ l of unlabeled t-SNARE vesicles and 5 μ l of fluorescent v-SNARE vesicles were preincubated overnight (12–15 hr) at 4°C in a 96-well microtiter plate (Nunc). The plate was placed into a 37°C fluorescent plate reader (Lab Systems Fluoroscan II), and the NDB fluorescence was monitored at 2 min intervals (excitation, 460 nm [half band width, 25 nm]; emission, 538 nm [half band width, 25 nm]). At the end of 120 min, 10 μ l of 2.5% (w/v) n-dodecylmaltoside was added to the reaction to determine the NDB fluorescence at infinite dilution. The kinetic fusion data was converted into percent fluorescence as described (Weber et al., 1998). In all cases, the first

4 min of each curve were removed to eliminate the decrease in NBD fluorescence due to the temperature change.

Rounds of fusion were obtained by utilizing a calibration curve that determines the fold lipid dilution for a given fluorescent percentage. A detailed description of this calibration procedure will be published elsewhere (Parlati et al., 1999), but a brief description follows. Vesicles were made that contained mixtures of labeled and unlabeled lipid corresponding to 0.5-, 1-, 2-, 4-, or 8-fold dilution of donor fluorescent lipid. The NBD fluorescence of these vesicles relative to the n-dodecyl maltoside signal was determined. This data was used to generate an equation that describes the relationship between fold lipid dilution and percent n-dodecyl maltoside signal. Use of this calibration curve allows the conversion of percent fluorescence into fold dilution of donor lipid. Fold dilution of donor lipid is identical to round of fusion if one assumes that v-SNARE and t-SNARE vesicles are approximately the same size and that all vesicles have an equal probability of fusion. The equation used to convert percent dodecylmaltoside signal to fold lipid dilution (rounds of fusion) in Figure 2, which used 1.5 mole percent of each fluorescent lipid, is $Y = [0.49666 * e^{(0.036031 * X)} - [0.50597 * e^{(-0.053946 * X)}]$ where Y is the fold lipid dilution and X is the percent dodecylmaltoside signal at a given time interval. An independent calibration curve was generated for Figure 3 where 2.0 mole percent of each fluorescent lipid was used. This equation was $Y = [0.78043 * e^{(0.031519 * X)} - [0.77885 * e^{(-0.031478 * X)}]$.

Acknowledgments

We would like to thank Drs. Frank Parlati, Christine Hughes, and Walter Nickel for critically reading the manuscript and many stimulating discussions. This work was supported by grants from the National Institutes of Health to J. E. R. and postdoctoral fellowships from the Swiss National Science Foundation and European Molecular Biology Organization (to T. W.) and the National Institutes of Health (to J. A. M.).

Received June 2, 1999; revised July 2, 1999.

References

- Aalto, M.K., Ruohonen, L., Hosono, K., and Keranen, S. (1991). Cloning and sequencing of the yeast *Saccharomyces cerevisiae* SEC1 gene localized on chromosome IV. *Yeast* 7, 643–650.
- Advani, R.J., Bae, H.R., Bock, J.B., Chao, D.S., Doung, Y.C., Prekeris, R., Yoo, J.S., and Scheller, R.H. (1998). Seven novel mammalian SNARE proteins localize to distinct membrane compartments. *J. Biol. Chem.* 273, 10317–10324.
- Baumert, M., Maycox, P.R., Navone, F., De Camilli, P., and Jahn, R. (1989). Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. *EMBO J.* 8, 379–384.
- Bennett, M.K., Calakos, N., and Scheller, R.H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* 257, 255–259.
- Bennett, M.K., Garcia-Araras, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuka, C.D., and Scheller, R.H. (1993). The syntaxin family of vesicular transport receptors. *Cell* 74, 863–873.
- Block, M.R., Glick, B.S., Wilcox, C.A., Wieland, F.T., and Rothman, J.E. (1988). Purification of an n-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc. Natl. Acad. Sci. USA* 85, 7852–7856.
- Chen, Y.A., Scales, S.J., Patel, S.J., Doung, Y.C., and Scheller, R.H. (1999). SNARE complex formation is triggered by Ca^{2+} and drives membrane fusion. *Cell* 97, 165–174.
- Clary, D.O., Griff, I.C., and Rothman, J.E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* 61, 709–721.
- Cowles, C.R., Emr, S.D., and Horazdovsky, B.F. (1994). Mutations in the VPS45 gene, a SEC1 homologue, result in vacuolar protein sorting defects and accumulation of membrane vesicles. *J. Cell Sci.* 107, 3449–3459.
- Halachmi, N., and Lev, Z. (1996). The Sec1 family: a novel family of proteins involved in synaptic transmission and general secretion. *J. Neurochem.* 66, 889–897.
- Hanson, P.I., Heuser, J.E., and Jahn, R. (1997a). Neurotransmitter release—four years of SNARE complexes. *Curr. Opin. Neurobiol.* 7, 310–315.
- Hanson, P.I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J.E. (1997b). Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* 90, 523–535.
- Harbury, P.A. (1998). Springs and zippers: coiled coils in SNARE-mediated membrane fusion. *Structure* 6, 1487–1491.
- Hay, J.C., and Scheller, R.H. (1997). SNAREs and NSF in targeted membrane fusion. *Curr. Opin. Cell Biol.* 9, 505–512.
- Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T.C., and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J.* 13, 5051–5061.
- Hoekstra, D., and Duzgunes, N. (1993). Lipid mixing assays to determine fusion in liposome systems. *Methods Enzymol.* 220, 15–32.
- Hohl, T.M., Parlati, F., Wimmer, C., Rothman, J.E., Söllner, T.H., and Engelhardt, H. (1998). Arrangement of subunits in 20 S particles consisting of NSF, SNAPs, and SNARE complexes. *Mol. Cell* 2, 539–548.
- Kee, Y., Yoo, J.-S., Hazuka, C.D., Peterson, K.E., Hsu, S.-C., and Scheller, R.H. (1997). Subunit structure of the mammalian exocyst complex. *Proc. Natl. Acad. Sci. USA* 94, 14438–14443.
- Lazar, T., Gotte, M., and Gallwitz, D. (1997). Vesicular transport: how many Ypt/Rab-GTPases make a eukaryotic cell? *Trends Biochem. Sci.* 22, 468–472.
- Lin, R.C., and Scheller, R.H. (1997). Structural organization of the synaptic exocytosis core complex. *Neuron* 19, 1087–1094.
- Linial, M. (1997). SNARE proteins—why so many, why so few? *J. Neurochem.* 69, 1781–1792.
- Martinez, O., and Goud, B. (1998). Rab proteins. *Biochim. Biophys. Acta* 1404, 101–112.
- Nakajima, H., Hirata, A., Ogawa, Y., Yonehara, T., Yoda, K., and Yamasaki, M. (1991). A cytoskeleton-related gene, *uso1*, is required for intracellular protein transport in *Saccharomyces cerevisiae*. *J. Cell Biol.* 113, 245–260.
- Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T.E., and Warren, G. (1995). Characterization of a cis-Golgi matrix protein, GM130. *J. Cell Biol.* 131, 1715–1726.
- Nakamura, N., Lowe, M., Levine, T.P., Rabouille, C., and Warren, G. (1997). The vesicle docking protein p115 binds GM130, a cis-Golgi matrix protein, in a mitotically regulated manner. *Cell* 89, 445–455.
- Nichols, B.J., and Pelham, H.R. (1998). SNAREs and membrane fusion in the Golgi apparatus. *Biochim. Biophys. Acta* 1404, 9–31.
- Novick, P., and Zerial, M. (1997). The diversity of Rab proteins in vesicle transport. *Curr. Opin. Cell Biol.* 9, 496–504.
- Orci, L., Perrelet, A., and Rothman, J.E. (1998). Vesicles on strings: morphological evidence for processive transport within the Golgi stack. *Proc. Natl. Acad. Sci. USA* 95, 2279–2283.
- Parlati, F., Weber, T., McNew, J.A., Söllner, T.H., and Rothman, J.E. (1999). Rapid and efficient fusion of phospholipid vesicles by the α -helical core of a SNARE complex in the absence of an N-terminal regulatory domain. *Proc. Natl. Acad. Sci. USA*, in press.
- Pevsner, J., Hsu, S.C., and Scheller, R.H. (1994). n-Sec1: a neural-specific syntaxin-binding protein. *Proc. Natl. Acad. Sci. USA* 91, 1445–1449.
- Poirier, M.A., Xiao, W., Macosko, J.C., Chan, C., Shin, Y.K., and Bennett, M.K. (1998). The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nat. Struct. Biol.* 5, 765–769.
- Qiao, H., Pelletier, S.L., Hoffman, L., Hacker, J., Armstrong, R.T., and White, J.M. (1998). Specific single or double proline substitutions in the “spring-loaded” coiled-coil region of the influenza hemagglutinin impair or abolish membrane fusion activity. *J. Cell Biol.* 141, 1335–1347.
- Sacher, M., Jiang, Y., Barrowman, J., Scarpa, A., Burston, J., Zhang,

- L., Schieltz, D., Yates, J.R., 3rd, Abeliovich, H., and Ferro-Novick, S. (1998). TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion. *EMBO J.* *17*, 2494–2503.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
- Sapperstein, S.K., Walter, D.M., Grosvenor, A.R., Heuser, J.E., and Waters, M.G. (1995). p115 is a general vesicular transport factor related to the yeast endoplasmic reticulum to Golgi transport factor Uso1p. *Proc. Natl. Acad. Sci. USA* *92*, 522–526.
- Schimmoller, F., Simon, I., and Pfeffer, S.R. (1998). Rab GTPases, directors of vesicle docking. *J. Biol. Chem.* *273*, 22161–22164.
- Schulze, K.L., Littleton, J.T., Salzberg, A., Halachmi, N., Stern, M., Lev, Z., and Bellen, H.J. (1994). rop, a *Drosophila* homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release in vivo. *Neuron* *13*, 1099–1108.
- Shultz, G.E., and Schirmer, R.H. (1988). *Principles of Protein Structure*, C.R. Cantor, ed. (New York: Springer-Verlag).
- Skehel, J.J., and Wiley, D.C. (1998). Coiled coils in both intracellular vesicle and viral membrane fusion. *Cell* *95*, 871–874.
- Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993a). A protein assembly–disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* *75*, 409–418.
- Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993b). SNAP receptors implicated in vesicle targeting and fusion. *Nature* *362*, 318–324.
- Sonnichsen, B., Lowe, M., Levine, T., Jamsa, E., Dirac-Svejstrup, B., and Warren, G. (1998). A role for giantin in docking COPI vesicles to Golgi membranes. *J. Cell Biol.* *140*, 1013–1021.
- Steegmaier, M., Yang, B., Yoo, J.S., Huang, B., Shen, M., Yu, S., Luo, Y., and Scheller, R.H. (1998). Three novel proteins of the syntaxin/SNAP-25 family. *J. Biol. Chem.* *273*, 34171–34179.
- Struck, D.K., Hoekstra, D., and Pagano, R.E. (1981). Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* *20*, 4093–4099.
- Sudhof, T.C., Baumert, M., Perin, M.S., and Jahn, R. (1989). A synaptic vesicle membrane protein is conserved from mammals to *Drosophila*. *Neuron* *2*, 1475–1481.
- Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* *395*, 347–353.
- TerBush, D.R., Maurice, T., Roth, D., and Novick, P. (1996). The exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *EMBO J.* *15*, 6483–6494.
- Trimble, W.S., Cowan, D.M., and Scheller, R.H. (1988). VAMP-1: a synaptic vesicle-associated integral membrane protein. *Proc. Natl. Acad. Sci. USA* *85*, 4538–4542.
- Waters, M.G., Clary, D.O., and Rothman, J.E. (1992). A novel 115-kD peripheral membrane protein is required for intercisternal transport in the Golgi stack. *J. Cell Biol.* *118*, 1015–1026.
- Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T.H., and Rothman, J.E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* *92*, 759–772.
- Yang, B., Gonzalez, L., Jr., Prekeris, R., Steegmaier, M., Advani, R.J., and Scheller, R.H. (1999). SNARE interactions are not selective. Implications for membrane fusion specificity. *J. Biol. Chem.* *274*, 5649–5653.