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The N-terminal Domain of the t-SNARE Vam3p Coordinates Priming and Docking in Yeast Vacuole Fusion

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Homotypic fusion of yeast vacuoles requires a regulated sequence of events. During priming, Sec18p disassembles *cis*-SNARE complexes. The HOPS complex, which is initially associated with the *cis*-SNARE complex, then mediates tethering. Finally, SNAREs assemble into *trans*-complexes before the membranes fuse. The t-SNARE of the vacuole, Vam3p, plays a central role in the coordination of these processes. We deleted the N-terminal region of Vam3p to analyze the role of this domain in membrane fusion. The truncated protein (Vam3ΔN) is sorted normally to the vacuole and is functional, because the vacuolar morphology is unaltered in this strain. However, in vitro vacuole fusion is strongly reduced due to the following reasons: Assembly, as well as disassembly of the *cis*-SNARE complex is more efficient on Vam3ΔN vacuoles; however, the HOPS complex is not associated well with the Vam3ΔN *cis*-complex. Thus, primed SNAREs from Vam3ΔN vacuoles cannot participate efficiently in the reaction because *trans*-SNARE pairing is substantially reduced. We conclude that the N-terminus of Vam3p is required for coordination of priming and docking during homotypic vacuole fusion.

INTRODUCTION

Transport between organelles is mediated by vesicles that bud from a donor membrane, are transported to and finally fuse with their target membrane (Rothman and Wieland, 1996; Mellman and Warren, 2000). In recent years, conserved sets of membrane proteins designated as SNAREs (soluble NSF attachment protein receptors) were identified in all eucaryotes as key players in docking and fusion (Rothman, 1994; Jahn and Südhof, 1999; Lin and Scheller, 2000). They have characteristic coiled-coil domains, which tightly interact in a parallel four-helix bundle (Katz *et al.*, 1998; Poirier *et al.*, 1998; Sutton *et al.*, 1998; Weimbs *et al.*, 1998). Initially, SNAREs are found in *cis*-complexes on both vesicle and target membranes (Walch-Solimena *et al.*, 1995; Otto *et al.*, 1997; Holthuis *et al.*, 1998; Ungermann *et al.*, 1998a). After disassembly by the ATPase NSF and its cofactor α-SNAP, SNAREs on opposing membranes interact in a *trans*-complex (Ungermann *et al.*, 1998b; Weber *et al.*, 1998). This process is considered to be a central event in membrane fusion (Hanson *et al.*, 1997; Chen *et al.*, 1999). Because different intracellular compartments have distinct SNARE proteins in their membranes, it is believed that their distinct interactions also account for the specificity of intracellular membrane traffic (McNew *et al.*, 2000; Scales *et al.*, 2000). Furthermore, in an in vitro liposome fusion assay, it was shown that isolated SNAREs are able to fuse lipid bilayers, suggesting that these proteins directly mediate fusion (Weber *et al.*, 1998).

The in vitro homotypic fusion of yeast vacuoles represents an ideal system to study membrane fusion and the role of SNARE proteins in the context of an authentic organelle fusion reaction (Nichols *et al.*, 1997; Wickner and Haas, 2000). The vacuolar SNAREs Vam3p, Nyv1p, Vti1p, Ykt6p, and Vam7p are initially found in a *cis*-complex on the isolated organelle (Ungermann *et al.*, 1999). This complex is dissociated through the action of Sec18p, Sec17p, and LMA1 (Mayer *et al.*, 1996; Ungermann *et al.*, 1998a; Xu *et al.*, 1998). After this priming step, docking is initiated by the interaction of the Rab protein Ypt7p with Vam7p and the *ho*motypic *f*usion and *p*rotein sorting (HOPS) complex (Price *et al.*, 2000a; Seals *et al.*, 2000; Ungermann *et al.*, 2000). The HOPS complex, consists of Vam2p and Vam6p (Price *et al.*, 2000a) and of the class C Vps proteins Vps11p, Vps16p, Vps18p, and Vps33p (Rieder and Emr, 1997; Seals *et al.*, 2000; Wurmser *et al.*, 2000). Formation of a *trans*-SNARE complex triggers downstream reactions that require Ca^{2+}, calmodulin, protein phosphatase 1, and the VO ATPase proteolipid to mediate complete membrane fusion (Peters and Mayer, 1998; Peters *et al.*, 1999, 2001).

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Abbreviations used: HOPS, homotypic fusion and protein sorting; SNARE, SNAP (soluble NSF attachment protein) receptor.

The t-SNARE that is structurally best characterized is the neuronal syntaxin 1a. It consists of three interaction domains: the transmembrane anchor that can interact with synaptobrevin (Laage *et al.*, 2000), the C-terminal helix 3 (H3), which contains the coiled–coil region and provides the platform for SNARE complex assembly (Kee *et al.*, 1995; Sutton *et al.*, 1998; Jahn and Südhof, 1999), and the N-terminal helices A, B, and C (H_{ABC}), which independently fold into a three-helix-bundle (Fernandez *et al.*, 1998). Biochemical studies have shown that the N-terminal domain of syntaxin can bind to its C-terminal domain, resulting in a closed conformation that can act as an inhibitor of SNARE complex formation (Dulubova *et al.*, 1999; Misura *et al.*, 2000). Furthermore, Sec1 proteins can interact with syntaxin-like t-SNAREs by recognizing the closed conformation (Jahn and Südhof, 1999). Even though the function is still not understood, the interaction of Sec1 proteins and t-SNAREs appears to be essential for fusion and in most cases seems to involve the N-terminal domain (Banta *et al.*, 1990; Ossig *et al.*, 1991; Wu *et al.*, 1999; Jahn, 2000; Verhage *et al.*, 2000; see discussion).

The yeast vacuolar t-SNARE Vam3p is structurally related to syntaxin, but does not adopt a closed conformation (Dulubova *et al.*, 2001). We prepared mutant yeast strains lacking the N-terminal domain of Vam3p to monitor the interactions and to characterize the function of this domain during the fusion of intact organelles. Truncation of the N-terminus leads to significantly reduced vacuole fusion. Although priming occurs more efficiently in the absence of the N-terminus, *trans*-SNARE complex formation is less than half of that of the wild-type Vam3p. We observe that HOPS/Vps33p is not recruited efficiently to the *cis*-complex in the absence of the N-terminal domain of Vam3p, suggesting that this domain is required to ensure the robust transition from priming to docking.

MATERIALS AND METHODS

Reagents and Yeast Strains

Antibodies against Vam3p and Vti1p were increased in New Zealand white rabbits, α-Vam3p by injecting recombinant full-length His_6-tagged Vam3p, α-Vti1p with a GST-fusion protein containing the complete cytoplasmic domain of Vti1p (von Mollard *et al.*, 1997; Ungermann *et al.*, 1998b)

Deletion mutants of *VAM3* were introduced into yeast strains BJ3505 (MATa *pep4::HIS3 prb1-Δ1.6RHIS3 lys-208 trp1-Δ101 ura3-52 gal2 can*) and DKY6281 (MATa *leu2-3 leu2-112 ura3-52 his3-Δ200 trp1 Δ901 lys2-Δ901 lys2-801 suc2-Δ9 pho8::TRP1*) by transformation and loop in-loop out at the chromosomal *VAM3* locus of the plasmid pRS406 Vam3ΔN containing a *URA3*-marker and encoding the N-terminal deleted Vam3p. Ura^+ transformants were selected. Ura^- clones that were generated in a second selection with 5-fluoroorotic acid were then tested for loss of the wild-type *VAM3* sequence by PCR and the size shift by immunoblotting (Ungermann *et al.*, 1999). The strains BJ3505 *VPS33::ProtA* and BJ3505 *VAM3ΔN VPS33::ProtA* were created by transformation of a PCR fragment containing the sequence encoding Protein A and a *kanMX6* selection marker with flanking regions of the *VPS33* 3′-region and the downstream sequence of the terminator (Knop *et al.*, 1999). Colonies that grew on YPD+Geneticin plates were restreaked and analyzed for the Protein A tag by PCR and immunoblotting.

Vacuole Fusion

Vacuole fusion is measured by a biochemical complementation assay (Conradt *et al.*, 1992; Haas, 1995). Vacuoles from DKY6281 have normal proteases but lack the membrane protein alkaline phosphatase. Vacuoles from BJ3505 accumulate alkaline phosphatase in the unprocessed and catalytically inactive "pro" form because of the deletion of the gene encoding the protease Pep4p. Incubation of a mixture of these vacuoles in reaction buffer at 26°C in the presence of cytosol and ATP leads to fusion, content mixing, and processing of proalkaline phosphatase by Pep4p. The active alkaline phosphatase is measured in a colorimetric assay at the end of the fusion reaction.

Yeast cells from 1 liter logarithmically growing culture were spheroblasted with recombinant lyticase and lysed by addition of DEAE dextran and heat shock as described (Haas, 1995). Vacuoles were purified by flotation in a discontinuous Ficoll gradient. The isolation procedure enriches vacuoles 45- to 50-fold with respect to total cell protein in the gradient. Only trace amounts of ER and cytosol are recovered by the procedure (Haas, 1995). Vacuoles were used immediately after isolation. The standard fusion reaction (30 μl) contained 3 μg of each vacuole type (BJ3505 and DKY6281) in reaction buffer (10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol, 150 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$), 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. For preclustering, vacuoles were centrifuged for 4 min at 10,000 × *g* and briefly resuspended in reaction buffer. To reduce proteolysis in the coimmunoprecipitation experiments, only the protease A–deficient BJ3505 vacuoles were analyzed.

Immunoprecipitation

After the reaction, vacuoles were pelleted (8000 × *g*, 5 min, 4°C), washed with 500 μl of reaction buffer, and reisolated as before. Vacuoles were detergent-solubilized by the addition of 1 ml of 1% NP-40, 125 mM NaCl, 10 mM Tris/HCl, pH 7.4, 0.5× PIC, and 1 mM PMSF and incubated on a nutator at 4°C for 10 min. Nonsolubilized material was removed by centrifugation (20,000 × *g*, 10 min, 4°C). A fraction (5%) of the clarified supernatant was removed, and proteins were precipitated by the addition of TCA (13% vol/vol). The remaining detergent extract was added to Protein A–Sepharose beads containing the coupled antibodies (Ungermann *et al.*, 1999) and incubated on a nutator at 4°C for 2 h or overnight. The beads were reisolated by brief centrifugation and were washed three times with 1 ml of lysis buffer containing 0.1% NP-40 for 10 min. Retained proteins were eluted by the addition of 1 ml of 0.1 M glycine/HCl, pH 2.5, 0.025% NP-40. Proteins were precipitated by TCA, washed with 1 ml of ice cold 100% acetone, briefly dried, and dissolved in SDS-sample buffer. Analysis of protein complexes was done by SDS-PAGE and Western blotting.

Protein A Purification

Protein A–tagged Vps33p was purified with IgG-Sepharose Fast-Flow (Amersham-Pharmacia, Freiburg, Germany). Vacuoles from the respective strains (300 μg) were pelleted by centrifugation (10000 × *g*, 10 min), solubilized in 2 ml of 0.1% TX100, 50 mM NaCl, 20 mM HEPES/KOH, pH 7.9, 0.5× PIC, and 1 mM PMSF, and incubated on a nutator at 4°C for 20 min. Nonsolubilized material was removed by centrifugation (20,000 × *g*, 10 min, 4°C). The supernatant was loaded on a Qiagen (Hilden, Germany) 5 ml polypropylene column and incubated with 100 μl of IgG-Sepharose for 2 h on a nutator. The column was drained by gravity and washed three times with 10 mM HEPES/KOH, pH 7.9, 50 mM NaCl and once with 10 mM HEPES/KOH, pH 7.9, 100 mM NaCl. Protein was eluted with 1 ml 0.1 M glycine, pH 2.6, and precipitated with TCA (as described above).

Analysis of trans-*SNARE Complexes*

Trans-SNARE pairing was analyzed with vacuoles prepared from DKY6281 *vam3*Δ, BJ3505 *nyv1*Δ, and BJ3505 *nyv1*Δ *VAM3*Δ*N*. Vacuoles (120 μg each) were mixed in a 750 μl reaction with cytosol and ATP and incubated for 45 min at 26°C. A 30-μl aliquot was used to measure alkaline phosphatase activity. Vacuoles were collected by centrifugation (16000 × *g*, 4°C, 10 min), washed with 10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol, and solubilized with 1 ml 20 mM Tris/Cl, pH 7.4, 0.1% Triton X-100, 150 mM NaCl for 10 min, and Nyv1p was coimmunoprecipitated with α-Vam3p antibodies as described (Ungermann *et al.*, 1998b).

RESULTS

Although the function of the H3 coiled-coil domain of t-SNAREs is well described, the role of the N-terminus has been a matter of debate. To study the function of the N-terminal domain in membrane fusion, we deleted amino acids 1–145 of Vam3p, preserving the complete SNARE domain, the transmembrane domain, and the di-leucine sorting signal from N154 to L160 (Darsow *et al.*, 1998), resulting in Vam3ΔN (Figure 1A). We introduced the truncated gene by homologous recombination into our tester strains, where it replaced the wild-type gene and was under the control of the original promoter (see MATERIALS AND METHODS). As a first test for functionality, we analyzed vacuolar morphology of these strains by labeling the cells with the lipophilic dye FM4–64 (Vida and Emr, 1995). Missorting or complete absence of Vam3p results in severe fragmentation of vacuoles (Figure 2, E and F; Darsow *et al.*, 1997; Nichols *et al.*, 1997). In contrast to this, vacuoles of the strain expressing Vam3ΔN were similar to vacuoles from the wild-type strain (Figure 2, C and D, and A and B, respectively), indicating that the truncated protein is properly sorted and functional in homotypic fusion. Furthermore, the N-terminal deleted Vam3p is found on the vacuoles at a level comparable to the wild-type protein (Figure 1B). Also, sorting of vacuolar alkaline phosphatase was unaltered in the mutant strain (our unpublished observations).

Homotypic fusion of vacuolar vesicles can be reconstituted in vitro by incubating isolated vacuoles in the presence of ATP and cytosol (Conradt *et al.*, 1992; Haas *et al.*, 1994; see MATERIALS AND METHODS). We used this assay to map the stage at which the N-terminus might function during membrane fusion. To our surprise, and in contrast to recent findings by Wang *et al.* (2001), we observed a pronounced decrease in fusion activity of vacuoles prepared from the mutant strain, indicating that the N-terminal domain is required for efficient fusion (Figure 3A). Relative to wild-type control, diminished fusion was observed consistently over a period of 90 min (Figure 3B). Extending the standard incubation time from 90 to 240 min did not increase the overall fusion of the Vam3ΔN vacuoles (our unpublished observations). This may be due to consumption or decay of critical components over time (Figure 3C and our unpublished observations). Furthermore, we found that the N-terminus does not function to inactivate Vam3p, which has been postulated for syntaxin (Jahn and Südhof, 1999). When vacuoles from both tester strains were incubated separately and mixed at different time points, the fusion signal declined equally in the wild-type and the mutant strain (Figure 3C).

We therefore used established assays (Wickner and Haas, 2000) to investigate the function of the amino terminus of Vam3p and to understand why fusion is reduced in its absence. Homotypic vacuole fusion can be separated into distinct subreactions termed priming, docking, and fusion (Wickner and Haas, 2000). We asked if Vam3ΔN vacuoles were defective at any of those stages. First, we analyzed whether the deletion of the N-terminus of Vam3p alters the composition of the *cis*-SNARE or its disassembly during priming. Vacuoles were pretreated with or without ATP, and SNARE complexes were isolated from detergent extracts by coimmunoprecipitation with antibodies against the SNARE Vti1p (Figure 4). Indeed, wild-type as well as truncated Vam3p is in a *cis*-SNARE complex with the SNAREs, Vti1p, Nyv1p, Ykt6p, and Vam7p, in the absence of ATP. However, in all experiments, two to three times more SNAREs were found in precipitations from Vam3ΔN vacuoles than in wild-type (Figure 4A, lane 1 vs. 3). Furthermore, priming, as measured by the ATP-dependent disassembly of the *cis*-SNARE complex, was much more efficient on Vam3ΔN vacuoles (Figure 4B; lane 2 vs. 4), suggesting that

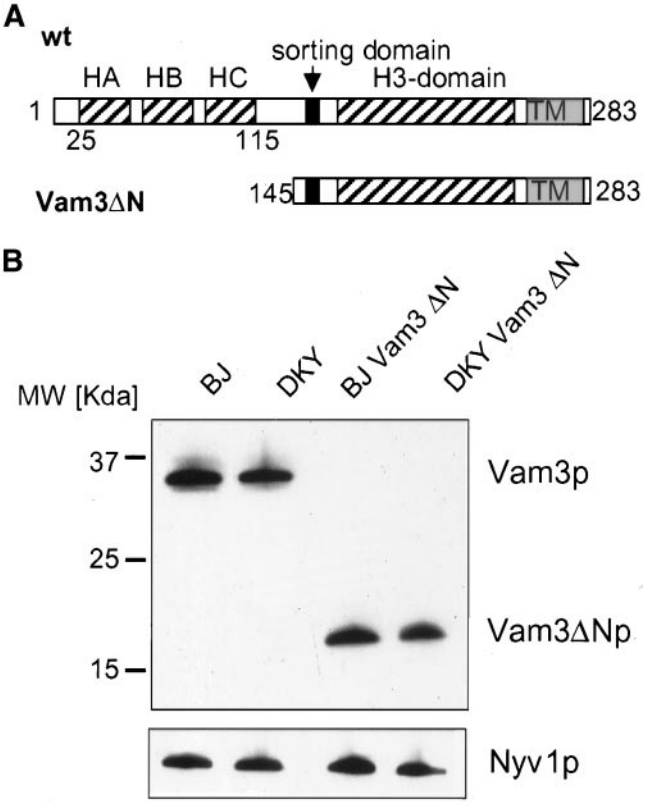

Figure 1. Deletion of the N-terminal domain of Vam3p. (A) Domain structure of wild-type Vam3p according to coiled-coil prediction (SwissProt Expasy "coils") and the NMR structure (Dulubova *et al.*, 2001). The approximate sites of coiled-coil domains are indicated by striped boxes, transmembrane domains (TM) by gray boxes, and the sorting domain (amino acids 154–160) by black boxes. Vam3ΔN is deleted from amino acids 1–145. (B) Expression and sorting of the truncated Vam3p. Vacuoles were prepared as described and a fraction (10 μg) of each wild-type and mutant tester strain was analyzed by SDS-PAGE followed by Western blotting. Immunoblots were decorated with antibodies to Vam3p and Nyv1p.

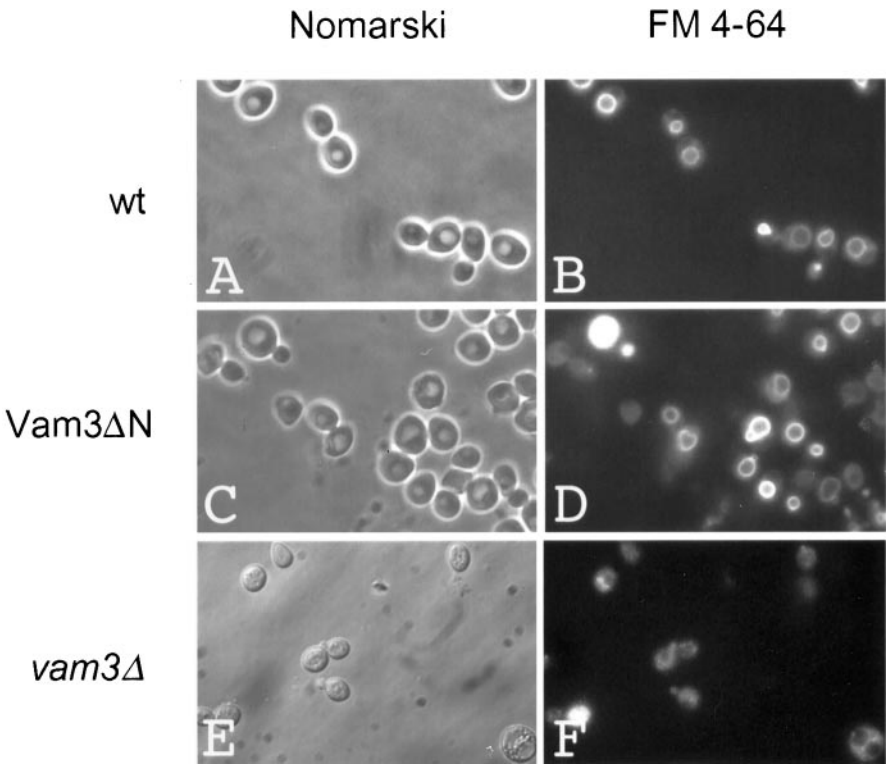

Figure 2. Vacuolar morphology is unaltered in the Vam3ΔN mutant. Wild-type or mutant BJ3505 strains were incubated with 10 μM of the dye FM4–64 (Molecular Probes, Eugene, OR) for 20 min at 30°C in YPD. Cells were centrifuged briefly (1 min at 5000 $\times$ *g*), washed twice with YPD medium, and chased for 15 min at 30°C (Vida and Emr, 1995). Stained vacuoles were analyzed in a standard fluorescence microscope. (A and B) BJ3505 wild-type cells; (C and D) BJ3505 *VAM3ΔN* cells; (E and F) BJ3505 *vam3Δ* cells as a negative control.

SNARE complexes containing Vam3ΔN are either less stable or more accessible to Sec18p than those with wild-type Vam3p. Thus, removal of the N-terminus of Vam3p obviously alters the dynamics of the SNARE complex.

Next we asked if the observed alteration in the *cis*-SNARE complex would influence the sensitivity of the vacuoles to known inhibitors and activators (Figure 5). Both types of vacuoles were equally sensitive to Gdi1p, a docking inhibitor (Haas *et al.*, 1995), as well as the fusion inhibitor BAPTA (Peters and Mayer, 1998) and were similarly stimulated by coenzyme A (CoA; Haas and Wickner, 1996; Veit *et al.*, 2001). Interestingly, addition of Sec18p, which enhances priming and thus fusion of wild-type vacuoles by ~150%, had virtually no stimulatory effect on the fusion of Vam3ΔN vacuoles (Figure 5, B and C). Sec18p promotes disassembly of *cis*-SNARE complexes (Mayer *et al.*, 1996; Ungermann *et al.*, 1998a), resulting most likely in more activated SNAREs that can participate in the docking step. If added in excess, Sec18p can also inhibit fusion, probably by continuously activating SNAREs and delaying their entry into the reaction (Ungermann *et al.*, 1998b). Because SNARE complexes containing Vam3ΔN disassemble more easily than those containing wild-type Vam3p (Figure 4), Sec18p cannot stimulate, but rather inhibits fusion (Figure 5B; Ungermann *et al.*, 1998b). It is possible that Sec18p associates more tightly with the *cis*-SNARE complex of Vam3ΔN vacuoles, an explanation that is suggested by preliminary experiments (our unpublished observations).

The stages of vacuole fusion can be kinetically separated in a time-of-addition experiment (Mayer *et al.*, 1996). We questioned whether the fast disassembly of the SNARE complex of Vam3ΔN influences any of these stages. A standard reaction was started in the presence of cytosol and ATP. At each time point, aliquots were withdrawn and incubated for the remaining time in the presence of the indicated inhibitors (Figure 6). To directly compare the inhibition kinetics of wild-type and Vam3ΔN vacuoles (as shown in the inlets), the values were normalized by setting the units of fusion obtained at 90 min to 100% (Figure 6, A and B). The priming step, as monitored by the acquisition of resistance to antibodies to Sec18p, was not altered by the removal of the amino-terminus of Vam3p (Figure 6A), even though priming was very efficient (Figure 4). However, resistance to docking inhibitors such as Gdi1p or antibodies against Vti1p was moderately delayed when wild-type and Vam3ΔN vacuoles were compared (Figure 6B). Statistical analysis of the time at which 50% of total fusion in the presence of priming and docking inhibitors was reached confirmed that docking, but not priming was indeed significantly slower (Figure 6, D and F). This suggests a role of the N-terminal domain in the coordination of docking.

It seemed unlikely that this moderate delay in docking could alone account for the drastic effect seen in the fusion reaction (Figure 3). Therefore, we compared the amount of *trans*-SNARE complexes that form during a fusion reaction in the presence of the wild-type Vam3p or the truncated protein. We deleted the v-SNARE Nyv1p in both strains, isolated the vacuoles, and fused them with vacuoles derived from a strain that lacks Vam3p (DKY6281 *vam3Δ*). *Trans*-complexes between Nyv1p and Vam3p only form if vacuoles dock in an ATP-dependent manner (Ungermann *et al.*, 1998b), as analyzed by coimmunoprecipitation of Nyv1p with an antibody against Vam3p. Nyv1p interacts with Vam3p only after priming with ATP (Figure 7A). Complex

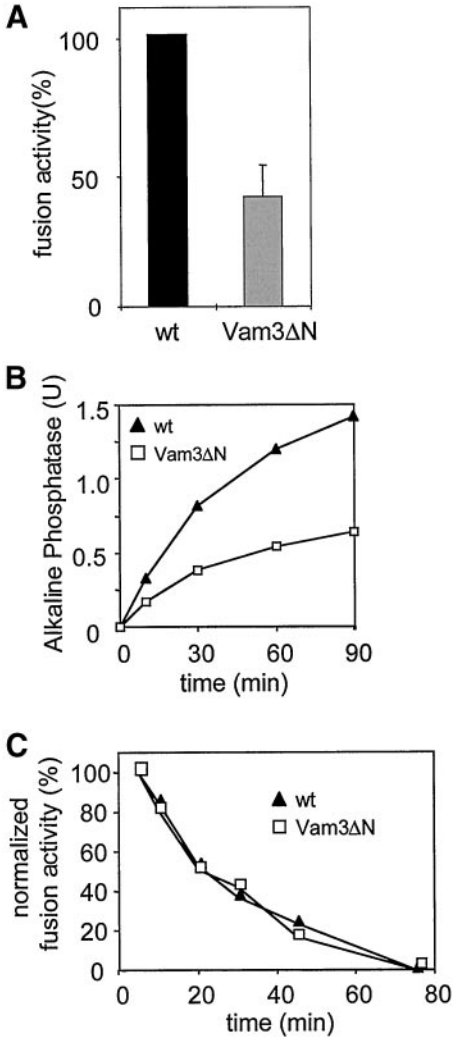

Figure 3. Decreased fusion of Vam3ΔN vacuoles. (A) Fusion activity of Vam3ΔN- compared with wild-type-vacuoles. Standard fusion reactions with cytosol and ATP were incubated for 90 min at 26°C, and alkaline phosphatase activity was determined as described in MATERIALS AND METHODS. To allow comparison of independent experiments, wild-type vacuole fusion was set to 100%. Fusion of Vam3ΔN vacuoles was reduced to 40 ± 11% (mean ± SEM, n = 14). (B) Time course of the fusion reaction. Standard fusion reactions (150 μl) containing wild-type or Vam3ΔN tester vacuoles were started with cytosol and ATP. At the indicated time points a 30-μl aliquot was removed and placed on ice to stop the reaction. Fusion activity was measured after 90 min. A representative experiment is shown. (C) Loss of fusion competence over time. Wild-type or mutant BJ3505 and DKY6281 vacuoles were separately incubated with cytosol and ATP at 26°C. At the indicated time points 20 μl of BJ3505 and DKY6281 vacuoles were mixed and incubated for an additional 70 min. Then fusion activity was assayed.

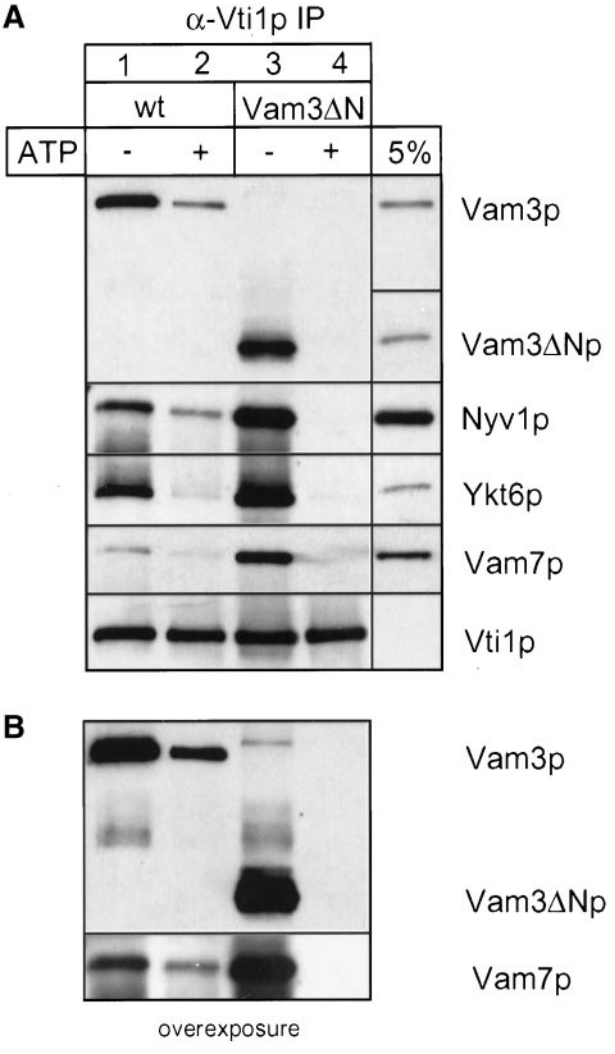

Figure 4. Vam3ΔN assembles into *cis*-SNARE complexes. Wild-type or mutant vacuoles (60 μg each) were incubated in reaction buffer with cytosol, with or without ATP at 26°C. After 10 min the vacuoles were collected by centrifugation. Solubilization and immunoprecipitation was performed as described in MATERIALS AND METHODS. Immunoprecipitation was done with a polyclonal anti-Vti1p antiserum covalently coupled to Protein A–Sepharose beads (Ungermann *et al.*, 1999). Proteins were eluted with 0.1 M glycine, pH 2.6, precipitated with TCA, separated on 15% SDS-polyacrylamide gels, and detected with the indicated antibodies (A). A fraction of the wild-type detergent extract (5%; for Vam3p, Vam7p, Vti1p, and Ykt6p) as well as a fraction of the mutant extract (Vam3ΔN) was precipitated and loaded as a controls. The amount of coprecipitated SNAREs was quantified densitometrically (NIH image 1.6): wild-type − ATP, 100 ± 16; wild-type + ATP, 46 ± 10; Vam3ΔN − ATP, 225 ± 14; Vam3ΔN + ATP, 24 ± 4 (mean density after background subtraction in arbitrary units ± SEM, n = 4). (B) An overexposure of the immunoblots decorated with anti-Vam3p and anti-Vam7p.

formation as well as fusion activity is strongly diminished if the truncated Vam3p is present on the tester vacuoles (Figure 7), showing that a decrease in fusion directly correlates with reduced *trans*-SNARE pairing. We consider the slight docking delay (Figure 6) unlikely to be responsible for this strong effect. Taken together, the data suggest that even though a high percentage of SNAREs is available for the reaction after priming (Figure 4), only a few enter the right pathway.

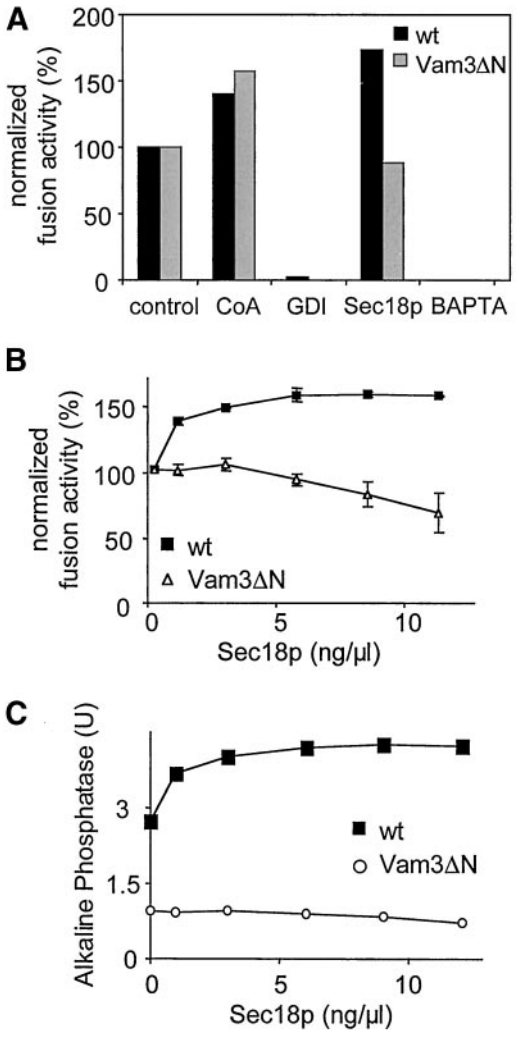

Figure 5. Effect of fusion inhibitors and activators. (A) Standard fusion reactions (30 μl) containing cytosol and ATP were incubated at 26°C for 90 min with or without the indicated inhibitors (64 ng/μl Gdi1p [GDI]; 10 mM BAPTA) or activators (10 μM CoA; 5 ng/μl Sec18p). To compare the results obtained for wild-type (black bars) and Vam3ΔN (gray bars) vacuoles, the fusion measured for control conditions of both strains was set to 100%. (B and C) Effect of Sec18p on the fusion reaction. Purified recombinant His_6-Sec18p (Haas and Wickner, 1996) was added at the indicated concentrations to 30 μl fusion reactions to stimulate priming. The reaction mixture was incubated at 26°C for 90 min and then fusion was measured. (A and C) Representative experiments; (B) average values of three independent experiments ± SEM expressed as percentage.

This lack of coordination between priming and docking suggested that the N-terminus of Vam3p might be necessary to recruit tethering factors to the *cis*-SNARE complex. We previously reported that the HOPS complex is associated with the SNARE complex on isolated vacuoles (Price *et al.*, 2000a). HOPS is released from the *cis*-SNARE complex upon priming and interacts with Ypt7p to initiate vacuole tethering (Price *et al.*, 2000a). We therefore asked whether SNARE complexes containing Vam3ΔN are able to bind HOPS. Vps33p, one component of the HOPS complex and a Sec1 homologue, was C-terminally tagged with Protein A in both strains. We then purified the tagged Vps33p via IgG Sepharose from vacuolar detergent extracts and probed for bound Vam3p by Western blotting. Although full-length Vam3p copurifies well with Vps33p, little Vam3ΔN was bound to Vps33 (Figure 8A). Only after overexposure did a small fraction of Vam3ΔN become visible (Figure 8B). The integrity of HOPS was maintained during the purification, because Vps11p, another member of the HOPS complex (Sato *et al.*, 2000; Seals *et al.*, 2000), was in a complex with Vps33p in both strains (Figure 8C). Thus, the N-terminal domain of Vam3p is essential to efficiently recruit HOPS/Vps33p to the *cis*-SNARE complex.

The reduced recruitment of HOPS to the *cis*-SNARE complex could explain the uncoordinated transition from priming to tethering. We asked if we could support this transition and promote fusion of Vam3ΔN vacuoles by enhancing physical contact between the vacuoles. We therefore preclustered wild-type and Vam3ΔN vacuoles by centrifugation, briefly resuspended them in reaction buffer containing ATP and cytosol, and incubated them for 90 min. Strikingly, this precentrifugation step only slightly stimulated fusion of wild-type vacuoles, but supported Vam3ΔN vacuoles such that both vacuole types showed virtually the same fusion activity (Figure 9A). Fusion was authentic, because it was dependent on ATP and sensitive to antibodies against Vam3p (Figure 9A). We expected that this rescue could only function early in the reaction, because it was initiated by vacuole priming. This is indeed the case. Figure 9B shows the ratio of fusion between Vam3ΔN and wild-type vacuoles. Centrifugation can rescue the mutant only when performed at early time points in the reaction (Figure 9B). This suggests that increased physical contact can overcome the docking defect of Vam3ΔN vacuoles. We also tested whether we could rescue the *trans*-SNARE pairing by preclustering the vacuoles. However, mutant vacuoles with wild-type Vam3p as well as with Vam3ΔN were too sensitive and did not show any activity after a precentrifugation step (our unpublished observations).

In sum, the N-terminal domain of Vam3p is preserving the physical interaction of the SNAREs with the HOPS complex within the *cis*-SNARE complex, thereby ensuring a coordinated transition from priming to tethering.

DISCUSSION

The role of the amino terminus of t-SNAREs has been analyzed before with purified proteins (Fernandez *et al.*, 1998; Parlati *et al.*, 1999; Misura *et al.*, 2000; Munson *et al.*, 2000). With the use of vacuole fusion as a model system, we were able to show that the N-terminus of Vam3p acts as a central domain by 1) recruiting the HOPS complex to the *cis*-SNARE complex and 2) by allowing coordinated docking. In the absence of this domain only a fraction of the vacuoles enters the right pathway, as shown by the reduced *trans*-SNARE pairing and fusion. This suggests that the N-terminus is needed for the processes and interactions that coordinate docking before membrane fusion.

The deletion of the N-terminal region of Vam3p obviously alters the composition and function of the SNARE complex, whereas transport to the vacuole and the basic interaction

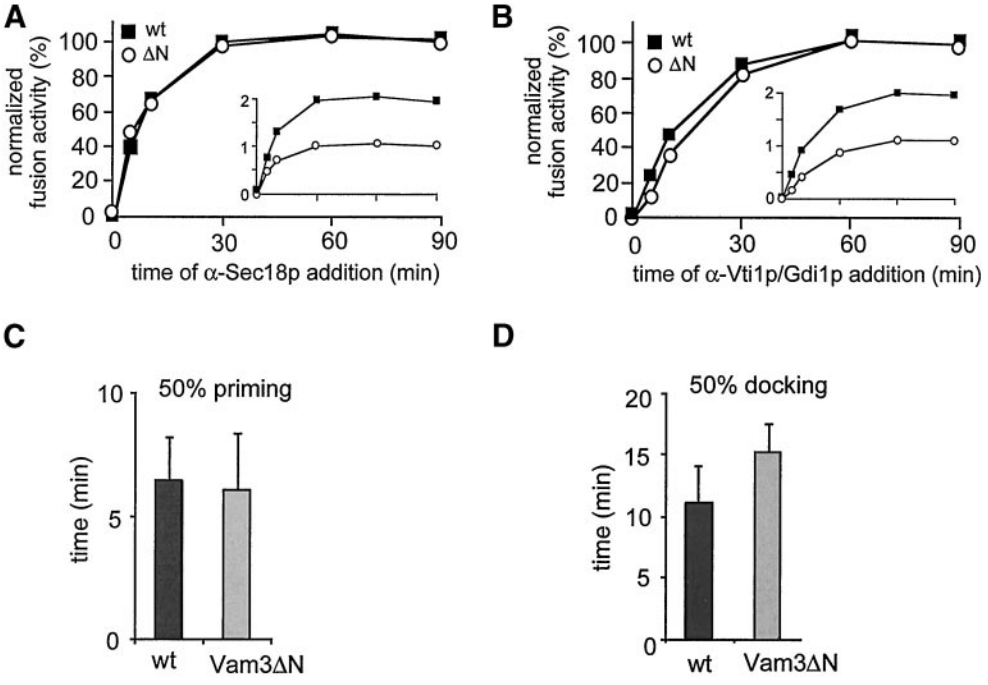

Figure 6. Vam3ΔN vacuoles show a delay in docking. Standard fusion reactions (200 μl), containing wild-type or Vam3ΔN vacuoles, were incubated in the presence of cytosol and ATP; at the indicated time points a 30-μl aliquot was mixed with an inhibitor and incubation at 26°C was continued for a total of 90 min. Concentration of antibodies to Sec18p and Vti1p IgGs was 0.1 μg/μl, Gdi1p was used at 64 μg/ml. (A) Sensitivity to α-Sec18p of wild-type and Vam3ΔN vacuoles. Priming kinetics obtained were normalized by setting fusion at 90 min to 100%. An inlet shows the alkaline phosphatase units before normalization. Average values of nine independent experiments are shown. (B) Sensitivity to α-Vti1p or Gdi1p of wild-type and Vam3ΔN vacuoles. Average values of 20 independent experiments are shown. (C) Time point, where fusion inhibition by α-Sec18 is 50%. Wild-type 6.5 ± 1.7 min (SEM), n = 9; Vam3ΔN 6.2 ± 2.2 min (SEM), n = 9. Difference not significant (Student's *t* test = 0.4). (D) Time point, where fusion inhibition by α-Vti1 or Gdi1p is 50%. Wild-type 11.5 ± 2.9 min (SEM), n = 20; Vam3ΔN 15.3 ± 2.4 min (SEM), n = 20. Difference is highly significant (Student's *t* test < 0.00001).

with the other SNAREs on the vacuole (Vti1p, Ykt6p, Vam7p, and Nyv1p) was unaffected. *Cis*-SNARE complexes precipitated from mutant strains contained substantially more SNAREs than those isolated from wild-type vacuoles (Figure 4). Removal of the N-terminus may reduce steric hindrance and could allow for a better assembly of the SNARE complex. In reverse, the lack of this domain may permit a more efficient access of Sec18/17p, which is suggested by preliminary experiments (our unpublished observations). Both explanations would be consistent with our finding that complexes containing Vam3ΔN are more abundant and disassemble more efficiently. In agreement with this, stimulation of SNARE disassembly by addition of Sec18p has no stimulatory effect on the fusion of Vam3ΔN vacuoles, but boosts the fusion of wild-type vacuoles (see Figure 5). Even though more SNARE complexes are found in *cis* and are primed, fusion is reduced, indicating a lack in coordination of the reaction.

A recent study addressing the role of the N-terminus of Vam3p reported no alteration of SNARE complexes on Vam3ΔN vacuoles (Wang *et al.*, 2001). However, it is possible that this can only be seen if the complex is precipitated with antibodies to a well-accessible vacuolar SNARE, because the SNARE complex becomes unstable during purification (unpublished observations). Nevertheless, our observation correlates with in vitro studies of isolated exocytotic SNARE complexes. Here, deleting the N-terminal region of either syntaxin or yeast Sso1 allowed faster or more efficient assembly of SNARE complexes (Nicholson *et al.*, 1998; Parlati *et al.*, 1999; Munson *et al.*, 2000).

Vacuoles with Vam3ΔN recruit less HOPS to the *cis*-SNARE complex (Figure 8), even though the amount of Vps33p on the vacuole, which is one of the subunits of the complex, is not altered (our unpublished observations). HOPS is required for the initial contact of vacuoles, termed tethering, where it associates with Ypt7p upon release from the SNARE complex (Price *et al.*, 2000a, 2000b; Sato *et al.*, 2000; Seals *et al.*, 2000). Emr and colleagues suggested that HOPS exclusively interacts with the disassembled Vam3p after priming (Sato *et al.*, 2000) and serves as a chaperone for *trans*-SNARE pairing. This is possible and supported by our observations that lack of the N-terminus of Vam3p results in less *trans*-SNARE pairs and slightly delayed docking (Figures 6D and 7A). We think, however, that the main interaction between Vam3p—and here in particular the N-terminus—and HOPS is required in the context of the *cis*-SNARE complex (Figure 8A). This may occur during the reformation of the *cis*-SNARE complex. From recent experiments it appears that *cis*-SNARE complexes are not necessarily a result of a previous fusion event. Vacuoles containing Vam3p with mutations in the coiled-coil domain still have *cis*-SNARE complexes, but do not show fusion (Wang *et al.*, 2001). Vam3p may be involved in the recruitment of HOPS while it is not yet complexed with other SNAREs, which would explain the contradictory results. Furthermore, it was reported that Vps33p, added as a lysate from a Vps33p overproducing yeast strain, was binding equally efficient to recombinant Vam3p with or without the N-terminus, although the coiled-coil domain was essential for this interaction (Dulubova *et al.*, 2001). It is difficult to compare this observation with ours, because the interaction of HOPS and Vam3p most likely does not reflect the recruitment of HOPS to the *cis*-SNARE complex. The interaction of Vam3p with HOPS requires Vps18 (Sato *et al.*, 2000). It is possible that

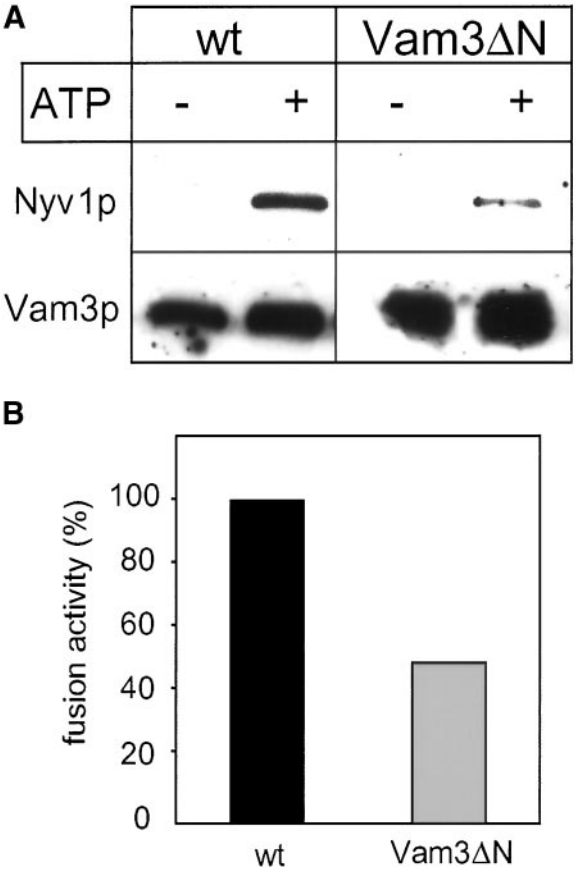

Figure 7. Vam3ΔN vacuoles form less *trans*-SNARE complexes. (A) Coimmunoprecipitation of Nyv1p with anti-Vam3p. Vacuoles from BJ3505 *nyv1*Δ or BJ3505 *nyv1*Δ *VAM3*Δ*N* were fused with DKY6281 *vam3*Δ as described in MATERIALS AND METHODS. After the incubation vacuoles were collected by centrifugation, solubilized and immunoprecipitated with α-Vam3p. Eluted proteins were separated by SDS-PAGE and detected by Western blotting with antibodies to Nyv1p and Vam3p. The amount of coprecipitated SNAREs was quantified densitometrically (NIH image 1.6): wild-type, 100 ± 10; Vam3ΔN, 34 ± 16 (mean density after background subtraction in arbitrary units ± SEM, n = 3). (B) A 30 μl aliquot was removed from the identical fusion reaction described in (A) and incubated for 90 min at 26°C to measure fusion activity. Fusion of BJ3505 *nyv1*Δ with DKY6281 *vam3*Δ (black bar) was set to 100%.

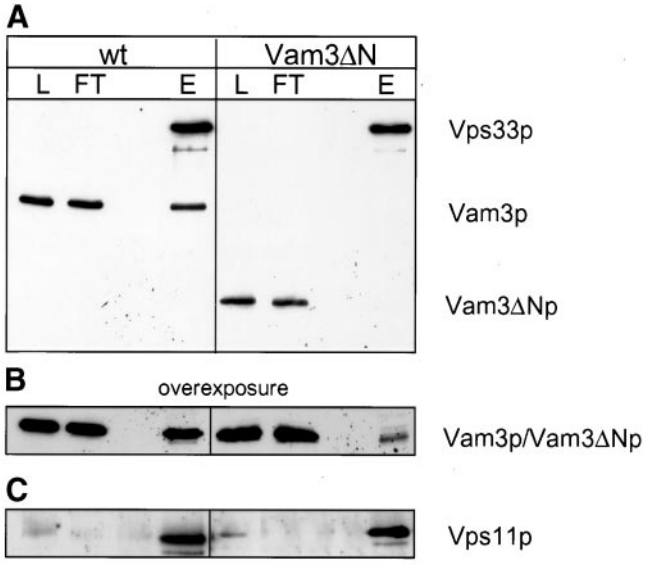

Figure 8. Efficient recruitment of HOPS/Vps33p to Vam3p depends on the N-terminal domain. Vacuoles (300 μg) prepared from BJ *VPS33:: ProtA* or BJ *VAM3*Δ*N VPS33:: ProtA* strains, were solubilized, and Protein A–tagged Vps33p was purified as described in MATERIALS AND METHODS. Fractions of the Protein-A-purification were precipitated with TCA, separated on a 15% SDS gel and blotted onto nitrocellulose. The Western blot probed with antibodies against Vam3p (A and B) or Vps11 (C). L = 1% of total protein loaded on the column; FT = 1% of the flow-through; E = 30% of the eluate. The left panel shows the isolation from vacuoles with full length Vam3p and the right panel the isolation from Vam3ΔN vacuoles (The high-molecular-weight band is Vps33p, which was detected by the secondary antibody because of the Protein A–tag).

only the monomeric Vps33p was binding to the recombinant Vam3p because it was overexpressed in yeast and may not have been part of the HOPS complex. It will therefore be important to analyze the assembly of the *cis*-SNARE complex in greater detail to evaluate these discrepancies.

Our data suggest that the Vam3ΔN *cis*-SNARE complex assembles and disassembles more efficiently, but because the interaction with HOPS is strongly reduced, only a fraction of primed SNAREs is able to pair in a *trans*-configuration, leading to fusion. Presumably the remaining SNAREs eventually assemble back in *cis*. Preclustering overcomes the necessity to physically tether the vacuoles and may allow the mutant SNAREs to bypass the coordinated HOPS requirement, although a function of HOPS during the fusion of Vam3ΔN is very likely.

The N-terminus of syntaxins/t-SNAREs has attracted much interest, mainly because of the interaction with Sec1p and its ability to fold back onto the coiled-coil domain (Kee *et al.*, 1995; Fernandez *et al.*, 1998; Nicholson *et al.*, 1998; Munson *et al.*, 2000; Misura *et al.*, 2000; Yang *et al.*, 2000). Recently, it was reported that the requirement for a cofactor of Munc18/nSec1p, Munc13, can be bypassed if a mutant syntaxin with a constitutively open conformation is expressed in *Caenorhabditis elegans* (Richmond *et al.*, 2001). Such a bypass has not been reported for Munc18 itself; in fact, neurons from *munc18* null mice do not show any synaptic exocytosis (Verhage *et al.*, 2000), suggesting that Sec1 proteins are essential in exocytosis. Even though there is a basic interaction of t-SNAREs with Sec1 proteins, there is also a lot of variability of this theme, as shown by a few examples: Novick and coworkers found that the yeast exocytic t-SNARE Sso1p only interacts with Sec1p when assembled into SNARE complexes (Carr *et al.*, 1999), whereas syntaxin binds the neuronal Sec1p in a stoichimetric 1:1 complex (Hata *et al.*, 1993; Misura *et al.*, 2000). Bryant and James (2001) recently showed that the yeast t-SNARE of the late Golgi, Tlg2p, interacts specifically with the Sec1 homolog Vps45p via its N-terminus, although it is not clear if this occurs in the context of the SNARE complex. Here, Vps45p function is essential to stabilize Tlg2p. In contrast to

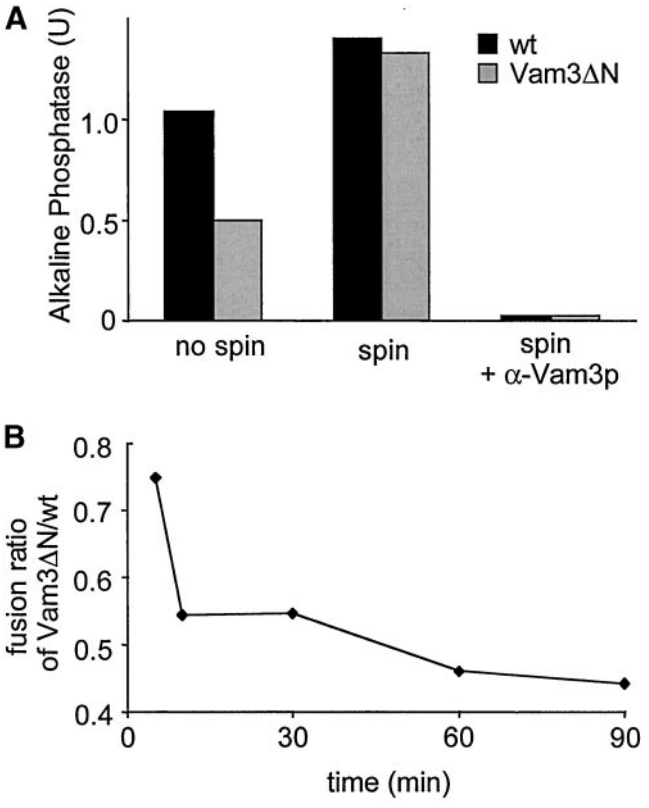

Figure 9. Preclustering rescues the reduced fusion of Vam3ΔN vacuoles. (A) Vacuoles prepared from wild-type or Vam3ΔN tester strains were incubated in a standard fusion reaction with cytosol and ATP at 26°C. Alternatively, tester vacuoles were mixed and centrifuged for 4 min at 9000 × *g* at 4°C, the supernatant was removed and the vacuoles were briefly resuspended in reaction buffer containing cytosol and ATP and incubated for 90 min at 26°C. Where indicated, IgGs to Vam3p (0.1 $\mu g/\mu l$) were added to the reaction. Fusion activity of wild-type vacuoles was set to 100%. (B) Time course of preclustering by centrifugation. Standard fusion reactions with wild-type or Vam3ΔN vacuoles where started by incubation at 26°C, at the indicated time points an aliquot was withdrawn, centrifuged for 4 min, 9000 × *g*, 4°C and resuspended, incubation at 26°C was continued to a total of 90 min. To illustrate the time-dependent effect of centrifugation, the ratio of fusion signals obtained for Vam3ΔN versus wild-type vacuoles is shown.

the wild-type Tlg2p, the truncated, but nonfunctional Tlg2p forms SNARE complexes even in the absence of Vps45p, suggesting that the Sec1 protein stabilizes the t-SNARE by binding to its N-terminus (Bryant and James, 2001). The Sec1p-homologue required for vacuole fusion, Vps33p, is part of the HOPS complex, which interacts with the *cis*-SNARE complex implying that it also binds to assembled SNAREs and not to uncomplexed Vam3p (Price *et al.*, 2000a). Deletion of the N-terminal domain leads to a drastic decrease in binding to the HOPS complex in vivo (Figure 8), indicating that this domain is involved in the interaction.

The variety of the folding of the N-termini of t-SNAREs and the context in which Sec1-like proteins interact with them makes it challenging to derive common principles. All seem to interact with t-SNAREs either in a complex with other SNAREs such as Sec1p or Vps33p or in a dimeric complex such as Munc18 (Hata *et al.*, 1993). This interaction occurs in part via the N-terminus, demonstrated by functional assays or direct binding studies (this study; Kee *et al.*, 1995; Bryant and James, 2001). Furthermore, Sec1 proteins are observed in tethering complexes (Burd *et al.*, 1997; Tall *et al.*, 1999; Price *et al.*, 2000a; Sato *et al.*, 2000; Wurmser *et al.*, 2000). They may function as a chaperone for the t-SNARE as suggested by Bryant and James (2001), and they might act as late as the final fusion step as described by Grote *et al.* (2000). Although much of this remains to be resolved, our results allow a clear assignment of the N-terminus of Vam3p through the analysis of an authentic fusion reaction. Our data suggest that although the C-terminal H3 domain of Vam3p is mainly required for direct "SNARE" interactions, the N-terminal domain is needed for interacting with SNARE effectors and regulators before and during the tethering and docking steps.

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