Structures of yeast vesicle trafficking proteins

TOMER TISHGARTEN,¹ FANG FANG YIN,¹ KEITH M. FAUCHER,² RICHARD A. DLUHY,²
TRACY R. GRANT,¹ GABRIELE FISCHER VON MOLLARD,^{3,4} TOM H. STEVENS,³
AND LEIGH ANN LIPSCOMB^{1,2}

¹Department of Biochemistry & Molecular Biology, University of Georgia, Athens, Georgia 30602

²Department of Chemistry, University of Georgia, Athens, Georgia 30602

³Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

⁴Georg-August-Universität Göttingen, Biochemie II, Gosslerstr. 12d, 37073 Göttingen, Germany

(RECEIVED May 3, 1999; ACCEPTED August 6, 1999)

Abstract

In protein transport between organelles, interactions of v- and t-SNARE proteins are required for fusion of protein-containing vesicles with appropriate target compartments. Mammalian SNARE proteins have been observed to interact with NSF and SNAP, and yeast SNAREs with yeast homologues of NSF and SNAP proteins. This observation led to the hypothesis that, despite low sequence homology, SNARE proteins are structurally similar among eukaryotes. SNARE proteins can be classified into two groups depending on whether they interact with SNARE binding partners via conserved glutamine (Q-SNAREs) or arginine (R-SNAREs). Much of the published structural data available is for SNAREs involved in exocytosis (either in yeast or synaptic vesicles). This paper describes circular dichroism, Fourier transform infrared spectroscopy, and dynamic light scattering data for a set of yeast v- and t-SNARE proteins, Vti1p and Pep12p, that are Q-SNAREs involved in intracellular trafficking. Our results suggest that the secondary structure of Vti1p is highly α -helical and that Vti1p forms multimers under a variety of solution conditions. In these respects, Vti1p appears to be distinct from R-SNARE proteins characterized previously. The α -helicity of Vti1p is similar to that of Q-SNARE proteins characterized previously. Pep12p, a Q-SNARE, is highly α -helical. It is distinct from other Q-SNAREs in that it forms dimers under many of the solution conditions tested in our experiments. The results presented in this paper are among the first to suggest heterogeneity in the functioning of SNARE complexes.

Keywords: α -helix; circular dichroism; coiled-coil; FTIR; light scattering; Pep12p; Q-SNAREs; R-SNAREs; SNAREs; Vti1p

In eukaryotic cells, each organelle requires a specific protein composition to perform its tasks. To maintain the correct protein composition within a given organelle, vesicles must transport proteins between organelles with high fidelity. Proteins to be transported accumulate in vesicles forming on the donor organelle surface. The vesicles, with their accompanying protein cargo, are released from the donor organelle in a process called budding (Rothman & Orci, 1996). After budding, interactions of v- and t-SNARE (SNAP

receptor) proteins on the surface of the vesicle and acceptor organelle play a role in targeting the vesicle to the appropriate organelle (Rothman, 1996; Rothman & Wieland, 1996). Upon fusion, vesicle cargo is delivered to the target organelle. Recent work has suggested that neuronal v- and t-SNARE proteins alone may be responsible for vesicle fusion (Weber et al., 1998). SNARE proteins have a large cytosolic domain and in most cases contain a C-terminal transmembrane domain or are membrane-anchored by isoprenylation. They contain sequences capable of forming α -helical coiled-coils near the C-terminus (Chapman et al., 1994; Gerst, 1997). These putative coiled-coils are involved in interaction between various SNARE proteins.

Various SNARE (SNAP receptor) proteins critical to intracellular membrane fusion are believed to operate by similar mechanisms in eukaryotic organelles (Rothman & Wieland, 1996; Rossi et al., 1997; Gotte & Fischer von Mollard, 1998). Operation by a similar mechanism would imply structural similarity, regardless of a lack of sequence conservation among evolutionarily distant members of the SNARE family. This structural similarity has been observed between mammalian SNARE proteins and some yeast

Reprint requests to: Leigh Ann Lipscomb, Department of Biochemistry & Molecular Biology, University of Georgia, Athens, Georgia 30602; e-mail: lipscomb@linus.bmb.uga.edu.

Abbreviations: CD, circular dichroism; DLS, dynamic light scattering; FTIR, Fourier transform infrared; Pep12, peptidase deficient gene; Sed5, suppressor of the *erd2* deletion mutant; Snc1, suppressor of the null allele of the cyclase-associated protein; SNAP, soluble N-ethylmaleimide sensitive fusion attachment protein; SNAP-25, synaptosomal-associated protein of 25 kDa; Sso1, suppressor of Sec1; v, vesicle; t, target; Vti1p, vps10 interacting; Vam3, vacuolar morphogenesis; SNARE, soluble N-ethylmaleimide sensitive fusion attachment protein receptor; NSF, N-ethylmaleimide sensitive fusion protein; HPLC, high-performance liquid chromatography.

homologues. Structural similarity between yeast v-SNARE Vti1p and its human homologue is suggested by the recent result that the human homologue can functionally replace Vti1p in two vesicle transport pathways (Fischer von Mollard & Stevens, 1998).

To date, structural/biophysical data have been described for SNAREs and SNARE complexes, including the following: (1) synaptobrevin II with syntaxin I and SNAP-25B (for exocytosis in synaptic vesicles) (Fasshauer et al., 1997a; Sutton et al., 1998), (2) Snc1p with Sso1p and Sec9p (for exocytosis in yeast) (Rice et al., 1997), (3) Bet1p and Bos1p (Stone et al., 1997), (4) SNAP-25 (Fasshauer et al., 1997b), and (5) Sec9p (Rice et al., 1997). These analyses have revealed striking differences. V-SNARE proteins synaptobrevin II and Snc1p are both monomeric with little secondary structure in the absence of appropriate t-SNARE binding partners (Fasshauer et al., 1997b). Extensive α -helical structure is induced when these proteins bind t-SNAREs. It has been suggested that this conformational change is linked to the close approach of vesicle and target membrane that leads to vesicle fusion (Hanson et al., 1995; Weber et al., 1998). Similarly, SNAP-25 (Fasshauer et al., 1997b) and Sec9p (Rice et al., 1997) appear to be unfolded, with α -helicity induced upon complex formation. Among t-SNAREs, syntaxin 1 (neuronal) (Fasshauer et al., 1997b) and Sso1p (yeast) (Rice et al., 1997) are both monomeric with a high percentage of α -helix. However, v-SNAREs Bet1p and Bos1p have α -helical character in the absence of t-SNARE binding partners (Stone et al., 1997).

It is possible to divide v-SNAREs into subgroups that may be structurally different. The X-ray structure of the synaptobrevin II-syntaxin 1-SNAP25B complex (Sutton et al., 1998) reveals a parallel four-helix bundle as well as ionic interactions between

arginine 56 of synaptobrevin II, and three glutamines of syntaxin 1, and the SNAP-25B fragments. In t-SNAREs, these glutamines are generally conserved, thus leading to their classification as Q-SNAREs (Fasshauer et al., 1998). Most v-SNAREs have a conserved arginine residue at the position analogous to Arg56 of synaptobrevin II. This group of v-SNAREs, classified as R-SNAREs (Fasshauer et al., 1998), includes synaptobrevin II and Snc1p. In a second group of v-SNAREs, proteins have a conserved glutamine at this position, and like t-SNAREs, they are classified as Q-SNAREs. Vti1p, Bos1p, and Bet1p are Q-SNAREs. The yeast exocytic complex probably also consists of a parallel four-helix bundle (Rice et al., 1997; Rossi et al., 1997). However, much of the limited structural data available is for exocytic SNARE complexes and not for SNARE complexes of intracellular trafficking steps. For v-SNAREs, much of the structural data available is for R-SNAREs not Q-SNAREs. It is presently unclear whether exocytic SNARE complexes and SNARE complexes of intracellular trafficking steps are structurally similar or different. Structural differences between Q- and R-SNAREs are poorly understood.

We describe here the biophysical characterization of yeast v- and t-SNARE proteins Vti1p and Pep12p. Vti1p-Pep12p interactions are required for fusion of late (trans)-Golgi-derived vesicles with the prevacuolar compartment. Vti1p, a Q-SNARE, is a 24 kDa (Fig. 1), integral membrane v-SNARE protein that has been localized to the late-Golgi and prevacuolar compartments (Fischer von Mollard et al., 1997). Pep12p, a Q-SNARE, is a 33 kDa, integral membrane t-SNARE protein on the prevacuolar compartment (Becherer et al., 1996). In addition to binding Pep12p, Vti1p also interacts with Sed5p (Hardwick & Pelham, 1992; Fischer von Mollard et al., 1997) in traffic to the early-Golgi. This paper gives

Fig. 1. The amino acid sequences of (A) Vti1p and (B) Pep12p. Secondary structure predictions were performed with the fold recognition based on prediction-based threading algorithm at the European Molecular Biology website (<http://www.embl-heidelberg.de/predictprotein/predictprotein.html#PP1TOPITS>). Regions predicted to be α -helical (reliability index of prediction was 7 or greater) are underlined. Subcloning into the pET28b expression vector resulted in a 20-residue, N-terminal tag that is shown in lower case letters. Every tenth residue number is given in the line below. The conserved glutamine, 138 for Vti1p and 229 for Pep12p, that is thought to participate in ionic interactions with t-SNAREs is marked with an arrow. Alignments with other SNAREs have been published previously (Weimbs et al., 1998).

results from circular dichroism (CD) and FTIR spectroscopy that suggest both Vti1p and Pep12p have a high percentage of α -helix. In this respect, Vti1p secondary structure appears to be different from that of R-SNAREs previously characterized, yet similar to that of some Q-SNAREs previously characterized. Results from dynamic light scattering demonstrate that both proteins form multimers under appropriate solution conditions. Vti1p and Pep12p are unique among SNARE proteins whose oligomerization states have been characterized. The results presented in this paper are the first to suggest significant structural or oligomerization differences among eukaryotic v-SNAREs or among t-SNAREs. They suggest that vesicle-localized Q-SNAREs are α -helical alone while vesicle-localized R-SNAREs are α -helical in complexes with t-SNARE binding partners.

Results

Vti1p and Pep12p were expressed in bacteria and purified to greater than 90% homogeneity. Purity was assessed by HPLC, mass spectrometry, and Coomassie and silver-stained SDS-PAGE. Recombinant Vti1p and Pep12p contained the complete soluble domain but no transmembrane region (see Fig. 1).

Vti1p and Pep12p are highly α -helical

CD spectra of Vti1p and Pep12p suggest that both proteins are highly α -helical (see Fig. 2). In FTIR spectra, frequencies of peptide backbone vibrational modes are consistent with high α -helix content for Vti1p and Pep12p. Two peptide backbone vibrational modes, the Amide I and II, are useful in secondary structure assignments for Vti1p and Pep12p. The Amide I band represents the normal mode involving mainly the (C=O) stretching vibration of the peptide backbone (see Jackson & Mantsch, 1995; Siebert, 1995 for excellent reviews). The Amide II band is a less pure vibrational mode involving mainly in-plane bending of the N-H bond. The C-N, C-C stretching, and C=O in-plane bending are involved to lesser extents in Amide II. Frequencies of both Amide I and II are consistent with α -helix for Vti1p and Pep12p (see Figs. 3, 4). In H₂O, the vibrational lines at 1,651 cm⁻¹ (Vti1p) and 1,649 cm⁻¹

Fig. 2. CD spectra of Vti1p expressed with (●) and without (▲) chaperones, and Pep12p (■). Protein concentrations were 5 μ M in 10 mM KH₂PO₄ (pH 8) at 25°C.

Fig. 3. FTIR spectra of Vti1p (~1.5 mg/mL) in (A) H₂O and (B) D₂O.

(Pep12p) appear to be the Amide I. The insensitivity of their frequencies to deuterated water is consistent with their assignment as the Amide I. Vibrational lines at 1,547 cm⁻¹ (Vti1p) and 1,551 cm⁻¹ (Pep12p) appear to be the Amide II. They display the approximate 100 cm⁻¹ shift expected for Amide II in D₂O.

These results are consistent with those from secondary structure prediction programs that suggest extended α -helical regions in both Vti1p and Pep12p (Fig. 1). They are also consistent with the recently reported X-ray structure of the neuronal synaptobrevin II·syntaxin 1·SNAP-25B complex (Sutton et al., 1998) that shows extended α -helical conformations of all proteins in the complex.

The α -helicity of Vti1p in the absence of t-SNARE binding partners is a striking result, and it is possible that co-expression of chaperones GroEL/ES (Yasukawa et al., 1995) alters the structural properties of the protein. To test this effect, we expressed Vti1p in the presence and absence of high amounts of chaperones. In both cases, the α -helical content of purified Vti1p was measured with CD. In both cases, Vti1p has a high percentage of α -helix (see Fig. 2). The α -helicity calculated for Vti1p co-expressed without chaperones is about 53%. The α -helicity calculated for Vti1p expressed with chaperones varied between 34 and 76% among preparations. For Vti1p expressed in the presence of large amounts of chaperones, five preparations gave α -helicity values of 34, 54, 65, 70, and 76%.

Vti1p was typically concentrated to ~40 mg/mL prior to storage at -80°C. It seemed reasonable to ask whether concentration of the protein-induced stable intermolecular associations leading to

Fig. 4. FTIR spectra of Pep12p (~1.5 mg/mL) in (A) H₂O and (B) D₂O.

oligomerization. To address this issue, we purified and stored Vti1p under dilute conditions. In this experiment, the concentration of Vti1p was less than ~1 mg/mL, typically ~0.5 mg/mL, at all steps of the purification protocol. Vti1p from 1 L of cell culture was purified by metal affinity chromatography as described under Materials and methods. Fractions containing Vti1p were dialyzed versus the buffer desired for CD (10 mM KH₂PO₄ pH 8). CD and dynamic light scattering data were obtained as described under Materials and methods. The DLS data gave an average molecular weight of 135 kD for Vti1p purified under dilute conditions (as compared to the 127 kD result obtained for Vti1p purified under concentrated conditions). CD showed Vti1p purified under dilute conditions to have significant α -helix content. The α -helix content (~34%) of Vti1p in this experiment is lower than that obtained for Vti1p purified and stored under more concentrated conditions (54–76%), but it is still significantly higher than that published previously for v-SNAREs of exocytosis. In summary, the oligomerization of Vti1p does not seem to be a result of the concentrations at which the protein was purified and stored. Vti1p has high α -helicity in all preparations of the protein performed in our laboratory, using a range of concentrations for purification and storage.

Denaturation

We have monitored CD signals for Vti1p as it is denatured with heat. CD signals as a function of temperature (Fig. 5A) indicate a

Fig. 5. CD spectra of Vti1p as a function of temperature. The Vti1p concentration was 5 μ M in 10 mM KH₂PO₄ (pH 8). **A:** Heating of Vti1p from 25 to 80 °C. **B:** Cooling of Vti1p from 80 to 25 °C.

substantial decrease in α -helicity when the temperature is raised from 40 to 60 °C. The spectrum obtained at 40 °C is different from that obtained at 25 °C. Slight shifts in the minima are observed (compare the 25 °C CD spectrum with minima at about 206 and 218 nm with the 40 °C spectrum that has minima at about 208 and 222 nm). These shifts probably reflect structural perturbations as a result of heating Vti1p to 40 °C. The spectra suggest that a small amount (~3%) of Vti1p's α -helicity is lost when the protein is heated from 25 to 40 °C.

After denaturation of Vti1p with 80 °C heat, we monitored CD signals as the sample was cooled. Comparison of the 25 °C CD spectra prior to (Fig. 5A) and after (Fig. 5B) heating indicates a substantial decrease in intensity of the 222 nm peak. This spectral change suggests partial irreversibility in unfolding of Vti1p. The 25 °C spectrum in Figure 5B suggests that Vti1p (unheated) has about 13% more α -helix than Vti1p after the heating and cooling renaturation step. The isodichroic point in Figure 5B (~204 nm) indicates that renaturation of Vti1p is a two-state process. Two conformations are likely involved in renaturation.

CD spectra suggest that α -helicity of Vti1p is maintained in 0.5 M guanidinium hydrochloride, but that it disappears entirely in 2.0 M guanidinium hydrochloride (data not shown). This result suggests unfolding of Vti1p at concentrations between 0.5–2.0 M guanidinium hydrochloride. Attempts to renature Vti1p by removal of guanidinium hydrochloride resulted in extensive aggregation of the protein.

We have also monitored CD signals for Pep12p as the protein was denatured with 80 °C heat and then cooled to 25 °C (data not shown). Spectra obtained for Pep12p at 25 and 40 °C were virtually identical, and that obtained at 60 °C suggested that the protein was unfolded at this temperature. CD spectra obtained when cooling Pep12p indicate that the original conformation of Pep12p could not be regained. When the protein was cooled from 80 to 25 °C, the spectra at 80, 60, 40, and 25 °C were similar and suggestive of unfolded protein. Apparently heat denaturation of Pep12p is irreversible under these conditions.

Oligomerization of Vti1p and Pep12p

Results from dynamic light scattering (DLS) suggest that Vti1p forms multimers under a variety of solution conditions (Table 1). We have tested the effects of glycerol, pH, and salt on the oligomerization of both Vti1p and Pep12p. For Vti1p at pH 8 in 200 mM NaCl, the results suggest that oligomerization is more extensive with 15% glycerol (average molecular weight ~327 kDa) than in the absence of glycerol (average molecular weight ~169 kDa). They also suggest that, with 5% glycerol and 200 mM NaCl, the formation of multimers is decreased at pH 5.6 as compared to pH 8 (average molecular weight 211 vs. 386 kDa). A striking result is that the presence of salt promotes formation of multimers (see Table 1) in the presence of 5% glycerol at pH 8. Optimization of the solution conditions in terms of solubility and decreased oligomerization gave an average molecular weight for Vti1p of 117 kDa in 5% glycerol (no salt, no buffer, standard deviation = 20 kDa). Based on the expected molecular weight of about 24 kDa for monomeric Vti1p, Vti1p appears to form tetramers or pentamers (or a mixture of both) in 5% glycerol. The average molecular weight of Vti1p was 127 kDa (standard deviation = 15 kDa) under the conditions used to obtain the CD spectra (10 mM KH₂PO₄, pH 8). Vti1p eluted in the void volume on a gel filtration column (molecular weight cutoff 75 kDa data not shown). These results are consistent with the formation of multimers.

Pep12p appears to form dimers under many of the solution conditions tested (Table 2) and, as compared to Vti1p, seems to be less prone to formation of multimers. The molecular weight of Pep12p was monitored for solution conditions of varying pH, glycerol, and salt concentrations, and it is not statistically different under the majority of the conditions tested. Based on the expected molecular weight of about 33 kDa for monomeric Pep12p, Pep12p appears to form dimers or a mixture of monomers and dimers under the first five conditions listed in Table 2. For the seventh condition (175 mM CaCl₂, 10% glycerol, 25 mM HEPES pH 8.0), the average molecular weight of 78 kDa is intermediate between that expected for a dimer (about 64 kDa), and that expected for a trimer (about 96 kDa). Pep12p may form a mixture of dimers and trimers under these conditions. Only one condition (5% glycerol, in the absence of salt or buffer) yielded extensive oligomerization of Pep12p (average molecular weight 257 kDa). The average molecular weight of Pep12p was 77 kDa under the conditions used for obtaining the CD spectra (10 mM KH₂PO₄, pH 8). The mobility of Pep12p on native gels and gel filtration columns is consistent with formation of a dimer or trimer (data not shown).

Interaction of Vti1p and Pep12p

We investigated interactions of Vti1p and Pep12p with DLS. No solution condition could be identified in which both proteins were soluble without extensive oligomerization. DLS revealed that incubation of the two proteins generally led to formation of aggregates of high molecular weight (on the order of 10⁴ kDa) (data not shown). Interaction of the two proteins was not observed by gel filtration chromatography or by native gel electrophoresis.

Discussion

We have investigated structural properties of two SNARE proteins from *Saccharomyces cerevisiae* using CD, FTIR spectroscopy, and dynamic light scattering. The results indicate that both Vti1p and Pep12p have high α -helical content. Previous work in this field has led to the hypothesis (Rice et al., 1997; Sutton et al., 1998) that v-SNAREs are generally unfolded unless in a complex with appropriate t-SNAREs. The results presented here indicate that this hypothesis is not universally applicable. They suggest that not all SNARE complexes are similar in form and function. Below we

Table 1. Dynamic light scattering results for Vti1p

Variable	Buffer	Average radius (nm)	Average molecular weight (kDa)	MW standard deviation (kDa)
Glycerol	0% glycerol, 200 mM NaCl, 25 mM HEPES pH 8	5.35	169	08.2
	10% glycerol, 200 mM NaCl, 25 mM HEPES pH 8	6.00	224	26
	15% glycerol, 200 mM NaCl, 25 mM HEPES pH 8	7.01	327	22
pH	25 mM Na citrate pH 5.6, 200 mM NaCl, 5% glycerol	5.51	211	38
	25 mM Tris pH 7, 200 mM NaCl, 5% glycerol	6.48	269	20
	25 mM Tris pH 8, 200 mM NaCl, 5% glycerol	7.49	386	75
NaCl	0 mM NaCl, 5% glycerol, 25 mM Tris pH 8	4.89	137	22
	200 mM NaCl, 5% glycerol, 25 mM Tris pH 8	6.50	273	40

Table 2. Dynamic light scattering results for Pep12p

Variable	Buffer	Average radius (nm)	Average molecular weight (kDa)	MW standard deviation (kDa)
Glycerol	0% glycerol, 175 mM KCl, 25 mM HEPES pH 8	3.37	55.4	7.2
	5% glycerol, 175 mM KCl, 25 mM HEPES pH 8	3.55	62.8	7.0
	10% glycerol, 175 mM KCl, 25 mM HEPES pH 8	3.55	62.6	8.3
pH	25 mM Na cacodylate pH 6.5, 175 mM KCl, 10% glycerol	3.10	45.1	12
	25 mM HEPES pH 8, 175 mM KCl, 10% glycerol	3.55	62.6	8.3
NaCl	0 mM KCl, 10% glycerol, 25 mM HEPES pH 8	3.78	75.1	27
	175 mM KCl, 10% glycerol, 25 mM HEPES pH 8	3.63	66.8	18
	175 mM CaCl ₂ , 10% glycerol, 25 mM HEPES pH 8	3.88	77.5	2.1

compare the structural properties of Vti1p and Pep12p with those of other SNARE proteins. The comparison suggests that the α -helical content of uncomplexed Vti1p, a vesicle-localized Q-SNARE, is higher than that of vesicle-localized R-SNAREs studied previously by CD (Rice et al., 1997; Sutton et al., 1998). Vti1p and Pep12p appear to be similar to other eukaryotic Q-SNAREs in terms of α -helical content. However, Pep12p seems to be unique in that it forms dimers/trimers in solution. Structural similarities among Q- or R-SNAREs are expected to be greater than those among v- or t-SNAREs.

Vti1p

The results presented in this paper suggest that α -helicity is a structural feature common among some Q-, but not R-, SNAREs. The group of v-SNAREs classified as R-SNAREs (Fasshauer et al., 1998) includes synaptobrevin II and Snc1p. Vti1p, Bos1p, and Bet1p are among the group of v-SNAREs classified as Q-SNAREs. Interactions between v- and t-SNAREs are expected to be different depending on whether the v-SNARE is an R- or Q-SNARE. Much of the v-SNARE structural data (X-ray, CD) (Rice et al., 1997; Stone et al., 1997; Sutton et al., 1998) available in the literature is for R-SNAREs. Our data on Vti1p provide much needed structural information for a v-SNARE belonging to the Q-SNARE subfamily.

The α -helical content of uncomplexed Vti1p appears to be higher than that of R-SNAREs previously characterized, including synaptobrevin II and Snc1p. Vti1p, unlike neuronal R-SNARE synaptobrevin II, is α -helical in the absence of appropriate t-SNARE binding partners. CD results indicate that synaptobrevin II is unfolded unless interacting with syntaxin 1 and SNAP-25B. Synaptobrevin II appears monomeric by dynamic light scattering analysis, while Vti1p has a tendency to form multimers. Differences in structural properties of Vti1p and synaptobrevin II may reflect differences in protein transport mechanisms for exocytosis and intracellular trafficking steps. The α -helicity of Vti1p is also higher than that of yeast v-SNARE Snc1p. Like synaptobrevin II, Snc1p is an R-SNARE and appears to be unfolded unless interacting with appropriate t-SNAREs (Rice et al., 1997). Snc1p is largely α -helical in a ternary complex with Sso1p and Sec9p. Light scattering results suggest that Snc1p is monomeric. The Snc proteins are believed to be

involved in transport of vesicles to the plasma membrane in *S. cerevisiae* (Protopopov et al., 1993). Differences in structural properties of Vti1p and Snc1p suggest differences in protein transport mechanisms for exocytosis and intracellular trafficking steps in yeast.

The data presented here and elsewhere suggest that some Q-SNAREs are α -helical alone while R-SNAREs appear to form α -helices in a complex with t-SNAREs. CD data previously described (Stone et al., 1997) for Q-SNAREs Bet1p and Bos1p has revealed α -helicity that is similar to that of Vti1p.

It seems most likely that structural differences among v-SNAREs are due primarily to differences in amino acid composition (i.e., Q- or R-SNAREs). However, as compared to uncomplexed synaptobrevin II and Snc1p, it is possible that the larger size of Vti1p contributes to its higher percentage of α -helix in the absence of t-SNAREs. V-SNAREs are integral membrane proteins, and all X-ray (Sutton et al., 1998) and CD experiments reported thus far (this paper, (Rice et al., 1997; Sutton et al., 1998)) have been conducted on the cytosolic domains of these proteins. The cytosolic domains of synaptobrevin II and Snc1p have 95 residues, while that of Vti1p has 195 residues. The larger size of Vti1p's cytosolic region may contribute to its ability to fold as an independent domain. In the X-ray structure of the synaptobrevin II-syntaxin 1-SNAP25B complex (Sutton et al., 1998), about 55 residues of each SNARE protein interact to form a four-helix bundle. According to sequence alignments, amino acid residues 130–187 of Vti1p are predicted to interact with t-SNAREs (Fischer von Mollard et al., 1997). A larger portion (~58%) of synaptobrevin II (and probably Snc1p) is involved in interaction with t-SNAREs as compared to Vti1p (~29%). However, the observation that Q-SNARE Bet1p, with 144 residues, has α -helical domains (Stone et al., 1997), suggests that size may not be an important issue and that amino acid composition (Q- or R-SNAREs) may have greater effects on structure.

The experiments here do not reveal which portions of Vti1p are structured in the unbound state. However, the results from structure-based threading programs indicate a high propensity for α -helix formation in particular regions of Vti1p (see Fig. 1). In particular, Vti1p's t-SNARE binding site (residues 130–187) has a high propensity for α -helix formation. These results suggest that the t-SNARE binding site of Vti1p has more secondary structure than that of synaptobrevin II.

It is unclear what structural features of Vti1p contribute to oligomerization. In this paper we have presented data to show that increasing glycerol or sodium chloride concentration promotes formation of Vti1p homo-oligomers. It seems that increasing glycerol or salt concentration would result in fewer water molecules surrounding the protein. Thus, oligomerization may be favored under dehydrating conditions. For salt, electrostatic effects could also be involved. Vti1p is the first vesicle-localized Q-SNARE that has been studied by light scattering. It is tempting to speculate that oligomerization is a structural feature common among vesicle-localized Q-SNAREs. It may be that intracellular trafficking relies on homo-oligomerization of vesicle-localized Q-SNAREs to organize SNARE complexes whereas fusion at the synapse may utilize other organizers (possibly synaptotagmin or munc-13).

Pep12p

Pep12p is a Q-SNARE and has the α -helicity expected for this class of proteins. Its α -helical content ($\sim 50\%$) is also similar to that of other eukaryotic t-SNARE proteins. CD spectra of Q-SNARE syntaxin 1 indicate that this neuronal protein is α -helical. We have estimated about 40% α -helix in syntaxin 1 based on data published by Fasshauer et al. (1997b). CD spectra of yeast Sso1p (also a Q-SNARE) indicate that this t-SNARE is highly α -helical as well (estimated 53%) (Rice et al., 1997; Rossi et al., 1997).

However, an important difference between Pep12p, syntaxin 1, and Sso1p is the oligomerization state. Light scattering results indicate that syntaxin 1 and Sso1p are monomeric, while the results presented here suggest that Pep12p is dimeric/trimeric under several of the conditions tested. This result may suggest differences in protein transport mechanisms involving these three t-SNAREs.

Oligomerization of Vti1p and Pep12p suggests a biological role for Vti1p·Vti1p and Pep12p·Pep12p interactions. The role of oligomerization in vivo, if any, is unclear. On native gels of yeast lysates, it is difficult to distinguish homo-oligomers from SNARE complexes. It may be that the stoichiometry of the Vti1p·Pep12p complex is not 1:1 (multiple copies of either or both proteins are present in the complex). Alternatively Vti1p·Vti1p or Pep12p·Pep12p interaction could link different SNARE complexes. It has been suggested that formation of a fusion pore requires a ring of SNARE complexes connecting the vesicle with the target membrane (Hanson et al., 1997; Weber et al., 1998).

Interaction of Vti1p and Pep12p

Our rough estimate of the equilibrium binding constant ($K_{eq} = [Vti1p \cdot Pep12p] / [Vti1p][Pep12p]$) for the following reaction

suggests that it is below about 10^2 M^{-1} . Vti1p and Pep12p were incubated and the mixture was analyzed by native gel electrophoresis and gel filtration chromatography. The Vti1p·Pep12p complex was not observed by either technique. The binding constant calculation is based on the assumption that about $1.0 \mu\text{g}$ of the Vti1p·Pep12p complex should be observable by gel electrophoresis and Coomassie Blue stain. Using the starting concentrations of Vti1p and Pep12p that were loaded on the gel, we have estimated a binding constant for the Vti1p·Pep12p complex below 10^2 M^{-1} . Our estimate assumes the simplest possible model of

1:1 stoichiometry for the Vti1p·Pep12p complex. If the interaction between Vti1p and Pep12p is 1:1, the absence of monomeric Vti1p and Pep12p in solution may have prevented formation of the 1:1 complex.

It may be that Vti1p and Pep12p bind strongly in the presence of additional proteins, perhaps additional components of the SNARE complex that have not yet been identified. The X-ray structure of the synaptobrevin II·syntaxin 1·SNAP-25B complex revealed four helix bundles with a central interaction between one arginine and three glutamines (Sutton et al., 1998). An arginine-containing SNARE would be absent from the Vti1p·Pep12p complex; perhaps arginine is contributed by a third protein. However, a SNAP-25B·syntaxin 1 complex has been observed (Fasshauer et al., 1997a, 1997b). In this complex containing only t-SNAREs, the four helix bundles are believed to interact via glutamines only. The complex containing only t-SNAREs is of a stability decreased relative to the synaptobrevin II·syntaxin 1·SNAP-25B complex. The interactions that stabilize SNARE complexes of intracellular trafficking steps may be different from those stabilizing exocytic SNARE complexes.

Previously we have described co-immunoprecipitation of Vti1p and Pep12p from yeast extracts (Fischer von Mollard et al., 1997). In that study the Vti1p·Pep12p complex probably includes other proteins. It is well established that binary SNARE complexes have affinities much lower than ternary ones (Sacher et al., 1997; Stone et al., 1997). Observation of protein–protein complexes on native gels or gel filtration columns requires high affinity interactions.

Materials and methods

Cloning and expression

We have prepared separate bacterial expression systems each for Vti1p and Pep12p. Recombinant Vti1p and Pep12p were constructed to contain only the cytosolic domain. Both proteins lack a single, C-terminal membrane-spanning α -helix that is present in the full-length proteins.

Recombinant Vti1p and Pep12p were expressed in *Escherichia coli* using the pET-28b expression vector (Novagen, Inc., Madison, Wisconsin). The proteins have a N-terminal hexa-histidine tag, with a thrombin cleavage site between the histidine tag and the first amino acid residue of the protein. The polymerase chain reaction (PCR) was used to subclone the cDNA. Forward and reverse primers were designed to correspond to the 5' and 3' sequence of the cDNA. For Vti1p, the forward primer contained the NdeI restriction site to the 5' side of the DNA coding for the N-terminal histidine. The reverse primer contained a stop codon and a XhoI restriction site. The Pep12p gene was subcloned analogously. The resulting plasmids were transformed first into a cloning host and then into BL21(DE3).

High levels of expression were obtained for both Vti1p and Pep12p with this system. For Vti1p, about half of expressed protein was in a soluble form. The remaining Vti1p was expressed as insoluble aggregates (inclusion bodies). In an effort to maximize yields of soluble protein, we co-expressed chaperones (Yasukawa et al., 1995). Vti1p co-expressed with GroES/EL had purification and solubility properties different from those of the Vti1p obtained with our original expression system. Vti1p co-expressed with chaperones was eluted from the metal affinity column with about 200 mM imidazole and could be concentrated to $\sim 40 \text{ mg/mL}$. Vti1p from the original expression system was eluted with

500 mM imidazole from the column and could typically be concentrated to 10–15 mg/mL. Pep12p was also co-expressed with chaperones. For protein expression, cells were grown at 37 °C under kanamycin and chloramphenicol selection. The temperature of the media was lowered to 30 °C just prior to induction with 1 mM IPTG for 2 h.

Purification

All chemicals were of the highest purity commercially available. Cells obtained from 1–6 L of culture were suspended in 80 mL of ice-cold buffer containing 50 mM HEPES pH 8.0, 0.5 M KCl, 5 mM imidazole, 10% glycerol, and 0.1% NP-40 (buffer A). Magnesium chloride (80 μ L of a 1 M solution), DNase (3–5 mg), and PMSF (800 μ L of a 100 mM solution) were added to the cell suspension. The cells were lysed by french press and spun at 18K rpm for 45 min at 4 °C. The supernatant was 0.45 μ m filtered and loaded onto a nickel agarose matrix (resin from Qiagen, Chatsworth, California) equilibrated with buffer A. The nickel column was washed with at least 10 column volumes of buffer A. Vti1p was eluted with a gradient linear in imidazole concentration between 5–500 mM. Pep12p was eluted with a gradient linear in imidazole concentration between 5–125 mM. Fractions containing protein were identified with SDS-PAGE. EDTA (pH 8.0) was added to these fractions to a final concentration of about 5 mM. The samples were concentrated to a volume of about 5 mL and dialyzed twice vs. 4–8 L of the desired buffer solution. For Vti1p, we used 25 mM HEPES pH 8.0, 200 mM NaCl, and 15% glycerol, while for Pep12p we used 25 mM HEPES pH 8.0, and 10% glycerol. Proteins were concentrated to near-saturation prior to long-term storage at –80 °C. Vti1p was typically concentrated to about 40 mg/mL, and Pep12p was typically concentrated to about 25 mg/mL.

Circular dichroism

Protein concentrations were determined by the bicinchoninic assay (Smith et al., 1985) prior to acquisition of CD spectra. Ultraviolet CD data were collected using a Jasco J-710 Spectropolarimeter and a 1 mm cylindrical quartz cell. The spectropolarimeter scanned five sets of spectra for each sample at 20 nm/min with a step resolution 0.2 nm/data between 190–240 nm. The percent α -helicity was calculated as

$$\% \alpha\text{-helicity} = [(\Theta_{222 \text{ nm}} * 100)/40,000(1 - 2.5/n)]$$

where $\Theta_{222 \text{ nm}}$ represents the molar ellipticity at 222 nm, and n represents the number of amino acid residues (Scholtz et al., 1991). For the heating/cooling experiments, samples were allowed to equilibrate for 15 min at the desired temperature prior to collection of CD spectra.

FTIR data acquisition

Infrared spectra were acquired using a Bio-Rad FTS-40 FTIR spectrometer equipped with a wide band MCT-B detector and Bio-Rad Win-IR (version 4.0) software. All spectra were acquired at 1,024 scans, 4 cm^{-1} resolution, triangular apodization, and one degree of zero filling. A CIRCLE cell ATR accessory with a 1/8" germanium micro rod (Spectra Tech, Shelton, Connecticut) was used to obtain spectra of protein sample solutions. Spectral base-

line correction and spectral subtraction were performed using the Bio-Rad Win-IR software.

Prior to use, the germanium micro rod was cleaned by sonication in a chloroform:methanol:water solution (6:4:1) for 15 min. The micro rod was additionally sonicated two more times for 15 min in distilled water. After the clean micro rod was allowed to dry, it was placed in the CIRCLE cell so that a background spectrum could be obtained. The buffer solution was then added into the CIRCLE cell, and an absorbance spectrum was acquired. Next the protein sample solution, at a concentration of 1–2 mg/mL, was added into the CIRCLE cell, and another absorbance spectrum was collected. Finally, the absorbance spectra of the buffer and protein sample solutions were subtracted from one another so that a difference spectrum from 1,800–1,000 cm^{-1} could be obtained. It was essential that the temperatures of the buffer and protein sample be as close as possible for proper subtraction.

Dynamic light scattering (DLS)

DLS data were acquired with a Protein Solutions DynaPro Microsizing instrument with a temperature controller (model MSTC). The temperature was maintained at 20 °C throughout the course of the experiments. The concentration of Vti1p and/or Pep12p was 2 mg/mL. The protein samples were passed through 0.2 and 0.1 μ m filters prior to collection of DLS data. At least three protein samples were prepared for each data point shown in Tables 1 and 2. The DLS signals were typically measured 10–15 times for each sample. DLS spectra were corrected for scattering of the solvent, and the average molecular weight was estimated from hydrodynamic radius. Standard deviation was calculated as follows:

$$\sigma = [\Sigma(M_{\text{avg}} - M_i)^2/N - 1]^{1/2}$$

where σ represents standard deviation, M_{avg} represents the average molecular weight, M_i represents the molecular weight of a single measurement, and N represents the number of measurements.

Interaction of Vti1p and Pep12p

Vti1p and Pep12p were incubated at 4 °C in equimolar ratios in a buffer containing 25 mM HEPES pH 8.0, 12.5% glycerol, and 100 mM NaCl. Incubation periods were ranged from about 30 min to 3 days and did not affect the results of the experiments.

Acknowledgments

The authors would like to acknowledge Drs. Leon Dure and John Brewer for discussion and critical reading of the manuscript.

References

- Becherer KA, Rieder SE, Emr SD, Jones EW. 1996. Novel syntaxin homologue, Pep12p, required for the sorting of luminal hydrolases to the lysosome-like vacuole in yeast. *Mol Biol Cell* 7:579–594
- Chapman ER, An S, Barton N, Jahn R. 1994. SNAP-25, a t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled coils. *J Biol Chem* 269:27427–27432.
- Fasshauer D, Bruns D, Shen B, Jahn R, Brünger A. 1997a. A structural change occurs upon binding of syntaxin to SNAP-25. *J Biol Chem* 272:4582–4590.
- Fasshauer D, Otto H, Eliason WK, Jahn R, Brünger AT. 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, Sutton R, Brnger A, Jahn R. 1998. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci USA* 95:15781–15786.
- Fischer von Mollard G, Nothwehr SF, Stevens TH. 1997. The yeast v-SNARE Vti1p mediates two vesicle transport pathways through interactions with the t-SNAREs Sed5p and Pep12p. *J Cell Biol* 137:1511–1524.
- Fischer von Mollard G, Stevens TH. 1998. A human homolog can functionally replace the yeast vesicle-associated SNARE Vti1p in two vesicle transport pathways. *J Biol Chem* 273:2624–2630.
- Gerst J. 1997. Conserved alpha-helical segments on yeast homologs of the synaptobrevin/VAMP family of v-SNAREs mediate exocytic function. *J Biol Chem* 272:16591–16598.
- Gotte M, Fischer von Mollard G. 1998. A new beat for the SNARE drum. *Trends Cell Biol* 8:215–218.
- Hanson P, Heuser J, Jahn R. 1997. Neurotransmitter release—four years of SNARE complexes. *Curr Opin Neurobiol* 7:310–315.
- Hanson P, Otto H, Barton N, Jahn R. 1995. The N-ethylmaleimide-sensitive fusion protein and alpha-SNAP induce a conformational change in syntaxin. *J Biol Chem* 270:16955–16961.
- Hardwick KG, Pelham HRB. 1992. SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J Cell Biol* 119:513–521.
- Jackson M, Mantsch HH. 1995. The use and misuse of FT-IR spectroscopy in the determination of protein structure. *Crit Rev Biochem Mol Biol* 30:95–120.
- Protopopov V, Govindan B, Novick P, Gerst J. 1993. Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. *Cell* 74:855–861.
- Rice LM, Brennwald P, Brnger AT. 1997. Formation of a yeast SNARE complex is accompanied by significant structural changes. *FEBS Lett* 415:49–55.
- Rossi G, Salminen A, Rice L, Brnger A, 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, Sec 9. *J Biol Chem* 272:16610–16617.
- Rothman JE. 1996. The protein machinery of vesicle budding and fusion. *Protein Sci* 5:185–194.
- Rothman JE, Orci L. 1996. Budding vesicles in living cells. *Sci Am* 37:70–75.
- Rothman JE, Wieland FT. 1996. Protein sorting by transport vesicles. *Science* 272:227–233.
- Sacher M, Stone S, Ferro-Novick S. 1997. The synaptobrevin-related domains of Bos1p and Sec22p bind to the syntaxin-like region of Sed5p. *J Biol Chem* 272:17134–17138.
- Scholtz J, Qian H, York E, Stewart J, Baldwin R. 1991. Parameters of helix-coil transition theory for alanine-based peptides of varying chain lengths in water. *Biopolymers* 31:1463–1470.
- Siebert F. 1995. Infrared spectroscopy applied to biochemical and biological problems. *Methods Enzymol* 246:501–526.
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76–85.
- Stone S, Sacher M, Mao Y, Carr C, Lyons P, Quinn AM, Ferro-Novick S. 1997. Bet1p Activates the v-SNARE Bos1p. *Mol Biol Cell* 8:1175–1181.
- Sutton RB, Fasshauer D, Jahn R, Brnger AT. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 resolution. *Nature* 395:347–353.
- Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE. 1998. SNAREpins: Minimal machinery for fusion. *Cell* 92:759–772.
- Weimbs T, Mostov K, Low SH, Hofmann K. 1998. A model for structural similarity between different SNARE complexes based on sequence relationships. *TICB* 8:260–262.
- Yasukawa T, Kanei-Ishii C, Maekawa T, Fujimoto J, Yamamoto T, Ishii S. 1995. Increase of solubility of foreign proteins in *Escherichia coli* by coproduction of the bacterial thioredoxin. *J Biol Chem* 270:25328–25331.