Vam7p, a vacuolar SNAP-25 homolog, is required for SNARE complex integrity and vacuole docking and fusion

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The vacuole v-t-SNARE complex is disassembled by Sec17p/ α -SNAP and Sec18p/NSF prior to vacuole docking and fusion. We now report a functional characterization of the vacuolar SNARE Vam7p, a SNAP-25 homolog. Although Vam7p has no hydrophobic domains, it is tightly associated with the vacuolar membrane. Vam7p is a constituent of the vacuole SNARE complex and is released from this complex by the Sec17p/Sec18p/ATP-mediated priming of the vacuoles. Even in the absence of the vacuolar v-SNARE Nyv1p, a subcomplex which includes Vam7p and the t-SNARE Vam3p is preserved. Vam7p is necessary for the stability of the vacuolar SNARE complex, since vacuoles from mutants deleted in *VAM7* do not have a Vam3p–Nyv1p complex. Furthermore, Vam7p alone, in the absence of Nyv1p and Vam3p, cannot mediate fusion with wild-type vacuoles, whereas vacuoles with only Nyv1p or Vam3p alone can fuse with wild-type vacuoles in the absence of the other two SNAREs. Thus, Vam7p is important for the stable assembly and efficient function of the vacuolar SNARE complex and maintenance of the vacuolar morphology. This functional characterization of Vam7p suggests a general role for SNAP-25 homologs, not only on the plasma membrane but along the secretory pathway.

Keywords: SNARE complex/SNAP-25/vacuole fusion/Vam7p

Introduction

SNAREs (soluble NSF attachment protein receptors) are membrane proteins with large cytosolic domains that provide an important layer of specificity to vesicle fusion within a eukaryotic cell (Ferro-Novick and Jahn, 1994; Rothman, 1994; Pfeffer, 1996; Hay and Scheller, 1997). Vesicle-associated SNAREs (v-SNAREs) are thought to interact specifically with their counterparts on the target membrane (t-SNAREs) upon docking (Rothman, 1994). Prior to docking, SNAREs are mainly in complexes with each other on the target and, possibly, on the vesicle membrane (Tagaya *et al.*, 1995, 1996; Walch-Solimena *et al.*, 1995; Holthuis *et al.*, 1998; Ungermann *et al.*, 1998). Some t-SNAREs have been recovered in a complex with Sec1-like proteins which may stabilize them in an active conformation (Hata *et al.*, 1993; Sogaard *et al.*, 1994). Thus, SNAREs appear to be largely complexed prior to their action (Holthuis *et al.*, 1998; Ungermann

et al., 1998). A 20S complex has been described in synaptic extracts that consists of the membrane-associated proteins VAMP/synaptobrevin (a v-SNARE), syntaxin and SNAP-25 (both members of the t-SNARE family), and two soluble proteins, the *N*-ethylmaleimide-sensitive factor (NSF) and the soluble NSF attachment protein (α -SNAP) (Söllner *et al.*, 1993a). This 20S SNARE complex is disassembled by NSF through ATP hydrolysis (Söllner *et al.*, 1993a,b) and may prepare the SNAREs for docking and fusion (Ungermann *et al.*, 1998).

In addition to SNAREs, the Ypt proteins, Rab-like GTPases which are regulated through dissociation factors and GTP hydrolysis activators, may contribute to the specificity of membrane fusion (Novick and Zerial, 1997). For example, Ypt1 protein may activate the Golgi t-SNARE Sed5p through direct interaction (Lupashin and Waters, 1997). However, the precise function of Ypt proteins in trafficking is still unknown.

We are studying homotypic vacuole fusion, the last step of yeast vacuole inheritance. This fusion reaction can be assayed *in vitro* (Conradt *et al.*, 1993; Haas *et al.*, 1994; Haas, 1995) and depends on proteins that are similar to those identified in other trafficking reactions, including a v-SNARE (Nyv1p), a t-SNARE (Vam3p; Nichols *et al.*, 1997), a Rab protein (Ypt7p; Haas *et al.*, 1995), α -SNAP and NSF homologs (Sec17p and Sec18p; Haas and Wickner, 1996), and a novel soluble trafficking factor LMA1 (Xu and Wickner, 1996; Xu *et al.*, 1997). Homotypic vacuole fusion can be dissected into priming, docking and fusion subreactions. During priming, Sec17p and Sec18p dissociate and activate a pre-existing SNARE complex on the vacuole membrane (Ungermann *et al.*, 1998). This priming reaction is essential for the subsequent docking and fusion of the vacuoles (Mayer and Wickner, 1997; Ungermann *et al.*, 1998). LMA1, a dimer of thioredoxin and I^B₂, initially is bound to vacuoles through Sec18p and is delivered during priming to a complex containing the t-SNARE Vam3p (Xu *et al.*, 1998). LMA1 is released late in the reaction. Ypt7p is required for docking of vacuoles but is not involved in the initial dissociation of the vacuole SNARE complex (Mayer and Wickner, 1997; C.Ungermann and W.Wickner, unpublished). Finally, the fusion reaction is sensitive to the phosphatase inhibitor microcystin LR and to GTP γ S.

Recently, by alignment of their coiled-coil domains, syntaxin-like proteins have been defined as a novel family of proteins, the t-SNARE family (Weimbs *et al.*, 1997). Both the vacuolar t-SNARE Vam3p and the yeast protein Vam7p belong to this family. We now present functional and structural evidence that Vam7p is an additional member of the vacuolar SNARE complex. This protein was identified in a pioneering genetic screen which sought to identify proteins involved in the maintenance of vacuole morphology (Wada *et al.*, 1992). Vam7p is a SNAP-25

A

B

Fig. 1. (A) Vam7p is homologous to SNAP-25. Alignment of the protein sequence of Vam7p and SNAP-25 was done with the ALIGN algorithm (Genestream Search, CRBM Montpellier, France). **(B)** Vam7p co-purifies with Vam3p. Vacuoles were isolated and extracted as described in Materials and methods. P is the pellet fraction, S stands for supernatant. Each two lanes (P and S) contain equivalent material to the vacuole (vac) lane.

homolog tightly bound to the vacuole membrane. It is in a tight complex with the t-SNARE Vam3p and the v-SNARE Nyv1p, is essential for the stability of this complex and functions at the same time in the reaction as these previously identified SNAREs. These data establish that a SNAP-25 homolog can function on a membrane other than the plasma membrane and thereby establish SNAP-25-like proteins as general factors of intracellular vesicle trafficking.

Results

Vam7p is a vacuolar SNAP-25 homolog

In yeast, both the Sec9p SNARE of the plasma membrane (Brennwald *et al.*, 1994) and Vam7p belong to a distinct set of t-SNARE proteins with homology to SNAP-25 (Weimbs *et al.*, 1997; Figure 1A). Deletions in *VAM7* or in the vacuolar t-SNARE *VAM3* caused vacuole fragmentation, but these vacuoles contained normal amounts of matured carboxypeptidase Y (Wada *et al.*, 1992, 1997; Nichols *et al.*, 1997). The phenotype of the Vam7p deletion

mutant led us to ask whether it is a SNAP-25 homolog in the vacuolar SNARE complex. As shown in Figure 1B, Vam7p remains with the vacuoles during their isolation. Though Vam7p does not have a transmembrane domain, it is not extracted by urea or carbonate, conditions which remove Sec17p (Figure 1B). SNAP-25-like proteins are anchored in the membrane via a palmitoyl residue covalently attached to cysteinyl residues (Veit *et al.*, 1996). Vam7p contains a single cysteine at position 207 which may serve as the palmitoylation site.

Vam7p function depends on Sec18p and docking

To assay vacuole fusion *in vitro*, we mix vacuoles which lack proteases, and thus have catalytically inactive pro-alkaline phosphatase, with vacuoles which have the proteases but lack the alkaline phosphatase altogether (Haas *et al.*, 1994). Upon incubation with ATP, the vacuoles are primed, dock to one another and fuse, allowing the protease access to the pro-phosphatase. The resulting mature phosphatase is then assayed colorimetrically. Using this assay, we find three lines of evidence which suggest that Vam7p is involved in homotypic vacuole fusion. First, antibodies to Vam7p, added at various times to *in vitro* vacuole fusion reactions, show kinetics of inhibition similar to those seen with antibodies to Vam3p (Figure 2A). In this experiment, samples of a complete fusion reaction were mixed with inhibitory antibodies or transferred to 0°C at the indicated times and incubation continued for a 90 min period. While all tested antibodies inhibit fully if added at the start of the incubation, the reaction rapidly acquires resistance to Sec17p antibodies as priming is completed and then become resistant to antibodies to Vam3p and Vam7p. This suggests that Vam7p and Vam3p may act at a similar stage of the reaction.

Secondly, vacuoles must dock to acquire resistance to Vam7p antibodies (Figure 2B). Vacuoles from each of the two fusion tester strains were incubated with ATP in separate tubes and combined at different times in the absence or presence of the indicated antibodies (Mayer *et al.*, 1996). Whereas resistance to an antibody to Sec17p is largely achieved after 10 min, Vam7p antibodies completely inhibit the reaction at any time of combination (Figure 2B), even though antibody resistance is achieved during these incubation times when the vacuoles are mixed from the start of the reaction (Figure 2A). Thus, vacuole-vacuole contact is essential to fulfill the Vam7p requirement as resistance to the antibody is only acquired when both vacuole populations are mixed from the start (Figure 2A).

Thirdly, Sec18p function is a prerequisite for Vam7p to fulfill its function and for the reaction to become resistant to antibodies to Vam7p (Figure 2C). A reaction was started in the presence ATP, GTP γ S and antibodies to Sec18p. After 30 min at 27°C, a time that normally is sufficient to achieve resistance to antibody to Vam7p (Figure 2A), vacuoles were reisolated, resuspended in the original volume of reaction buffer containing ATP and cytosol and divided into four aliquots. One was placed on ice, one received no additions, one was mixed with an antibody to Vam3p and one received an antibody to Vam7p. After 5 min at 27°C, Sec18 protein was added to each of the five samples to overcome the anti-Sec18p block. Antibodies to Vam7p and Vam3p efficiently inhibited any

further reaction (Figure 2C), indicating that Sec18p needs to act before Vam7p and Vam3p. As reported for Vam3p and Nyv1p (Nichols *et al.*, 1997; Ungermann *et al.*, 1998), the completion of Vam7p function, as judged by resistance to anti-Vam7p, depends on Sec18p and on vacuole docking.

Vam7p is part of the vacuolar SNARE complex

We have shown previously that the v-SNARE Nyv1p and the t-SNARE Vam3p are found in a complex with Sec17p on the vacuolar membrane (Ungermann *et al.*, 1998). This complex is disassembled by Sec18p upon ATP hydrolysis. Both the vacuolar localization of Vam7p and the effect of antibodies to Vam7p on the stages of our *in vitro* vacuole fusion reaction suggest that Vam7p might be part of this SNARE complex. We tested the association of Vam7p with Vam3p by co-precipitation with an antibody to Vam3p

from vacuole detergent extracts. Vam3p and Vam7p are found in a tight complex (Figure 3, lane 2), even when the detergent extract was prepared from vacuoles from a strain deleted for Nyv1p (lane 6). Vam3p and Vam7p were in the same proportion in this complex (lanes 2 and 6) as in the whole vacuoles (lanes 1 and 5), showing that this complex is the major state of these proteins. On both wild-type vacuoles and vacuoles deleted for Nyv1p, the Vam3p–Vam7p complex was disassembled when vacuoles were incubated with ATP prior to preparation of the detergent extract (lanes 3 and 7). As reported (Ungermann *et al.*, 1998), this disassembly is more efficient on vacuoles that lack Nyv1p. The SNARE disassembly reaction was inhibited by antibodies to Sec18p (Figure 3, lanes 4 and 8), which themselves do not co-precipitate the SNARE complex under these conditions (not shown). Indeed, Vam3p and Vam7p co-migrate in a 10S complex during glycerol gradient sedimentation (Figure 3B). When vacuoles were pre-incubated for 10 min at 27°C in the presence of ATP, both Vam3p and Vam7p were found near the top of the gradient (Figure 3B, bottom), indicating that the complex has been disassembled by ATP. A truncated version of Vam7p, which probably was generated during lysis, was not present in the SNARE complex and migrated at the monomeric position. About 20% of Vam3p was not recovered in the SNARE complex even in the absence of ATP. Thus, Vam7p and Vam3p form a t-SNARE subcomplex on the vacuole membrane which is disassembled by Sec17p/Sec18p to function in the subsequent reaction.

Using deletion mutants in the vacuolar SNAREs Nyv1p and Vam3p, we have shown that Sec17p is in a complex with Vam3p and that this complex does not require the v-SNARE Nyv1p (Ungermann *et al.*, 1998). However, Sec17p is not found in a complex with Nyv1p unless Vam3p is also present (Ungermann *et al.*, 1998). To map the interactions of the SNARE proteins with each other and with Sec17p, we generated mutants in which VAM7

Fig. 2. Sensitivity of stages of the vacuole fusion reaction to antibodies to Vam7p. (A) Coincident acquisition of resistance to anti-Vam7p and anti-Vam3p during the fusion reaction. A 30× scale fusion reaction was started in the presence of ATP at 27°C. Aliquots (30 µl) were removed at the indicated times, added to antibodies to Sec17p, Vam3p or Vam7p and incubated at 27°C or placed on ice. Fusion reactions were incubated for a total of 90 min at 27°C before being assayed for alkaline phosphatase activity. (B) Vam7p action depends on vacuole docking. Two 10-fold reactions with vacuoles from either BJ3505 or DKY6281 were incubated at 27°C in the presence of ATP. At the indicated times, 15 µl portions were removed from each reaction and combined in the presence of buffer and antibodies to either Sec17p, Vam3p or Vam7p, and incubated for 90 min at 27°C. One aliquot was combined in the presence of buffer and placed on ice for the remaining reaction period. After 90 min, all reactions were analyzed for fusion. (C) Sec18p action must precede that of Vam7p in the fusion assay. Two 6-fold reactions containing vacuoles from BJ3505 and DKY6281 were started in the presence of cytosol, ATP, 1.5 mM GTPγS and antibodies to Sec18p. A 30 µl portion was removed and placed on ice and the rest was incubated for 30 min at 27°C. Vacuoles were then reisolated, resuspended in the original volume of reaction buffer containing ATP and cytosol and divided into 30 µl portions. Where indicated, IgGs to Vam3p or Vam7p were added. One reaction was left on ice, the others were returned to 27°C. After 5 min, 400 ng of pure Sec18p were added to each, incubations were continued for 90 min at 27°C, and each was analyzed for fusion as described above. Lane 1 is the ice control in which both the first and second incubations were performed on ice. The background activity (0.23 U) was subtracted in all lanes.

Fig. 3. Vam7p and Vam3p form a subcomplex on the vacuolar membrane. **(A)** Vam7p disassembles from the vacuolar SNARE complex. Isolated vacuoles (100 μ g) from wild-type (v,t,s) or cells lacking the v-SNARE Nyv1p (t,s) were incubated in reaction buffer in the absence or presence of ATP for 10 min at 27°C. The reactions were diluted with 400 μ l of 10 mM PIPES-KOH pH 6.8, 200 mM sorbitol and vacuoles were sedimented (10 min, 8000 g, 4°C). Vacuoles were then solubilized by adding 1 ml of lysis buffer (1% digitonin, 10 mM HEPES-KOH, pH 7.4, 110 mM NaCl, 5 mM EDTA, 1 mM PMSF, 30 μ M pefablock SC, 30 ng/ml leupeptin, 15 μ M *o*-phenanthroline, 150 ng/ml pepstatin) and protein complexes were analyzed by co-immunoprecipitation with protein A-immobilized antibodies to Vam3p as described (Ungermann *et al.*, 1998). A portion (10%) of the total detergent extract was TCA precipitated. Proteins retained on the protein A-immunoglobulin beads were released by addition of 30 μ l of 2% SDS and heating for 2 min at 95°C. The supernatant was transferred to a new tube, 7 μ l of a 5-fold concentrated SDS sample buffer were added and the sample was heated for 4 min at 95°C. Proteins were analyzed by SDS-PAGE, transferred to nitrocellulose and decorated with antibodies to Vam3p and Vam7p. **(B)** Vam3p and Vam7p co-migrate on a glycerol gradient. Isolated wild-type vacuoles (300 μ g) were incubated in reaction buffer in the absence or presence of ATP for 10 min at 27°C and processed as described in (A). Vacuoles were solubilized for 10 min on ice in 0.5% Triton X-100, 10% glycerol, 20 mM HEPES-KOH pH 7.4, 50 mM NaCl, 1 mM PMSF, 0.5% PIC (Xu and Wickner, 1996). The detergent extract was centrifuged (10 min at 26 000 g) and the supernatant was loaded on a linear 10–34% glycerol gradient. The samples were centrifuged for 18 h at 4°C [40 000 r.p.m., SW41 (Beckman)]. Fractions (500 μ l) were collected from top to bottom, proteins were precipitated with TCA, analyzed by SDS-PAGE, transferred to nitrocellulose and decorated with antibodies to Vam3p and Vam7p. The bottom of the gradient did not contain any protein complexes and is not shown here.

is deleted. Deletion in *VAM7*, either alone or together with *VAM3* or *NYV1*, leads to fragmentation of the vacuoles (Wada *et al.*, 1992; our unpublished observations). However, all Δ vam7 mutant vacuoles contain bound Sec17p and have the vacuolar marker protein Vma1p (Figure 4A). In this figure and those which follow, the vacuole SNAREs are designated v (for the v-SNARE Nyv1p), t (for the t-SNARE Vam3p) and s (for the SNAP-25 homolog Vam7p). As Sec17p is part of the SNARE complex and is present on all SNARE deletion vacuoles, we used co-

Fig. 4. Both t-SNAREs are required for the correct assembly of the SNARE complex. **(A)** Vacuoles that lack certain SNAREs still contain Sec17p and the vacuolar marker Vma1p. Vacuoles from wild-type and mutant cells were isolated, solubilized in sample buffer and heated for 4 min at 95°C. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose and the blots were decorated with antibodies to the indicated proteins. 't' (the t-SNARE Vam3p), 's' (the SNAP-25-like protein Vam7p) and 'v' (the v-SNARE Nyv1p) refer to the SNAREs that are present on the analyzed vacuoles. **(B)** Composition of the vacuolar SNARE complex on vacuoles that lack certain SNARE proteins. Isolated vacuoles (100 μ g) from BJ3505 (v,t,s), and BJ3505 with deletion in the genes for Nyv1p, Vam3p or Vam7p were analyzed by co-immunoprecipitation with protein A-immobilized antibodies to Sec17p as described in Figure 3. Specifically retained proteins were analyzed by SDS-PAGE, transferred to nitrocellulose and decorated with antibodies to Vam3p, Vam7p, Nyv1p and Sec17p.

immunoprecipitation with anti-Sec17p to test the composition of the SNARE complex of those mutants (Figure 4B). Strikingly, Nyv1p was only found in a complex with Sec17p when both Vam3p and Vam7p were present (Figure 4B, lanes 7, 9 and 10), though Nyv1p was present on vacuoles lacking either Vam3p or Vam7p (lanes 3 and 4). Thus, the integrity of the SNARE complex depends on the presence of both the t-SNAREs, Vam3p and Vam7p. In contrast, Vam7p and Vam3p form a subcomplex with Sec17p in the absence of Nyv1p (Figure 4B, lane 8). Furthermore, even in the absence of Nyv1p and the other t-SNARE, both Vam7p and Vam3p co-precipitate separately with Sec17p (Figure 4B, lanes 11 and 12). Thus, both t-SNAREs are necessary for Nyv1p binding, but they can be found in a complex with Sec17p individually.

Sec17p appears to bind strongly to the t-SNARE-like proteins (Figure 4B). Sec18p is isolated with the SNARE complex at 50 mM salt in the solubilization buffer, but is only weakly retained with the SNARE complex at the salt concentration used in our experiments (Xu *et al.*, 1998). Antibodies to the t-SNARE Vam3p blocked the Sec17p release reaction (Ungermann *et al.*, 1998), whereas antibodies to the v-SNARE Nyv1p or Vam7p did not interfere with the Sec17p release (not shown). Sec17p binding to the vacuoles is unaltered on vacuoles that lack both t-SNAREs (Figure 4A). Furthermore, Sec17p was released in an ATP-dependent reaction from all mutant vacuoles investigated in this study (not shown). This was unexpected as it was suggested previously that Sec17p only binds

Fig. 5. Vacuoles need Vam3p or Nyv1p to fuse with their wild-type partner. Vacuoles from wild-type cells (DKY6281) and cells (BJ3505) deleted in the respective SNAREs were isolated. Vacuoles from the different strains were incubated with ATP and cytosol for 90 min at 27°C or on ice and assayed for fusion. The results are expressed as percentages of the wild-type (v,t,s) fusion signal and represent a typical result from three independent experiments. For each lane, a control sample was placed on ice and the value was subtracted from the fusion signal.

to membranes via complexes of the SNARE proteins (Rothman, 1994). Recent results from our laboratory suggest that Sec18p can bind Sec17p and mediate a 'futile' Sec17p release from liposomes in the presence of ATP (K.Sato and W.Wickner, submitted). We therefore suggest that Sec17p binds to the vacuolar membrane via two affinities, one for the t-SNAREs and a second for Sec18p.

We have shown previously that vacuoles which lack both Vam3p and Nyv1p cannot fuse with a wild-type partner (Nichols *et al.*, 1997). However, Vam7p was still present on the membranes of these vacuoles. We therefore asked whether vacuoles lacking Vam7p could fuse with a wild-type partner. As shown in Figure 5, vacuoles from strains deleted for both Vam3p and Nyv1p and bearing only the Vam7p 's'-SNARE are completely inactive in fusion with the wild-type, whereas those with both Nyv1p and Vam7p or those with Vam3p or Nyv1p alone still fuse. This suggests that the minimal set of SNAREs required for fusion are Nyv1p and Vam3p. We cannot exclude that other SNAREs might also be present on the vacuole and function in our assay; however, they would not compensate for the loss of both Vam3p and Nyv1p. We also asked whether Vam7p might be dispensable from both partners for fusion. The fusion signal obtained with the corresponding Δ vam7 vacuoles was drastically reduced (~3% compared with wild-type; not shown). In summary, Vam7p cannot function alone, but is required for the integrity of the SNARE complex.

Discussion

Our analysis of a vacuolar SNAP-25 homolog suggests that SNAP-25 proteins may have a general role in intracellular trafficking. Thus far, SNAP-25 proteins have only been studied on the plasma membrane during synaptic vesicle fusion, exocytosis and yeast secretion (Brennwald *et al.*, 1994; Südhof, 1995; Martin, 1997). On the synaptic membrane and on synaptic vesicles, SNAP-25 is found in a complex with syntaxin and synaptobrevin (Walch-

Solimena *et al.*, 1995; Tagaya *et al.*, 1996). This complex is disassembled by the action of NSF and α -SNAP (Otto *et al.*, 1997). Two independent lines of experimentation show that SNAP-25 has an important role in synaptic vesicle fusion. First, SNAP-25 is necessary for proper assembly of the synaptic SNARE complex. *In vitro* experiments with purified GST fusion proteins showed that SNAP-25 forms a subcomplex with syntaxin which serves as a high affinity binding site for synaptobrevin (Chapman *et al.*, 1994; Hayashi *et al.*, 1994; Pevsner *et al.*, 1994; McMahon and Südhof, 1995). α -SNAP will bind to either syntaxin or SNAP-25, and the complete complex provides an additional site for α -SNAP (Hayashi *et al.*, 1994). The *in vitro* assembled SNARE complex could then be dissociated by the addition of α -SNAP, NSF and ATP (Söllner *et al.*, 1993a,b; Hayashi *et al.*, 1995). Secondly, SNAP-25 has a critical role in membrane fusion. Botulinum toxin A cleaves 20 aminoacyl residues from the C-terminus of SNAP-25 and thereby blocks the fusion of synaptic vesicles with the presynaptic membrane and Ca^{2+} -dependent exocytosis (Blasi *et al.*, 1993; Banerjee *et al.*, 1996). However, this cleavage blocks neither the disassembly nor the formation of the SNARE complex *in vitro* (Otto *et al.*, 1995). Furthermore, peptides that mimic the C-terminal domain of SNAP-25 or antibodies to this region efficiently block Ca^{2+} -dependent exocytosis (Gutierrez *et al.*, 1995; Metha *et al.*, 1996), emphasizing the critical role of the C-terminus of SNAP-25 in membrane fusion. However, it is not understood how SNAP-25 functions in this reaction.

Vam7p belongs to the family of SNAP-25-like proteins: (i) it has extensive sequence homology with SNAP-25 (Figure 1A; Weimbs *et al.*, 1997); (ii) it is a membrane protein, probably anchored to the vacuolar membrane via palmitoylation; (iii) Vam7p is part of the vacuolar SNARE complex and disassembles from it upon ATP-dependent priming of the vacuoles; (iv) Vam7p is found in a t-SNARE subcomplex with Vam3p in the absence of the v-SNARE Nyv1p; and (v) Vam7p appears to interact directly with Sec17p as it is recovered in a complex with Sec17p in the absence of the other two identified vacuolar SNAREs. The fragmented vacuole phenotype of the VAM7 deletion mutant and the strong inhibition of our fusion assay by antibodies to Vam7p indicate that Vam7p has a critical role in this fusion event. The vacuolar SNARE complex is only assembled efficiently in the presence of both Vam7p and the t-SNARE Vam3p. Thus, the vacuole membrane has a t-SNARE complex that is required for fusion and maintenance of vacuolar integrity.

It is intriguing that Vam3p and Vam7p act as a t-SNARE pair. In general, syntaxin-like proteins appear to have a key role in organelle identity and specificity of trafficking (Pfeffer, 1996; Hay and Scheller, 1997). In yeast, deletions of t-SNAREs have a stronger effect than deletions of v-SNAREs. Consequently, the specificity of t-SNAREs in vesicle trafficking could be regulated by the addition of a SNAP-25-like protein. Biophysical analyses have indeed demonstrated that syntaxin and SNAP-25 undergo significant conformational changes when they bind to each other (Fasshauer *et al.*, 1997a,b; Rice *et al.*, 1997). The complex of syntaxin and SNAP-25 and the associated conformational change allows other proteins, like the v-SNAREs, to bind to this complex. Though SNAP-25

Fig. 6. Working model for the homotypic fusion of yeast vacuoles. Abbreviations are as follows: Sec17p (17), Sec18p (18), Nvy1p (v), Vam3p (t), Vam7p (s), pro-alkaline phosphatase (Pro-ALP), alkaline phosphatase (ALP). An asterisk on the t-SNARE indicates the activated state of the protein after priming. See text for details.

homologs are essential for SNARE complex assembly, their precise role in docking and fusion remains to be established. The interactions between SNAP-25-like proteins and other SNAREs upon docking may be quite similar to the assembly interactions that have been described *in vitro* (Chapman *et al.*, 1994; Hayashi *et al.*, 1994, 1995; Pevsner *et al.*, 1994; McMahon *et al.*, 1995), though we have little molecular understanding of the role of the SNAREs in the fusion event itself. In this context, our finding that Vam7p alone cannot mediate membrane fusion (Figure 5) suggests that it may act largely to stabilize the association of the other SNAREs and conformation at the docking/fusion site.

Our current working model (Figure 6) proposes that isolated vacuoles have a multisubunit SNARE complex which includes the t-SNARE Vam3p, the v-SNARE Nvy1p, the 's'-SNARE Vam7p, Sec17p, Sec18p, LMA1, and perhaps additional proteins as well. The 's'-SNARE

Vam7p is crucial for the stability of this complex. ATP hydrolysis by Sec18p drives the Sec17p release, SNARE complex disassembly, activation of the t-SNARE and transfer of LMA1 from Sec18p to the activated t-SNARE (Xu *et al.*, 1998). Docking is accompanied by v:t SNARE pairing (C.Ungermann, in preparation). The rearrangements of SNAREs upon fusion, and the reassembly of the initial SNARE complex, clearly needs further study.

One SNARE protein of the early secretory pathway in yeast has been suggested to have a SNAP-25-like function. Bet1p, a SNARE involved in endoplasmic reticulum (ER) to Golgi vesicle trafficking, has marginal sequence homology to SNAP-25 but belongs to the v-SNARE class of proteins (Pfeffer, 1996; Stone *et al.*, 1997; Weimbs *et al.*, 1997). Bet1p has been localized to the ER, to ER-derived vesicles and, recently, to the intermediate compartment between the ER and the Golgi (Newman *et al.*, 1992; Lian and Ferro-Novick, 1993; Hay *et al.*, 1997; Zhang *et al.*, 1997). Bet1p could, therefore, function with either Ufe1p, the t-SNARE on the ER, or Sed5p, the *cis*-Golgi t-SNARE. *In vitro* binding studies with purified proteins showed that Bet1p promotes binding of Bos1p, a v-SNARE on ER-derived vesicles, to the t-SNARE Sed5p (Stone *et al.*, 1997). Bet1p and Bos1p are found in a complex with Sed5p in detergent extracts (Søgaard *et al.*, 1994). Though the localization of Bet1p is not yet established, it recently has been shown that ER-derived vesicles required Bet1p for fusion with the Golgi (Cao *et al.*, 1998). Whether Bet1p also has a role in the retrograde transport from the Golgi to the ER remains to be tested.

Why do the vacuole and the plasma membrane depend on complexes of two t-SNAREs if the rest of the secretory pathway does not have a SNAP-25 homolog? Alternatively, have SNAP-25-like proteins been overlooked in other trafficking events of the secretory pathway? In yeast, two additional SNAP-25 homologous proteins have been characterized, the plasma membrane SNARE Sec9p and a Sec9 homolog SPO20. SPO20 is required for the formation of the prospore plasma membrane and replaces Sec9p in this process (Neiman, 1998). It therefore defines a developmentally regulated branch of the secretory pathway. Molecular details are better understood in the case of Sec9p. Surprisingly, the Sec9 protein is much larger than the other known SNAP-25 proteins and is not anchored to the membrane via palmitoylation, as shown by its facile extraction by carbonate (Brennwald *et al.*, 1994; Couves and Gerst, 1994). The localization of Sec9p to the plasma membrane and its recovery in a complex with the t-SNAREs Sso1/2p and the v-SNAREs Snc1/2p (Brennwald *et al.*, 1994) suggest a functional similarity to SNAP-25 of the synaptic membrane. A subcomplex of only Sso1p and Sec9p was able to bind the v-SNARE Snc1p *in vitro* (Rossi *et al.*, 1997). Thus, a t-SNARE complex is required on the yeast plasma membrane for the binding of the v-SNARE, as we find for the vacuolar membrane.

The vacuolar SNARE complex as described herein contains more subunits than we previously appreciated. Holthuis *et al.* (1998) recently reported that Vam3p also interacts with the v-SNARE Vti1p by co-precipitation (Fischer von Mollard *et al.*, 1997; Lupashin *et al.*, 1997). Recently, we have discovered that the homotypic vacuole

fusion reaction depends on Vti1p and that Vti1p is part of the vacuolar SNARE complex (C.Ungermann, G.Fischer von Mollard, T.H.Stevens and W.Wickner, unpublished). We do not yet know whether all these subunits are part of a large complex or whether we are looking at subcomplexes that catalyze separate fusion reactions of vacuoles with vesicles from other organelles. Nevertheless, multiple SNAREs are apparently involved in at least the homotypic vacuole fusion reaction.

Materials and methods

Materials and yeast strains

The sources of reagents are as described by Haas (1995), Mayer *et al.* (1996) and Haas and Wickner (1996). Yeast strains are described in Nichols *et al.* (1997). Deletions in Vam7p in those strains were generated by recombination with a PCR-generated cassette containing appropriate homologous regions of Vam7p and the *URA3* gene of *Saccharomyces cerevisiae*. The PCR primer contained 40 bases of identity to the regions flanking the open reading frame (5' primer sequence: 5'ACA AAA ACA ATA AAG TCA TAT AAG GGT TGA TAA TTG ATA TTG GAT TCC GGT TTC TTT GAA AT; 3' primer sequence: 5'ATA TAC TCT CAG CAT TTG TAA CCC GGA TAG TAA CTC ATT AAT TCT AAT TTG TGA GTT TAG TAT AC). Deletions were confirmed by PCR using primers that anneal to the promotor region of the *VAM7* gene (CGC CTA AAG ATT TGT ATC AA) and the coding region of the *URA3* gene (CCC AAT GCG TCT CCC TT) and isolated genomic DNA from the selected clones following a standard PCR protocol (Ausubel *et al.*, 1995) and immunoblotting (Harlow and Lane, 1989).

Biochemical methods

SDS-PAGE, immunoblotting using enhanced chemiluminescence (ECL; Haas *et al.*, 1995), purification of his₆-tagged Sec18p (Haas and Wickner, 1996) and assay of Sec17p release (Mayer *et al.*, 1996) were as described. Antibodies were generated in rabbits against two Vam7p peptides (residues 147–164 and 242–257). Peptides corresponding to regions of Nyv1p (Ungermann *et al.*, 1998) and Vam7p were immobilized on an Ultralink column (Pierce, Rockford, IL) and affinity purification of antibodies was performed as described (Haas and Wickner, 1996). Sec18p IgGs were affinity purified and concentrated according to Haas and Wickner (1996). IgGs to Vam3p, Vam7p, Nyv1p and Ypt7p were purified according to Harlow and Lane (1989), concentrated by ultrafiltration, diluted in PS buffer (10 mM PIPES, pH 6.8, 200 mM sorbitol) and concentrated to 5 mg/ml (Haas and Wickner, 1996). Aliquots (50 µl) were frozen in liquid nitrogen and stored at –20°C. Co-immunoprecipitations in digitonin were performed as described (Ungermann *et al.*, 1998).

Localization of Vam7p

Cell extracts were prepared from an overnight YPD culture. Cells (0.25 OD₆₀₀ units) were sedimented (3 min, 5000 g) and suspended successively in 1 ml of H₂O, 1 ml of 1 mM EDTA, 10 mM Tris–HCl, pH 8.0, and 500 µl of H₂O. Lysis cocktail [75 µl of 1.85 M NaOH, 1 M β-mercaptoethanol, 10 mM phenylmethylsulfonyl fluoride (PMSF)] was added, the sample was incubated for 10 min at 0°C, and 500 µl of cold 50% (w/v) trichloroacetic acid (TCA) was added. After 10 min on ice, the sample was centrifuged (10 min, 16000 g). The pellet was suspended in 1 ml of acetone and centrifuged (10 min, 16000 g). The pellet was dried for 5 min at 56°C and proteins were dissolved in SDS sample buffer (2% SDS, 50 mM Tris–HCl, pH 6.8, 10% glycerol, 0.01% bromophenol blue) and heated for 4 min at 95°C.

Vacuoles were isolated as described (Haas, 1995). Aliquots (30 µg) were diluted into 500 µl of 20 mM HEPES–KOH, pH 7.0, 1 mM PMSF, mixed with an equal volume of freshly prepared 200 mM Na₂CO₃ and the samples were incubated for 30 min at 0°C. Insoluble material was removed by centrifugation for 30 min at 100 000 g in a TLA 45 rotor, and proteins were precipitated by addition of TCA (13% w/v final) and acetone washed as described above. For urea extraction, vacuoles (30 µg) were diluted into 6 M urea, 20 mM Tris–HCl pH 7.5 (final concentration), and incubated for 30 min at 4°C. Pellet and supernatant fractions were separated and treated as above. Proteins in the pellet and supernatant fractions were solubilized in SDS sample buffer, heated to 95°C for 4 min, resolved by SDS-PAGE on 12% polyacrylamide gels, transferred to nitrocellulose and immunoblotted with antibodies to Vam3p, Vam7p and Sec17p (Harlow and Lane, 1989).

Vacuole fusion

Vacuoles were used immediately after isolation (Haas, 1995). The standard fusion reaction (30 µl) contained 3 µg of each vacuole type (BJ3505 and DKY6281) in reaction buffer (10 mM PIPES, pH 6.8, 200 mM sorbitol, 150 mM KCl, 0.5 mM MgCl₂, 0.5 mM MnCl₂, 0.5 mM ATP, 3 mg/ml cytosol, 3.5 U/ml creatine kinase, 20 mM creatine phosphate, and a protease inhibitor cocktail (PIC; Xu and Wickner, 1996) containing 7.5 µM pefabloc SC, 7.5 ng/ml leupeptin, 3.75 µM *o*-phenanthroline and 37.5 ng/ml pepstatin. To reduce proteolysis in the Sec17p release reaction and the co-immunoprecipitation experiments, only the protease A-deficient BJ3505 vacuoles were analyzed. One unit of fusion activity is defined as 1 µmol of *p*-nitrophenol phosphate hydrolyzed per min and per µg of BJ3505.

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