Homotypic vacuole fusion occurs in ordered stages of priming, docking, and fusion. Priming, which prepares vacuoles for productive association, requires Sec17p (the yeast homolog of α-SNAP), Sec18p (the yeast NSF, an ATP-driven chaperone), and ATP. Sec17p is initially an integral part of the cis-SNARE complex together with vacuolar SNARE proteins and Sec18p (NSF). Previous studies have shown that Sec17p is rapidly released from the vacuole membrane during priming as the cis-SNARE complex is disassembled, but the order and causal relationship of these subreactions has not been known. We now report that the addition of excess recombinant his6-Sec17p to primed vacuoles can block subsequent docking. This inhibition is reversible by Sec18p, but the reaction cannot proceed to the tethering and trans-SNARE pairing steps of docking while the Sec17p block is in place. Once docking has occurred, excess Sec17p does not inhibit membrane fusion per se. Incubation of cells with thermostressive Sec17-1p at nonpermissive temperature causes SNARE complex disassembly. These data suggest that Sec17p can stabilize vacuolar cis-SNARE complexes and that the release of Sec17p by Sec18p and ATP allows disassembly of this complex and activates its components for docking.

Intracellular membrane traffic is a highly conserved process. Different membranes bear related SNAREs,1 homologous integral membrane proteins that can associate through their coiled-coil helices (1). Associated SNAREs can be disassembled (2) by the ATP-driven chaperone Sec18p (NSF) and its co-chaperone, Sec17p (α-SNAP). GTP binding proteins of the Rab/Ypt family are also crucial for trafficking (3). Other regulatory factors, such as Rab effector protein complexes (4) and Sec1/Munc18 regulators of SNARE association (5), have important roles. The functional relationships between these factors in regulating membrane fusion are being studied in organisms from yeast to humans and in each trafficking stage of the endocytic and exocytic pathways.

We have studied the homotypic fusion of yeast vacuoles. This reaction occurs in obligately ordered steps of priming, docking, and fusion. Priming occurs on separate vacuoles and prepares them for docking (6). The purified vacuoles bear a cis-SNARE complex (7–9), which contains a (target)-SNARE (Vam3p),(vesicle)-SNAREs (Nyv1p, Vti1p, and Ykt6p), a homolog of the neuronal SNAP-23/25 (Vam7p), a Ypt/Rab effector complex (Vam2/6p), Sec18p, a small co-chaperone (LMA1), and Sec17p. During priming, driven by Sec18p hydrolysis of ATP, this cis-SNARE complex is disassembled (10), the Sec17p is released from the vacuole (6), the Vam3p is activated (10), and LMA1 is transferred from Sec18p to Vam3p to stabilize its active state (11). The order and causal relationships between these subreactions of priming have not been known. Priming is required for productive vacuole association, termed docking (12). Docking occurs in two ordered subreactions, reversible tethering and an irreversible trans-SNARE pairing (13). Tethering is initiated by the transfer of Vam2/6p, liberated from the cis-SNARE complex during priming, to Ypt7p, a vacuolar Rab-like GTP binding protein (14). Tethering leads to trans-SNARE pairing, thereby forming stably docked vacuoles (13). Docking induces a flux of calcium out of the vacuole (15), which interacts with a complex of calmodulin and protein phosphatase 1 to drive its vacuole association (16). While the target(s) of protein phosphatase 1 are not yet known, their dephosphorylation is needed for LMA1 release (11) and for fusion per se.

We now report that Sec17p can stabilize or even drive the reassembly of vacuolar cis-SNARE complexes, which are otherwise labile. These findings lead to a hypothesis that the displacement of Sec17p by the action of Sec18p may actually cause cis-SNARE complex disassembly rather than being a mere consequence of it.

**Experimental Procedures**

**Strains and Reagents—**Saccharomyces cerevisiae strain BJ5505 (MATα, pep4::HIS3, prb1Δ1Δ6, HIS3, lys2-208, trp1Δ101, ura3-52, gal2Δ can) and DKY6281 (MATα, leu2-3,112, ura3-52, his3Δ200, trp1Δ1001, lys2-801, suc2-39, pho8::TRP1) were obtained from Dr. D. Klionsky (University of California, Davis, CA). The ts mutant strain RSY387 sec17-2 and its parental wild type strain RSY725 (MATα, ura3-52, leu2-3,112) were obtained from Dr. Charles Barlowe (Dartmouth Medical School).

SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotting using ECL, affinity purification of antibodies, his-sec17p, and his-sec18p were as described by Haas (17) and Haas and Wickner (18). Quinacrine, cyclopiazonic acid, and apyrase (grade VII) were purchased from Sigma.

**In Vitro Vacuole Fusion—**Vacuoles were isolated as described previously (6, 19) and diluted as needed with PS buffer (10 mM Pipes/KOH, pH 6.8, 200 mM sorbitol). Standard vacuole fusion assays (30 μl) were as described (19). Fresh vacuoles from BJ5505 and DKY6281 (3 μg each) were mixed in fusion buffer (10 mM Pipes/KOH, pH 6.8, 200 mM sorbitol, 150 mM KAc, 5 mM MgCl2) ATP regenerating system (1 mM ATP, 40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase), proteinase inhibitors (3.3 μM Pefabloc SC, 0.1 mg/ml leupeptin, 16.6 μM o-phenanthroline, 16.6 ng/ml pepstatin; 20). Cytosol was prepared as described (17) and added to fusion reactions where indicated. One unit of fusion
activity is defined as 1 μmol of p-nitrophenyl phosphate hydrolyzed per minute per microgram of vacuole from Bj3505.

Immunoprecipitation—Vacuoles were solubilized in 500 μl of IP buffer (20 mM HEPES, pH 6.8, 50 mM KCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 30 μM Pefabloc SC, 30 ng/ml leupeptin, 15 μM o-phenanthroline, 150 ng/ml pepstatin, 1% Triton X-100) by rotating for 15 min at 4 °C. Unsolubilized material was removed by centrifugation (14,000 rpm, 10 min, 4 °C). A portion (5%) of the detergent extract was saved prior to immunoprecipitation. Protein A-agarose beads coupled with IgGs were added to the detergent extract and incubated on a Nutator rocking shaker for 2 h at 4 °C. The beads were twice re-isolated by a 30-s centrifugation at 14,000 rpm, resuspended each time with 500 μl of IP buffer and incubated for 10 min at 4 °C. Proteins were eluted from the beads by 0.1 M glycine-Cl (pH 2.2) and neutralized with 1 M Tris-Cl (pH 10). Eluates were mixed with SDS-sample buffer and heated at 95 °C for 3 min and analyzed by SDS-PAGE and immunoblotting.

RESULTS

To assay homotypic vacuole fusion, we isolate vacuoles from two yeast strains. Bj3505 is deleted for the genes encoding vacuole luminal proteases A and B and thus accumulates the catalytically inactive pro-alkaline phosphatase. Dky6281 contains normal vacuolar proteases but is deleted for the gene encoding alkaline phosphatase. Neither population of vacuoles has alkaline phosphatase activity. Upon vacuole fusion, the luminal contents mix and the pro-alkaline phosphatase is proteolytically activated. The active enzyme is assayed spectrophotometrically as a measure of membrane fusion (17, 19).

Recombinant Sec17p was added to the fusion assay. Very low levels of this protein caused a minor stimulation of fusion, but vacuole fusion was inhibited as increasing Sec17p was added (Fig. 1A). Standard fusion reactions have 6 μg of vacuoles, which bear 6 ng of Sec17p (Fig. 1B). To achieve 90% inhibition (Fig. 1A), recombinant Sec17p had to be added to approximately 15-fold greater level than the endogenous (6 ng endogenous versus 30 μl × 3 ng/μl added). To test whether this inhibition was specific, purified Sec18p was added to fusion reactions in the presence or absence of added Sec17p. High levels of Sec18p completely overcame the inhibition by excess Sec17p (Fig. 1C), whereas there was little stimulation of fusion by excess Sec18p alone. The ability of Sec18p (but not calmodulin or LMAM; data not shown) to overcome the inhibition by exogenous Sec17p suggests that the inhibitory effect of excess Sec17p is specific.

Inhibition by excess Sec17p occurs on the vacuole membrane (Fig. 1D). Vacuoles were preincubated with Sec17p and then re-isolated to remove unbound protein (lanes 4–6). Almost no fusion took place (lane 5) unless Sec18p was added (lane 6), whereas, in vacuoles preincubated with buffer, fusion was not significantly stimulated by Sec18p (lanes 2 and 3). This observation argues against a sequestration of soluble fusion components by excess Sec17p in solution but supports the idea that excess Sec17p inhibits by binding to vacuole membranes and blocking at a site that can be relieved by Sec18p.

Vacuole fusion occurs in ordered reactions of priming, docking, and bilayer fusion. Sec18p and Sec17p normally function together to disassemble the cis-SNARE complex during priming and are not required for the later subreactions of docking and fusion. The reaction requires resistance to inhibitors of priming before it becomes resistant to inhibitors of docking (6). Because priming does not require vacuole-to-vacuole contact, we asked whether the reaction becomes resistant to added Sec17p prior to vacuole mixing and found that it does not (Fig. 2A, triangles), even though it becomes resistant to antibody to Sec17p (filled squares). This suggests that the added excess Sec17p acts after the endogenous Sec17p is normally released. To determine whether Sec17p inhibited steps after priming, we measured the sensitivity of the reaction to inhibitors that were added at various times. This type of experiment tells us the latest stage that is sensitive to each inhibitor. A large fusion reaction was started and, at different times, aliquots were either transferred to ice as a measure of fusion that had occurred, were mixed with different antibodies, or were mixed
with excess Sec17p. Each incubation was then continued at 27 °C or on ice for a total of 60-min incubation (Fig. 2B). When added from the start, each inhibitor abolished fusion. After 10–15 min, the reaction was largely resistant to antibodies to Sec17p, indicating that Sec17p was no longer required after priming. The kinetic course of fusion sensitivity to excess Sec17p was coincident with that of anti-Vam3, and both were well separated from the ice curve, indicating that excess Sec17p did not inhibit bilayer fusion per se. However, under this reaction condition with a high vacuole concentration, docking had occurred quite soon after priming. To better distinguish between priming and docking, we employed the same assay but with diluted vacuoles (Fig. 2C). Docking, assayed by acquired resistance to anti-Vam3p or anti-Ypt7p, was slowed significantly while priming, measured by the early acquired resistance to anti-Sec17p and anti-Sec18p, was not affected. Resistance to added Sec17p was still acquired with the same kinetics as resistance to other inhibitors of docking. These data suggest that the latest stage of the reaction which is sensitive to excess Sec17p is docking. Because docking requires vacuole acidification (21), we measured the effect of Sec17p on the vacuole accumulation of quinacrine in response to its ATP-driven acidification (Fig. 2D) but found that there was no effect.

To confirm that the final, bilayer fusion stage of the reaction is not affected by excess Sec17p, a staging experiment was performed using the calcium chelator BAPTA (Fig. 3). BAPTA reversibly inhibits the bilayer fusion stage of the reaction without affecting priming and docking (15, 21). Vacuoles were incubated with ATP in the presence of BAPTA for 30 min to allow the completion of priming and docking, then centrifuged and resuspended to remove the BAPTA. Very little fusion had occurred by 30 min in the presence of BAPTA (Fig. 3, lane 7). Vacuoles retained significant ability to fuse (lane 6), and this fusion had become fully resistant to either anti-Vam3p (lane 10) or excess Sec17p (lane 9), though either inhibitor blocked fusion efficiently if added from the beginning of the reaction (lanes 4 and 5). Thus excess Sec17p does not inhibit the final step of bilayer fusion.

To determine whether subreactions of docking such as tethering and trans-SNARE pairing, which follow priming, can occur while the reaction is blocked by excess Sec17p, vacuoles were incubated for 15 min at 27 °C in the presence of excess Sec17p (Fig. 4, lanes 15–21). The blockade of the fusion reaction (lane 15) was largely overcome by the addition of Sec18 during second incubation (lane 16), and this rescue of the reaction by Sec18p required ATP (lane 17). Therefore, excess Sec17p did not allow the reaction to proceed beyond a stage that still

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**Fig. 2. Kinetics of sensitivity of fusion to excess Sec17p.** A, vacuole contact is required to acquire resistance to added Sec17p. Two 10× scale fusion reactions were started at 27 °C with ATP and cytosol, one with BJ505 vacuoles and the other with DKY6281 vacuoles. At indicated times, aliquots (15 µl) were removed from each reaction and combined, together with either control buffer (PS buffer) or different inhibitors. Fusion was continued at 27 °C or kept on ice for a total incubation of 90 min, and alkaline phosphatase activity was measured. Inhibitor concentrations were: his6-Sec17p, 20 ng/µl; anti-Sec17p (affinity-purified), 80 ng/µl; anti-Vam3 (IgG), 100 ng/µl. B and C, kinetics of acquiring resistance to inhibitors. A large scale standard fusion reaction was started at 27 °C with ATP and cytosol, 0.05% Triton X-100 in water. Quinacrine was assayed at OD 430.

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**Fig. 3.** Docking is not affected by excess Sec17p. A, docked vacuoles have acquired resistance to anti-Vam3p and anti-Ypt7p. Docking, assayed by acquired resistance to anti-Vam3p or anti-Ypt7p, was slowed significantly while priming, measured by the early acquired resistance to anti-Sec17p and anti-Sec18p, was not affected. Resistance to added Sec17p was still acquired with the same kinetics as resistance to other inhibitors of docking. These data suggest that the latest stage of the reaction which is sensitive to excess Sec17p is docking. Because docking requires vacuole acidification (21), we measured the effect of Sec17p on the vacuole accumulation of quinacrine in response to its ATP-driven acidification (Fig. 2D) but found that there was no effect.

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required priming. The reaction also had not fulfilled its requirements for Ypt7p (lane 18), for Vam2p (lane 20), which is a subunit of the Ypt7 effector complex (14), and for the t-SNARE Vam3p (lane 19), as judged by continued sensitivity to antibody to these proteins, though the need for these proteins would have been fulfilled by 15 min in the absence of his6-Sec17p (lanes 9–14).

To further resolve the mechanism of excess Sec17p inhibition of early reaction steps, we employed the biochemical assays of Sec17p release and cis-SNARE complex disassembly. Endogenous Sec17p underwent ATP-dependent release from the vacuoles into the supernatant (Fig. 5, lanes 1 and 2), and this release was not affected by excess Sec17p (lane 3). Because priming also entails the dissociation of cis-SNARE complexes (10), we used the co-immunoprecipitation of the v-SNARE Nyv1p by antibody to the t-SNARE Vam3p to measure the disassembly of cis-SNARE associations (Fig. 6). In contrast to the ATP-dependent disassembly under normal fusion conditions (lanes 1 and 2), cis-SNARE associations were preserved or re-formed in the presence of excess Sec17p (lane 3). These findings suggest that Sec17p normally stabilizes the cis-SNARE complex, that it must be displaced by Sec18p before the cis-SNARE complex can be disassembled, and that added exogenous Sec17p cannot prevent the release of endogenous Sec17p but can associate with the vacuole rapidly and either interrupt cis-SNARE complex disassembly or even drive reassembly.

Thermosensitive mutants in the SEC17 gene provide another means to explore the Sec17p function in cis-SNARE complex stability. Vacuoles were purified from wild-type cells or from the sec17-2 mutant, which had been grown at the permissive temperature of 23 °C. Immunoprecipitation analysis of detergent extracts of these vacuoles with antibodies to the v-SNARE Vti1p showed coprecipitation of Sec17p and Vam3p (Fig. 7, A and B, lane 1), indicating an intact SNARE complex. Preincubation of spheroplasts at 37 °C for 10 min (lane 2) or 20
SNARE complex disassembly is a necessary step of priming, the loss of integrity of this complex by thermal denaturation of the temperature-sensitive Sec17-2p did not promote the overall reaction but, rather, led to its diminution (data not shown). Thus, Sec17p both stabilizes the cis-SNARE complex and fulfills a positive function beyond the disassembly of this complex in the priming reaction.

**DISCUSSION**

SNAP proteins were discovered, and named, as soluble NSF attachment proteins (22, 23), and Sec17p was shown to be its yeast homologue (24). Further biochemical studies established that SNAP proteins bind to syntaxin, SNAP-25, or to trimeric 7S complexes of syntaxin, SNAP-25, and synaptobrevin (2, 25, 26). Association with α-SNAP causes conformational changes in these SNAP proteins (25, 27), and recent determination of the structure of Sec17p (28) has suggested models of NSF/SNAP/SNARE interactions. SNAP proteins are essential for the disassembly of the 7S SNARE oligomer mediated by NSF and ATP (2, 25, 29, 30) and stimulate the ATPase activity of NSF (31, 30). However, it has not been previously known whether the role of SNAP is limited to mediating the membrane attachment of NSF. Furthermore, the stability of the 7S complex of neural SNAREs to exposure to even heat or SDS (29, 27) has masked whether SNAP protein can also contribute directly to SNARE complex stability. α-SNAP has also been found to prevent the ATP-driven release of NSF from membranes, perhaps through mediating reattachment (32). The yeast cis-SNARE complex is labile, and our current studies suggest that Sec17p association contributes to its stability.

Our studies provide new insights into the cis-SNARE complex and the priming stage of vacuole fusion. Though purified recombinant neuronal SNAREs will spontaneously form a stable 7S complex that is resistant to SDS (33), and the associations of purified vacuolar SNAREs are far more labile. The isolable vacuolar cis-SNARE complex contains Sec17p (10, 34). We postulate that Sec17p association stabilizes the cis associations of vacuolar SNAREs and that the ATP-driven displacement of Sec17p by Sec18p causes the disassembly of the cis-SNARE complex by removing the Sec17p "glue," which had held it together. In addition, Sec17p has a positive role in making this disassembly reaction productive for the further steps that lead to fusion, possibly through activation of the t-SNARE, Sec17p can apparently associate with primed vacuoles, and may even promote re-assembly of some components of the cis-SNARE complex, but this can again be reversed by the action of Sec18p and ATP. The concentration of released Sec17p in our standard in vitro fusion reactions is far less than that needed to drive the reformation of cis-SNARE associations (Fig. 1). Priming releases endogenous Sec17p, and the ensuing tethering reaction is fully reversible (12, 13). Thus, even after priming is complete, the reversibility of tethering allows the addition of high levels of Sec17p to block the overall reaction (Fig. 1A). trans-SNARE pairing renders docking irreversible (13), and the reaction thereby becomes resistant to excess added Sec17p. Though the blockade by excess Sec17p is fully reversible by Sec18p (Figs. 1C, 1D, and 4), vacuoles blocked by excess Sec17p cannot complete even the Vam2/6p- and Ypt7p-
dependent tethering reaction until the block by Sec17p is reversed by the addition of Sec18p (Fig. 4). This model of Sec17p function is the simplest explanation for our current findings, though further studies are needed to establish whether this novel proposed function of Sec17p is physiologically important and whether it applies to other trafficking reactions. However, the physiological relevance of our findings is supported by the observation (Fig. 7) that a defective Sec17p causes lability of its cis-SNARE complex. This is in contrast to the stabilization of this complex by thermodenaturation of Sec18p (10), and the lack of effect of excess Sec18p on fusion (13) and suggests that Sec17p has a distinct role in stabilizing cis-SNARE complexes.

The cis-SNARE complex, which includes Sec17p, is not merely a residual complex of trans-SNARE associations that is converted to “cis” upon membrane fusion. Rather, it contains Sec17p, Sec18p (10), its bound LMA1 co-chaperone (11), and the multisubunit 38S Vam2/6p complex (14) as well as the SNARE proteins. Although our current study suggests that Sec17p has a major role in stabilizing this complex, it will be important to determine the physiological pathway of assembly of the cis-SNARE complex at normal levels of Sec17p and, with pure components, to recapitulate effects of Sec17p on this assembly pathway. The size of this complex, which has been estimated to be 65S (14), suggests that this will be a daunting task. Further studies will also be needed to determine whether each of the components that is released from the cis-SNARE complex during the normal fusion reaction can reassemble with the SNAREs upon excess Sec17p addition.

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