Genetic and morphological analyses reveal a critical interaction between the C-termini of two SNARE proteins and a parallel four helical arrangement for the exocytic SNARE complex

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In a screen for suppressors of a temperature-sensitive mutation in the yeast SNAP-25 homolog, Sec9, we have identified a gain-of-function mutation in the yeast synaptobrevin homolog, Snc2. The genetic properties of this suppression point to a specific interaction between the C-termini of Sec9 and Snc2 within the SNARE complex. Biochemical analysis of interactions between the wild-type and mutant proteins confirms this prediction, demonstrating specific effects of these mutations on interactions between the SNAREs. The location of the mutations suggests that the C-terminal H2 helical domain of Sec9 is likely to be aligned in parallel with Snc2 in the SNARE complex. To test this prediction, we examined the structure of the yeast exocytic SNARE complex by deep-etch electron microscopy. Like the neuronal SNARE complex, it is a rod ~14 nm long. Using epitope tags, antibodies and maltose-binding protein markers, we find that the helical domains of Sso, Snc and both halves of Sec9 are all aligned in parallel within the SNARE complex, suggesting that the yeast exocytic SNARE complex consists of a parallel four helix bundle. Finally, we find a similar arrangement for SNAP-25 in the neuronal SNARE complex. This provides strong evidence that the exocytic SNARE complex is a highly conserved structure composed of four parallel helical domains whose C-termini must converge in order to bring about membrane fusion.

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Introduction

Biochemical and genetic studies of membrane trafficking in eukaryotic cells have led to the identification of a group of proteins called SNAREs which are required for the correct docking and fusion of vesicles with their target membranes (Rothman, 1994). Selectivity in membrane docking and fusion is thought to arise from the specific interaction of a transmembrane protein present on a vesicle (v-SNARE) with cognate proteins on a target membrane (t-SNAREs) (Rothman, 1994). In yeast, the post-Golgi v-SNARE is composed of two closely related redundant proteins called Snc1 and Snc2, which are homologs of

the neuronal v-SNARE synaptobrevin (Protopov *et al.*, 1993). The plasma membrane t-SNARE is a heterodimer of two proteins called Sec9 and Sso1/2 (also a dual gene family), which are the yeast homologs of synaptic t-SNARE SNAP-25 and syntaxin, respectively (Aalto *et al.*, 1993; Brennwald *et al.*, 1994).

Although the components of the SNARE machinery have been identified in a number of eukaryotic systems, little is known about their mechanism of action. Biophysical studies of neuronal SNARE proteins revealed that SNAP-25 and synaptobrevin are largely unstructured, while syntaxin shows a significant degree of α -helicity (Fasshauer *et al.*, 1997b). Despite low similarity at the primary sequence level, yeast SNARE monomers have biochemical and structural features similar to those of the neuronal SNAREs, suggesting an evolutionarily conserved function (Rice *et al.*, 1997; Rossi *et al.*, 1997). SNARE proteins assemble spontaneously into a tightly packed ternary complex while undergoing considerable structural changes (Fasshauer *et al.*, 1997b; Rice *et al.*, 1997). In neurons, the SNARE complex is proposed to be a four helix bundle, with synaptobrevin (Snc1/2 homolog) and syntaxin (Sso1/2 homolog) contributing one helix each and SNAP-25 (Sec9 homolog) contributing two (Fasshauer *et al.*, 1998; Poirier *et al.*, 1998; Weimbs *et al.*, 1998). The synaptic SNARE complex visualized by electron microscopy is a 12–14 nm long rod, in which syntaxin and synaptobrevin are aligned in parallel with each other (Hanson *et al.*, 1997). The arrangement of the two helices of SNAP-25 within this complex is not known, and the overall structure and arrangement of proteins within the yeast SNARE exocytic complex has not been determined.

Using biochemical and genetic analysis, we previously had characterized a recessive temperature-sensitive mutant of *SEC9*, *sec9-7*. We had shown further that the *sec9-7* mutant exhibits a strong dominant-negative phenotype when overproduced in a wild-type background. Since *in vitro* binding assays demonstrated that the Sec9-7 protein has no detectable defect in the SNARE complex assembly, we concluded that it may inhibit secretion by forming non-productive complexes that saturate the SNARE machinery *in vivo* (Rossi *et al.*, 1997). To identify factors which may play a role in SNARE-mediated fusion, we used the *sec9-7* allele in a second site suppressor screen designed to uncover novel protein–protein interactions. Here we report the identification and characterization of a gain-of-function allele of *SNC2* that specifically suppresses the *sec9-7* mutant. The positions of the interacting residues suggest a specific arrangement for the helical domains of yeast and neuronal SNARE complex which we have visualized directly by quick-freeze/deep-etch electron microscopy. Using combinations of globular protein and epitope tags placed on the ends of the SNARE helices, we demonstrate that, as predicted, all four helical

elements are arranged in parallel with each other within the yeast exocytic SNARE complex. We show that SNAP-25 has the same orientation within the neuronal SNARE complex, which is therefore also composed of four parallel α -helices. This conserved arrangement has important implications for the evolution and mechanism of SNARE-mediated membrane fusion.

Results

Isolation and cloning of a dominant extragenic suppressor of sec9-7

In previous studies, we have mapped the sites of two temperature-sensitive alleles in the *SEC9* coding sequence and examined the effects of these mutations on the assembly of both heterodimeric t-SNARE complexes with Sso1, and heterotrimeric SNARE complexes with Snc1 and Sso1. One of these mutants, *sec9-4^{ts-}*, was found to encode a Gly to Asp mutation in the N-terminal helical domain (H1) of Sec9 (Brennwald *et al.*, 1994). Analysis of the Sec9-4 mutant protein demonstrated a severe defect in forming t-SNARE interactions with Sso1, yet was still able to form heterotrimeric SNARE complexes with Snc1, although at a diminished level (Rossi *et al.*, 1997). The second allele, *sec9-7^{ts-}*, encoded a Leu to His substitution near the end of the C-terminal helical domain (H2). Despite the lethality of this mutation at high temperature, we observed no detectable defect in the ability of Sec9-7 protein to form either t-SNARE or v-t SNARE complexes *in vitro*. Consistent with the notion that the Sec9-7 mutant protein can assemble into SNARE complexes *in vivo*, we found that when overexpressed, the *sec9-7* allele demonstrated a potent dominant-negative effect on growth at high temperature. In contrast, the *sec9-4* allele, which is highly defective in SNARE assembly *in vitro*, had no dominant inhibitory effect on growth when overexpressed under identical conditions (Rossi *et al.*, 1997). Taken together, these data suggest that the Sec9-7 mutant protein is able to assemble into complexes *in vivo*, but that the complexes containing the mutant protein are defective for some function which follows the initial heterotrimeric SNARE complex formation. We presumed that this defect involves the interaction of the C-terminus of Sec9 with some other factor, and that this interaction is necessary for the function of Sec9 in SNARE-mediated membrane fusion.

To identify the factor or factors affected by the *sec9-7* mutation, we initiated a screen for extragenic suppressors of the temperature-sensitive growth defect conferred by this mutation. We were especially interested in those extragenic suppressors which acted by restoring a functional interaction with the Sec9-7 protein. Such gain-of-function mutations are predicted to behave dominantly. To increase the likelihood of recovering dominant suppressors, we made use of a diploid strain that was homozygous for the *sec9-7* allele. Since it is extremely unlikely that two recessive mutations will appear simultaneously at both loci, virtually all of the suppressors obtained from a diploid strain are likely to be dominant.

Following a selection for growth at the restrictive temperature of 37.5°C for 3 days, ~20 spontaneously arising revertant colonies were isolated. Meiotic analysis of these diploids identified five strains out of the 20 which

both sporulated and showed 2:2 segregation for growth at 37.5°C—indicative of a single locus chromosomal mutation that conferred temperature resistance. Backcrossing of these suppressors to the wild-type strain revealed that four of the five suppressors were tightly linked to the *SEC9* locus. These were due to intragenic suppressing mutations and were not analyzed further. One revertant contained a suppressor mutation unlinked to the *SEC9* locus and thus represented a dominant extragenic suppressor. Further analysis revealed that while this suppressor could completely restore wild-type growth to the *sec9-7* strain, it had no ability to suppress several other temperature-sensitive alleles of *SEC9* including *sec9-4^{ts-}*, *sec9-104^{ts-}*, *sec9-123^{ts-}* and *sec9-201^{ts-}*, demonstrating that this extragenic suppressor is highly allele specific (data not shown). This is an extremely important characteristic since dominant allele-specific suppression is a hallmark of suppressors that act by restoring a physical interaction with the gene product they are suppressing. Therefore, the genetic properties of this suppressor strongly suggest that it codes for a specific gain-of-function mutation which results in a restored interaction of the suppressor with the mutated C-terminus of Sec9.

In order to determine the identity of the dominant extragenic suppressor, a genomic library was prepared from a *sec9-7*; *SUP+* strain. This library was used to clone the suppressing gene by the ability to confer temperature resistance to the *sec9-7* mutant. Chromosomal DNA was isolated from the *sec9-7*; *SUP+* strain, partially digested and subcloned into a single copy shuttle vector YCp50. The *sec9-7*; *SUP+* genomic library was introduced into the *sec9-7* strain, and ~10 000 transformants were screened by replica plating for growth at the restrictive temperature. Plasmid DNA was recovered from several *ts+* colonies and re-tested for the ability to rescue the *sec9-7* mutant following transformation. One plasmid fulfilled this property and was found to contain a 4.5 kb genomic insert. Sequence analysis of the insert demonstrated that it was from chromosome XV and included the entire open reading frame (ORF) of a protein known to interact physically with Sec9, the post-Golgi v-SNARE Snc2.

To determine if the suppressing activity of the cloned gene was contained within the *SNC2* coding sequence, we used a technique known as gap repair. In yeast, centromere-containing plasmids with a simple gap in the genomic insert will transform only following repair of the gap with the information from the chromosomal locus. Restriction digestion of the cloned suppressor in YCp50 was used to introduce a gap in the plasmid that spanned the entire *SNC2* ORF. The gapped plasmid was then used to transform each of two yeast strains: *sec9-7*; *sup-* or *sec9-7*; *SUP+*. After recovering plasmids repaired within each of the two strains, we found that plasmids repaired from the *SUP+* strain conferred temperature resistance when transformed into *sec9-7*, while plasmids repaired in the *sup-* strains did not. Sequencing of several recovered plasmids revealed that the plasmids gap-repaired in the *SUP+* strains differed from the plasmids gap-repaired in the *sup-* strains by a single base change which falls within the *SNC2* coding sequence. This base change results in an amino acid substitution of an alanine at position 63 of Snc2 to a threonine in the dominant suppressing allele which is now

Fig. 1. Identification of gain-of-function mutations in the v-SNAREs, *SNC2* and *SNC1*, which act as allele-specific dominant suppressors of a mutant t-SNARE encoded by *sec9-7^{ts}*. (A) Allele-specific suppression of *sec9-7* by *SNC2-1*. The *SNC2-1* allele was first identified as a chromosomal mutation that behaved as an extragenic dominant suppressor of *sec9-7*. The suppressing allele was cloned by rescue of *sec9-7^{ts}* using a genomic library prepared from a *sec9-7*; *SUP*⁺ strain. The cloned gene was shown by integration mapping to be linked tightly to the chromosomal suppressing locus and by sequence analysis to contain the *SNC2* locus. Gap repair of the *SNC2* locus from *Sup*⁺ and *sup*⁻ strains demonstrated that they differ by a single point mutation. This results in a single amino acid substitution of alanine to threonine at position 63 of *SNC2*. Each of the alleles shown was subcloned into a single copy *CEN* vector and introduced into a *sec9-4* or *sec9-7* strain by transformation. Four independent transformants of each were picked into microtiter wells, transferred onto YPD plates and incubated at the indicated temperature for 2–3 days. (B) Alignment of *SNC1* and *SNC2*; the sites of the suppressing mutations in *SNC2-1* and *SNC1-1* are indicated. (C) The analogous mutation in *SNC1* also acts as a dominant allele-specific suppressor of *sec9-7*. The mutation analogous to *SNC2-1*, *SNC1-1* (A64T), was introduced into the *SNC1* coding sequence by site-directed mutagenesis and tested for suppression. These results show that a single amino acid substitution in *SNC1* and *SNC2* is sufficient to rescue completely the temperature sensitivity of a specific mutation in the C-terminus of Sec9.

termed *SNC2-1* (Figure 1B). This position falls in the C-terminal one-third of the cytosolic domain of the Snc2 protein and corresponds to a region close to the C-terminal membrane anchor, a region predicted to be both helical and essential to the function of *SNC1/2* (Gerst, 1997).

To determine whether the cloned *SNC2-1* allele had the same genetic properties as the original chromosomal suppressor, we examined the allele specificity and strength of suppression by the clone in *sec9-7* and *sec9-4* strains. Figure 1A demonstrates that while a *CEN* plasmid con-

taining the *SNC2* gene has no ability to rescue the temperature sensitivity of a *sec9-7* strain at 37.5°C, the *SNC2-1* mutant can suppress this mutant as effectively as the *SEC9* gene itself (Figure 1A, top panel). However, both *SNC2* and *SNC2-1* failed to suppress the *sec9-4* strain (Figure 1A, bottom panel), demonstrating complete allele-specific suppression of the *sec9-7* defect. Therefore, the genetic properties of the cloned *SNC2-1* suppressor are the same as those of the original chromosomal suppressor, consistent with their probable identity.

As a final verification that the cloned *SNC2-1* allele was identical to the original chromosomal extragenic suppressor, we performed linkage analysis between *SNC2* and the suppressing locus. The wild-type *SNC2* gene was subcloned into the *URA3*-integrating vector pRS306. After cleavage at a unique site in the insert, the plasmid was directed into the *SNC2* chromosomal locus of a *sec9-7; ura3-52* strain. By crossing this strain to a *sec9-7; ura3-52; SUP+* strain, we could assess the linkage of the chromosomal suppressor (seen as growth at 37°C) to the integrated *SNC2* gene (seen as growth on –Ura media). Analysis of 36 tetrads from the cross demonstrated that all of the tetrads had the same segregation pattern of two *ura+*, *ts-* and two *ura-*, *ts+* segregants (data not shown). This indicates that the extragenic suppressor locus is linked tightly to the *SNC2* locus and gives an independent verification of the identity of this chromosomal suppressor as the dominant suppressing allele *SNC2-1*.

The homologous mutant form of *SNC1* also is a dominant allele-specific suppressor of *sec9-7*

Since *SNC2* is 79% identical to and functionally redundant with a second gene, *SNC1* (Protopov *et al.*, 1993), we examined whether the suppressing mutation in *SNC2-1* was unique to this gene or if a homologous change in *SNC1* would have a similar dominant suppressing phenotype. We created the *SNC1-1* mutant by changing an alanine at position 64 to threonine (Figure 1B) by site-directed mutagenesis. Analysis of this mutant, *SNC1-1*, demonstrated that it is indistinguishable from *SNC2-1* in its ability to suppress fully the *sec9-7* but not the *sec9-4* mutation at the restrictive temperature (Figure 1C).

Both helices of *Sec9* are required for SNARE complex assembly

Our previous work had demonstrated that the *Sec9-7* protein appeared to form t-SNARE and v-t SNARE complexes normally. In order to characterize further the effects of the *sec9-7* and *SNC2-1* mutations on the interaction of these proteins, we sought to develop an assay that would be more dependent on the affected regions for binding. *In vitro* studies with the neuronal SNARE complex have demonstrated that the N-terminal H1 helix of SNAP-25 is sufficient for the binary interaction with syntaxin, while the C-terminal H2 helix, when added separately along with the H1 helix, will form stable heterotrimeric SNARE complexes with synaptobrevin (Fasshauer *et al.*, 1997a, 1998; Poirier *et al.*, 1998). To test whether the two helical domains of *Sec9* can function as separate molecules, we expressed the H1 (amino acids 402–552) and H2 (amino acids 553–651) peptides in *Escherichia coli* and added them separately or in combination to SNARE assembly reactions (Figure 2A). In contrast to SNAP-25, *Sec9* binding to the t-SNARE *Sso1* is absolutely dependent on the presence of both H1 and H2 helices (Figure 2B), consistent with our observations that mutations in the H2 domain also affect *Sso1* binding (Rossi *et al.*, 1997; A.Salminen and P.Brennwald, unpublished data). As in the case of the neuronal SNARE complex, the assembly of complexes containing *Snc2* and *Sso1* is dependent on the presence of both H1 and H2 helices of *Sec9* (Figure 2C). This suggests that *Sec9* contributes two distinct helical domains to the SNARE

Fig. 2. Binding of both the N-terminal (H1) and C-terminal (H2) helical domains of *Sec9* is required for heterodimeric interactions with *Sso1* and for heterotrimeric interactions with *Sso1/Snc2*. (A) Schematic diagram of the regions of the *Sec9* protein used in this analysis. (B) Binding of recombinant *Sec9*-H1 (3 μ M) and *Sec9*-H2 (3 μ M) to GST or GST-*Sso1* (193–265). (C) Binding of *Sec9*-H1 (3 μ M) and *Sec9*-H2 (3 μ M) to GST or GST-*Snc2* in the presence of soluble recombinant truncated *Sso1* (2 μ M). Results show that both helical elements of *Sec9* are required for the formation of t-SNARE heterodimers and the SNARE heterotrimeric complex.

complex and gave us a way in which to examine the interaction of the H2 domain, which contains the *sec9-7* mutation, independently of the binding of the rest of *Sec9* to the SNARE complex.

Biochemical suppression of *Sec9-7* defect by *Snc2-1*

To determine whether we could observe a biochemical correlate of the genetic interaction of the *sec9-7* and *SNC2-1* mutants, we made use of the assay described above to examine separately the binding of the H1 and H2 domains of *Sec9*. In this way, we could measure the relative ability of the wild-type and *Sec9-7*-H2 domains to form heterotrimeric SNARE complexes. *Sec9*-H2 (i.e. wild-type H2 domain) or *Sec9-7*-H2 recombinant peptides were expressed, metabolically labeled with [^{35} S]methionine/cysteine in *E.coli* and purified (see Materials and methods). These radiolabeled proteins were then used in binding assays with GST-*Snc2* or GST-*Snc2-1* proteins immobilized on agarose beads. Experiments were performed using a constant amount of immobilized GST–

Fig. 3. The Sec9-7-H2 domain has a lower affinity for heterotrimeric complexes and this defect is suppressed by the presence of the Snc2-1 protein. **(A)** Saturation binding assays with recombinant metabolically labeled H2 helix. Sec9-H2 or Sec9-7-H2 proteins were labeled with [^{35}S]methionine/cysteine and added in increasing concentrations (0.05–10 μM) to the SNARE assembly reaction. Other components of the mix were kept at constant concentrations (GST-Snc2 or GST-Snc2-1 at 0.5 μM , Sso1 at 1 μM). Each panel is a representative example of three separate experiments. **(B)** The amount of bound peptide was quantitated using the ImageQuant software, and the EC_{50} was calculated using the GraphPad PRISM program. The EC_{50} values shown are the average of values determined from three separate experiments each done in duplicate. The standard deviation from these three experiments is shown. The results show that the Sec9-7-H2 protein has an ~ 3 -fold lower affinity for the SNARE complex in the presence of Snc2. This defect can be restored completely by Snc2-1 protein. This suggests a physical interaction between the two affected regions of Sec9 and Snc2.

Snc2, soluble Sso1 and soluble Sec9-H1, while the concentration of the Sec9-H2 domain was varied over a range of values. The amount of H2 domain bound after several washes was quantitated following SDS-PAGE and exposure to a phosphoimaging screen (Figure 3A). These data were used to plot binding curves for each combination of Sec9-H2 domain and GST-Snc2 protein. This was used to calculate an EC_{50} value for each combination, which gives a measure of the relative affinity of the H2 domain for the heterotrimeric complex.

Using this assay, we were able to observe a clear biochemical defect arising from the *sec9-7* mutation. Comparison of the ability of the wild-type Sec9-H2 domain and the Sec9-7-H2 domain to form heterotrimeric complexes on GST-Snc2 revealed a 3-fold increase in the EC_{50} (i.e. a 3-fold decrease in affinity) for the Sec9-7-H2 domain (Figure 3B). In contrast, when the suppressing form of Snc2, Snc2-1, was used in the assembly assay,

the EC_{50} of the Sec9-7-H2 domain was restored to wild-type levels (Figure 3B). Interestingly, the Snc2-1 mutation does not significantly affect the affinity of the wild-type Sec9-H2 domain. This is consistent with our observation that the *SNC2-1* mutant is fully functional as the only source of Snc protein in the cell (L.Katz and P.Brennwald, unpublished observation).

Structure and relative orientation of SNAREs within yeast and neuronal exocytic SNARE complexes examined by electron microscopy

The genetic and biochemical data strongly suggest that the *SNC2-1* mutant suppresses the temperature sensitivity of the *sec9-7* mutation by restoring a local physical interaction between the affected regions of each protein, and thus that there is a specific interaction between the residue mutated in *sec9-7*, Leu627, and the residue mutated in *SNC2-1*, Ala63. These residues are therefore likely to be in close proximity to each other within the assembled heterotrimeric SNARE complex. The yeast SNARE complex, like the neuronal complex, appears to consist of four α -helices (Figure 2; Fasshauer *et al.*, 1998; Poirier *et al.*, 1998). Each of the four helical elements is predicted to be approximately the 14 nm (70 residue) length of the neuronal complex (Hanson *et al.*, 1997), and is therefore likely to extend once through the complex. The mutations responsible for *sec9-7* and *SNC2-1* are near the C-terminal ends of their respective helices [the second helical domain of Sec9 (H2) and the helical domain of Snc1/2], and will therefore only be able to interact physically if the H2 domain of Sec9 is aligned within the complex in parallel with the helical domain of Snc1/2. This alignment is, however, not consistent with current models which suggest that the C-terminal (H2) helix of SNAP-25 runs anti-parallel with respect to the rest of the complex (Götte and Fischer von Mollard, 1998; Weimbs *et al.*, 1998).

In order to visualize the structural arrangement of components in the yeast exocytic SNARE complex and to determine how the H2 domain of Sec9 is arranged relative to Snc1/2, we examined platinum replicas of the complex in the electron microscope, as previously reported for the neuronal SNARE complex (Hanson *et al.*, 1997). Yeast SNARE complexes containing the cytoplasmic domains of Sso and Snc together with the SNAP-25-like domain of Sec9 appeared as ~ 14 nm long rods with tails hanging off one end, and were indistinguishable from neuronal SNARE complexes of syntaxin, synaptobrevin and SNAP-25 (compare Figure 4a and b). The tail in the yeast complex corresponds to N-terminal domains of Sso1 (amino acids 1–192), since deletion of these residues did not affect complex assembly but eliminated the tail (Figure 4a, center panel). Addition of a 41 kDa maltose-binding protein (MBP) marker to the N-terminus of Sso marked the end of the tail (Figure 4a, right panel with MBP shown in yellow), further confirming its identity. Similar structural assignments have been made for the neuronal complex, where the tail corresponds to N-terminal domains of syntaxin (Figure 4b; Hanson *et al.*, 1997).

Synaptobrevin and syntaxin are aligned in parallel with each other in the neuronal SNARE complex, and this arrangement is thought to play an important role in their ability to help fuse membranes (Hanson *et al.*, 1997; Weber *et al.*, 1998). To determine if this alignment is

conserved in the yeast complex, we added MBP and myc epitope tags to the ends of Sso and Snc and immunodecorated such complexes with IgG recognizing the myc epitope. In Figure 4c, complexes with MBP on the N-terminus of Snc and a myc tag on the C-terminus of Sso show that the N-terminus of Snc is on the opposite end of the rod from the C-terminus of Sso, and thus that Sso and Snc extend in parallel with each other through the complex. An additional combination in which N-termini of both Sso and Snc were tagged and found on the same end of the complex further confirmed their parallel arrangement (data not shown).

Because the amino acid mutated in *sec9-7* is in the second (H2) helix of Sec9p, we next determined how this helix was aligned within the complex. For this purpose, we prepared complexes in which either Sso or Snc were tagged on their N-terminus with MBP and Sec9 was tagged on its C-terminus with a myc epitope. After immunodecorating these complexes with IgG recognizing the myc epitope, we found that Sec9's C-terminus is on the opposite end of the complex from the N-termini of

both Sso and Snc (Figure 4d; data not shown), and thus that the H2 helix must extend in parallel with Sso and Snc from the N- to the C-terminus of the complex. Since this result was rather surprising, we prepared comparable neuronal complexes in which the C-terminus of SNAP-25 was tagged, and found the same arrangement (Figure 4f). The alignment of the C-terminal second helix of SNAP-25 in parallel with the single helix SNAREs is thus a conserved feature of SNARE complex structure.

With the second half (H2) of Sec9 aligned in parallel with Sso and Snc, how is the rest of the molecule incorporated into the complex? In the case of SNAP-25, there are cysteine residues in the region between the H1 and H2 helices that are palmitoylated and therefore constrain this area to a position near the membrane (Gonzalo and Linder, 1998). The simplest arrangement of SNAP-25's H1 helix anti-parallel to the rest of the complex is therefore unlikely. To determine how the N-terminal H1 helix of Sec9 is arranged, we tagged Sec9 with a myc tag on the N-terminus of its SNAP-25-like domain, and oriented this relative to MBP tags on Snc and Sso. Immunodecoration of these complexes revealed that the N-termini of all three molecules are found on one end of the complex (Figure 4e). A similar arrangement was seen for SNAP-25 after its

Fig. 4. Structure and parallel organization of yeast and neuronal exocytic SNARE complexes visualized by deep-etch electron microscopy. (a) Yeast SNARE complexes containing (left) the cytoplasmic domain of Sso1, the cytoplasmic domain of Snc1 and the domain of Sec9 homologous to SNAP-25; (center) the H3 domain of Sso1 together with the same Snc1 and Sec9; (right) Sso1 with the 41 kDa MBP attached to its N-terminus in a complex with Snc1 and Sec9. All electron microscopic images are of platinum replicas shown at 300 000 $\times$ and are entirely representative. The drawings shown to the right of the EM images represent the three components (and four helices) of the SNARE complexes, with the N-terminus of Sso extending outside of the core SNARE complex. For clarity, MBP markers are colored yellow. (b) Comparable images of the neuronal SNARE complex consisting of (left) the cytoplasmic domain of syntaxin 1a, the cytoplasmic domain of synaptobrevin II and full-length SNAP-25B; (center) the H3 domain of syntaxin in a complex with the same synaptobrevin and SNAP-25; (right) syntaxin with MBP attached to its N-terminus with synaptobrevin and SNAP-25. Similar images of the neuronal SNARE complex have been reported previously (Hanson *et al.*, 1997) and are shown here for direct comparison with the yeast complex. (c) Yeast SNARE complexes with MBP on Snc1's N-terminus reacted with myc IgG recognizing a myc epitope engineered onto the C-terminus of Sso1. The three sphere deep-etch image of an IgG is colored green. MBP and antibody are found on opposite ends of the SNARE complexes, demonstrating a parallel alignment for Sso1 and Snc1 in the complex. The antibody has captured symmetric SNARE complexes at each of its two antigen-binding sites, as indicated in the adjacent interpretive drawing. (d) Yeast SNARE complexes with MBP on Sso1's N-terminus and a myc epitope tag on Sec9's N-terminus reacted with myc IgG. Green and yellow markers are found on the same end of the SNARE complex rod, demonstrating that the N-termini of Sso and Sec9 are found on one end of the complex. (e) Yeast SNARE complexes with MBP on Sso1's N-terminus and a myc tag now on Sec9's C-terminus. Green and yellow markers are found on opposite ends of the complex, indicating that the C-termini of Sec9 and Sso1 must be on the same end of the complex and adjacent to the membrane. Given the separate binding of Sec9's H1 and H2 domains in the SNARE complex as well as their total length, these results imply that Sec9 contributes two parallel α -helices to the complex. (f) Neuronal SNARE complexes with MBP on syntaxin's N-terminus and a myc tag on SNAP-25's C-terminus. MBP and myc IgG markers appear on opposite ends of the complex, showing that also in this case the C-termini of syntaxin and SNAP-25 will be found on one end of the complex, and, therefore, that SNAP-25 must contribute to parallel α -helices to the complex.

Fig. 5. Model of SNARE complex re-arrangement during vesicular docking and fusion. Upon vesicle docking, the vesicle-bound v-SNARE Snc/syntaxin (in blue, transport vesicle above) interacts with the plasma membrane-bound t-SNAREs Sec9/SNAP-25 (pink) and Sso/syntaxin (yellow, plasma membrane below). As these proteins come together, they form a bridge between the two membranes (gray). The SNARE complex is a four helical bundle (which is flattened here to show each helical domain) of $\sim 14 \times 4$ nm, with Snc and Sso contributing one helical element each and Sec9 contributing two. Sso/syntaxin has an extended N-terminal tail that is not packed into a complex. The helical regions are depicted as cylinders. Two helices of Sec9/SNAP-25, H1 and H2, are connected by a long loop containing the plasma membrane attachment site, which for SNAP-25 is a cluster of palmitoylated cysteines immediately following the H1 helix. Sec9 lacks any cysteine residues, and the mechanism by which it associates with the plasma membrane is not known. The four helical elements are extended in parallel within the SNARE rod with their C-termini towards the membrane. *sec9-7* and *SNC2-1* mutations are indicated as asterisks. The position of the mutations is marked to scale with respect to their position in each helical domain. (A) Initial SNARE assembly/docking. During the early step of the SNARE complex assembly, v-SNARE Snc/syntaxin selectively recognizes and pairs with t-SNAREs Sec9/SNAP-25 and Sso/syntaxin. At that stage, the C-terminus of Snc would be at some distance from the Sec9/Sso heterodimer. The bend in Snc is exaggerated to highlight the convergence of the C-termini that must occur as the membranes come together. It is this packing together of the C-termini which appears to be defective in *sec9-7* and corrected by the *SNC2-1* mutation. (B) The SNARE complex just before fusion of the vesicle membrane with the plasma membrane. As two membranes are brought close together following complex assembly, the SNARE subunits would be predicted to 'zip up', packing the C-termini of the four helices in a much restricted space.

N-terminus was tagged either with a myc epitope or again with MBP (not shown). The simplest model that accommodates the positioning of the N- and C-termini of Sec9 and SNAP-25 at opposite ends of the SNARE complex is a parallel four helix bundle in which the first and second (H1 and H2) helices are connected by an inter-helical linker (Figure 5). Both the interhelical domain

(with the cysteine residues palmitoylated in SNAP-25) and the C-terminus of these two helix SNAREs will be found adjacent to the membrane. This arrangement, together with the high α -helical content of the complexes (Fasshauer *et al.*, 1997b; Rice *et al.*, 1997), predicts that the structural unit of both the neuronal and yeast exocytic SNARE complexes is a bundle of four parallel α -helices.

Discussion

SNAREs represent a highly conserved family of proteins that play a central role in both determining the specificity and catalyzing the fusion of intracellular membrane pairs. The original SNARE hypothesis suggested that specific vesicle docking and fusion events would follow proper pairing of v-SNAREs on the vesicle membrane with their cognate t-SNAREs on the target membrane (Rothman, 1994). Recent studies with SNARE-containing proteoliposomes suggest that the interaction of a v-SNARE with its cognate t-SNARE may itself be sufficient to drive membrane fusion (Weber *et al.*, 1998). Understanding the structural make-up of SNARE interactions both *in vitro* and *in vivo* is a key to understanding the molecular mechanism by which these proteins mediate specific membrane fusion events.

Previous studies have shown that the core of the neuronal SNARE complex is a 14×4 nm rod containing one helical element from the t-SNARE syntaxin and a second parallel helical element from the v-SNARE synaptobrevin co-assembled with SNAP-25 (Hanson *et al.*, 1997). The positioning of SNAP-25, however, has not been determined. Here we describe a specific interaction between the C-terminal ends of the yeast homologs of SNAP-25 and synaptobrevin, Sec9 and Snc1/2. This interaction appears to be critically important for assembly of functional SNARE complexes, although it is not necessary for their initial assembly.

The structure of the yeast and neuronal exocytic SNARE complexes

The data presented here demonstrate a high degree of conservation between SNARE complexes mediating exocytic fusion at the plasma membrane in yeast and neuronal cells. Previous work had hinted at this similarity (Brennwald *et al.*, 1994; Rice *et al.*, 1997; Rossi *et al.*, 1997), but we now provide direct visual evidence of the conservation of their overall topology. Not only are the dimensions of the two SNARE complexes superimposable as ~14 nm long rods, but the arrangements of the helical elements within them are highly conserved. In particular, both complexes are composed of two membrane-spanning SNAREs (syntaxin/Sso and synaptobrevin/Snc), which each contribute one helical element, together with a third SNARE, Sec9/SNAP-25, which lacks a transmembrane anchor but contributes two helices.

Recently, Brünger and co-workers have solved the crystal structure of the core of the synaptic SNARE complex, showing that it is indeed a parallel four helix bundle (Sutton *et al.*, 1998). This structure is consistent with our electron microscopy analysis of the full cytoplasmic neuronal and yeast SNARE complexes. Furthermore, the physical interaction between the C-termini of SNAP-25 and synaptobrevin predicted by the genetic analysis described here is well supported by the neuronal SNARE crystal structure in which the C-termini of SNAP-25 and synaptobrevin are in close physical contact with each other. With this in mind, the evidence for the overall three-dimensional arrangement of the SNAREs now comes from at least four distinct and complementary approaches: resolution by X-ray and NMR, analysis of *in vivo* genetic interactions and direct visualization by electron micro-

scopy. There can be little doubt that the final arrangement of exocytic, and perhaps all, SNARE complexes is a parallel four helix bundle whose structure, but not the primary sequence of its components, has been remarkably well conserved in organisms as divergent as yeast and mammals.

While the overall topology of the yeast and neuronal SNARE complexes are similar, the eventual delineation of a high resolution structure for the yeast SNARE complex will be critical to understanding the details of this conservation as well as the precise details of the *sec9-7-SNC2-1* interaction described here. The primary sequences of the C-termini of SNAP-25 and Sec9 are quite divergent and can be aligned in different ways, making it difficult to determine with certainty which residue in SNAP-25 is analogous to the Leu626 residue mutated in *sec9-7*. For example, in alignments designed to optimize primary sequence similarity in this region, the Leu626 residue falls after the C-terminal end of mouse SNAP-25 (Brennwald *et al.*, 1994). A more recent alignment based on repeating motifs present in all SNARE proteins aligns yeast Sec9-Leu626 with rat SNAP-25-Ile192 (Wiembs *et al.*, 1998). In this case, the analogous residues in the neuronal crystal structure (Ala67 in synaptobrevin II and Ile192 in SNAP-25) would be separated by one heptad repeat (D.Fasshauer, R.B.Sutton, A.T.Brünger and R.Jahn, submitted). This could suggest either a difference in the packing of the helices in the yeast complex relative to the neuronal complex or that the suppression of the *sec9-7* mutant by *SNC2-1* involves a more distant set of interactions in this region. Therefore, while we have found that the suppression results from restoration of a physical interaction between the mutated regions of each protein, the precise side chain interactions involved in that suppression will have to await the crystallography of the wild-type and mutant forms of the yeast SNARE complex.

Implications for the evolution of SNARE-mediated membrane fusion

The SNARE complex, which is composed of four parallel helical elements contributed by three distinct proteins, is a novel structure. Since all four helices have their C-termini toward the membrane, this suggests that the ancestral SNARE complex once may have been composed of four transmembrane proteins. The idea that Sec9 and SNAP-25 may have a transmembrane domain-containing ancestor is supported by the recent identification of membrane-anchored SNAREs more homologous to SNAP-25 than to syntaxin (e.g. syntaxin 6; Bock *et al.*, 1996). The prototypical SNARE may thus have contained a single helical domain and a C-terminal transmembrane anchor, which has evolved to meet special demands of particular fusion reactions. Indeed, SNAREs with the two helix structure of SNAP-25 and Sec9 have not been found on internal membranes such as the endoplasmic reticulum and Golgi apparatus, and it seems possible that the single helix SNAREs involved in these steps may be able to form effective four helix complexes from four rather than three molecular components.

Structural rearrangement of the SNARE complex: from docking to fusion

In addition to delineating the overall structure of the yeast SNARE complex, our data suggest that assembly of a

SNARE oligomer alone is not sufficient to fuse membranes. Our previous analysis of the Sec9-7 mutant protein demonstrated that its functional defect did not prevent assembly of the SNARE complex (Rossi *et al.*, 1997). The identification and genetic characterization of the *SNC2-1* allele, which fully suppresses the temperature sensitivity of *sec9-7*, demonstrates that the defect in *sec9-7* is in fact due to an impaired interaction within the SNARE complex itself. The C-terminus of Sec9-7 is unable to interact with the C-terminus of Snc1/2, in spite of initial assembly of a ternary SNARE complex. In many ways, the biochemical phenotype of Sec9-7 protein resembles the effect of the botulinum toxin A (BoNTA) on neuronal SNARE complexes. BoNTA removes the C-terminal nine amino acids of SNAP-25 and thereby impairs exocytosis without affecting assembly or subsequent disassembly of heterotrimeric SNARE complexes (Otto *et al.*, 1995). Since initial assembly of the SNARE complex occurs with the v-SNARE on one membrane and the heterodimeric t-SNARE on the other, it is likely that the C-termini of Sec9/SNAP-25 and Snc1/synaptobrevin would be at some distance from each other. In order for the vesicle and target membranes to fuse, their C-termini will have to pull tightly together all the way up to the membrane (see Figure 6). We suggest that it is in fact one step in the compaction of the C-termini which is defective in the Sec9-7 protein, a finding which underscores the dynamic rearrangement which the SNARE complex must undergo in order to catalyze the membrane fusion event.

Materials and methods

Plasmid constructions

All constructions were generated by PCR amplification with restriction sites incorporated in the PCR primers. The pGEX-4T1 expression vector (Pharmacia Biotech Inc.) was used in all cases. *EcoRI* and *SalI* sites were used to subclone Snc1 (1–116), Snc2 (1–115), Snc1-1 (1–116), Snc2-1 (1–115) and Sso1 (1–265). Sec9 (402–651), Sec9-H1 (402–552), Sec9-H2 (553–651) and Sec9-7-H2 (553–651) were subcloned using *BamHI* and *SalI* sites.

Myc epitope tags were added after amino acids 402, 533, 536 and 652 of Sec9. Design of the synthetic Sec9 is described elsewhere. For Sso1, tags were added at the N- and C-termini (positions 1 and 265) of the protein. MBP-Snc1 and MBP-Sso1 were generated by subcloning Sso1 (1–265) and Snc1 (1–93) into the pMAL-c2 vector (New England Biolabs).

Preparation of the genomic library

Genomic DNA was isolated from the *sec9-7; SUP+* strain as described by Philippsen *et al.* (1991) and Rose and Broach (1991). DNA (100 µg) was partially digested with 0.25 U/µg of *Sau3A* enzyme (New England Biolabs) and layered on top of a 38 ml sucrose density gradient. The gradient was centrifuged at 113 000 g for 20 h at 15°C. Fragments of 4–10 kb in size were pooled and extracted with 2-butanol. The shuttle vector YCp50 (35 µg) was digested with 240 U of *BamHI* enzyme (New England Biolabs), and treated with calf intestinal phosphatase (New England Biolabs). The ligation mix was transformed into DH10B competent cells (Life Technologies). A total of 10 000 clones were pooled, amplified in *E.coli* and recovered by the alkaline lysis plasmid purification method.

Suppression assays, gap-repair and linkage analysis

SNC1, *SNC1-1*, *SNC2* and *SNC2-1* sequences were subcloned into a *CEN-URA* vector pRS316. All plasmids were introduced into the *sec9-7* or *sec9-4* strain by lithium acetate transformation. Clones were selected for growth in the absence of uracil, replica plated on complete media (YPD) and grown for 2–3 days at the permissive or restrictive temperatures. For gap repair, the ORF of the *SNC2* gene was deleted from a clone recovered from the genomic library using *BssHII* and *MluI*

enzymes. The resulting plasmid was introduced into *sec9-7; sup-* or *sec9-7; SUP+* strains, and clones were selected for growth on plates lacking uracil. Recovered plasmids were sequenced with primers flanking the *SNC2* sequence. For linkage analysis, *SNC2* was subcloned into a *URA3*-integrating vector (pRS306). The plasmid was cleaved with *MluI*, a unique sites in the *SNC2* gene, prior to transformation of BY70 (*a, sec9-7; sup-; ura3-52*) to target the integration into the *SNC2* chromosomal locus. The resulting strain was crossed to α , *sec9-7; SUP+*; *ura3-52*. Meiotic progeny of the cross were scored for growth at permissive (25°C) and restrictive (37.5°C) temperatures, and in the presence and absence of uracil.

Site-directed mutagenesis

Single-stranded *SNC1* template was used for mutagenesis by the Kunkel method (Kunkel *et al.*, 1987). The oligonucleotide used for alanine to threonine substitution had the following sequence: CCATTGAAGATAA-AACTGATAACCTAGCGGTC.

Purification of recombinant proteins

GST fusion proteins encoding yeast SNAREs were purified as described by Rossi *et al.* (1997). Neuronal SNARE proteins with His₆ tags were purified as described by Hanson *et al.* (1997). MBP-Sso1, MBP-Snc1 and MBP-syntaxin1a proteins were purified using amylose resin (NEB) and eluted with 10 mM maltose as recommended by the manufacturer. Protein concentrations were estimated by BCA assay (Pierce) using bovine serum albumin (BSA) as a standard and by comparison with purified proteins of known concentration following SDS-PAGE and Coomassie staining.

Binding assays and complex preparation for electron microscopy

Immobilized GST fusion proteins at 1 µM were mixed with soluble proteins at 2 µM, unless indicated otherwise, in a binding buffer [10 mM HEPES-KOH pH 7.6, 140 mM KCl, 2 mM MgCl₂, 0.5% Triton X-100, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride and 1 mM phenyl-methylsulfonyl fluoride] and allowed to assemble overnight at 4°C on a nutator. Binding reactions were done in a 100 µl total volume with 20 µl of a 1:1 suspension of beads. The beads were washed three times in binding buffer and boiled in 90 µl of 2× sample buffer. To prepare complexes for electron microscopy, beads were washed three times in binding buffer and once in cleavage buffer (20 mM HEPES-KOH pH 7.6, 150 mM NaCl). Thrombin was added (0.5 U per 10 µl of bead volume) and cleaved for 3 h at room temperature. The supernatant was separated from the beads. The NaCl concentration in the supernatant was raised to 250 mM and thrombin was removed by incubation with benzamidine-Sepharose 6B (Pharmacia Biotech Inc.) with 10 µl of beads per 100 µl binding reaction. The protein concentration in the supernatant was measured by the Bradford assay using BSA as standard, and complexes were visualized by Coomassie staining. All complexes contained stoichiometric amounts of proteins. For SNARE complex immunodecoration, purified myc IgG (9E10; Evan *et al.*, 1985) was added to purified SNARE complex and incubated for 30–60 min. Samples were diluted to a final concentration of 15 µg/ml SNARE complex and 3 µg/ml IgG in 30 mM HEPES (pH 7.3), 70 mM KCl, 5 mM MgCl₂ and 3 mM EGTA for adsorption to mica flakes as below.

Metabolic labeling and affinity measurements

Cultures (80 ml) were grown to an optical density of 0.7 and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 30°C. At the time of induction, 2 mCi of [³⁵S]methionine/cysteine labeling mix was added to the cultures. Proteins were purified as described by Rossi *et al.* (1997). Complexes were assembled as described above except that soluble Sec9-H1 and Sso1 proteins were added at 1 µM, Sec9-H2 or Sec9-7-H2 were added at 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10 µM. Bound proteins were resolved by SDS-PAGE, and gels were dried and exposed to a phosphorimager screen. Band volume was quantified using the ImageQuant program, and the EC₅₀s were estimated using the GraphPad PRISM program. All experiments were carried out in duplicate.

Quick-freeze/deep-etch electron microscopy (EM)

SNARE complexes and SNARE complex-antibody mixtures described above were prepared for electron microscopic imaging by adsorption to mica chips, quick freezing, etching and platinum replication as previously described (Heuser, 1983, 1989; Hanson *et al.*, 1997). Representative molecules were selected from EM negatives photographed at 68 000×, enlarged to 300 000× and processed using Adobe Photoshop. Molecules shown were selected because they show that each antibody molecule

(with its two antigen-binding sites) recruits two tagged complexes that exhibit an identical arrangement. Antibodies that had recruited only one complex were also seen. No molecules exhibiting the opposite orientation were identified.

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References

- Aalto,M.K., Ronne,H. and Kernen,S. (1993) Yeast syntaxins, Sso1p and Sso2p, belong to a family of related membrane proteins that function in vesicular transport. *EMBO J.*, **12**, 4095–4104.
- Bock,J.B., Lin,R.C. and Scheller,R.H. (1996) A new syntaxin family member implicated in targeting of intracellular transport vesicles. *J. Biol. Chem.*, **271**, 17961–17965.
- Brennwald,P., Kearns,B., Champion,K., Kernen,S., Bankaitis,V. and Novick,P. (1994) Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. *Cell*, **79**, 245–258.
- Evan,G.I., Lewis,G.K., Ramsay,G. and Bishop,J.M. (1985) Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell. Biol.*, **5**, 3610–3616.
- Fasshauer,D., Bruns,D., Shen,B., Jahn,R. and Brtnger,A.T. (1997a) A structural change occurs upon binding of syntaxin to SNAP-25. *J. Biol. Chem.*, **272**, 4582–4590.
- Fasshauer,D., Otto,H., Eliason,W.K., Jahn,R. and Brtnger,A.T. (1997b) Structural changes are associated with soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. *J. Biol. Chem.*, **272**, 28036–28041.
- Fasshauer,D., Eliason,W.K., Brtnger,A.T. and Jahn,R. (1998) Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. *Biochemistry*, **37**, 10354–10362.
- Gerst,J.E. (1997) Conserved α -helical segments on yeast homologs of the synaptobrevin/VAMP family of v-SNAREs mediate exocytic function. *J. Biol. Chem.*, **272**, 16591–16598.
- Gonzalo,S. and Linder,M.E. (1998) SNAP-25 palmitoylation and plasma membrane targeting require a functional secretory pathway. *Mol. Biol. Cell*, **9**, 585–597.
- Gtte,M. and Fischer von Mollard,G. (1998) A new beat for the SNARE drum. *Trends Cell Biol.*, **8**, 215–218.
- Hanson,P.I., Roth,R., Morisaki,H., Jahn,R. and Heuser,J.E. (1997) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell*, **90**, 523–535.
- Heuser,J.E. (1983) Procedure for freeze-drying molecules adsorbed to mica flakes. *J. Mol. Biol.*, **169**, 155–195.
- Heuser,J.E. (1989) Protocol for 3-D visualization of molecules on mica via the quick-freeze, deep-etch technique. *J. Electron Microsc. Tech.*, **13**, 244–263.
- Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) Rapid and efficient mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.
- Otto,H., Hanson,P.I., Chapman,E.R. and Jahn,R. (1995) Poisoning by botulinum neurotoxin A does not inhibit formation or disassembly of the synaptosomal fusion complex. *Biochem. Biophys. Res. Commun.*, **212**, 945–952.
- Philippsen,P., Stoltz,A. and Scherf,C. (1991) DNA of *Saccharomyces cerevisiae*. *Methods Enzymol.*, **194**, 169–182.
- Poirier,M.A., Hao,J.C., Malkus,P.N., Chan,C., Moore,M.F., King,D.S. and Bennett,M.K. (1998) Protease resistance of syntaxin. SNAP-25-VAMP complexes. Implications for assembly and structure. *J. Biol. Chem.*, **273**, 11370–11377.
- Protopopov,V., Govindan,B. and Gerst,J.E. (1993) Homologs of the synaptobrevin/VAMP family of synaptic vesicle protein function on the late secretory pathway in *S.cerevisiae*. *Cell*, **74**, 855–861.
- Rice,L.M., Brennwald,P. and Brtnger,A. (1997) Formation of a yeast SNARE complex is accompanied by significant structural changes. *FEBS Lett.*, **415**, 49–55.
- Rose,M.D. and Broach,J.R. (1991) Cloning genes by complementation in yeast. *Methods Enzymol.*, **194**, 195–230.
- Rossi,G., Salminen,A., Rice,L.M., Brtnger,A.T. and Brennwald,P. (1997) Analysis of a yeast SNARE complex reveals remarkable similarity to the neuronal SNARE complex and a novel function for the C terminus of the SNAP-25 homolog, Sec9. *J. Biol. Chem.*, **272**, 16610–16617.
- Rothman,J.E. (1994) Mechanisms of intracellular protein transport. *Nature*, **372**, 55–63.
- Sutton,R.B., Fasshauer,D., Jahn,R. and Brtnger,A.T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 resolution. *Nature*, **395**, 347–353.
- Weber,T., Zemelman,B.V., McNew,J.A., Westermann,B., Gmachl,M., Parlati,F., Sllner,T. and Rothman,J.E. (1998) SNAREpins: minimal machinery for membrane fusion. *Cell*, **92**, 759–772.
- Weimbs,T., Mostov,K. and Low,S.H. (1998) A model for structural similarity between different SNARE complexes based on sequence relationships. *Trends Cell Biol.*, **8**, 260–262.

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