

# Trans-SNARE interactions elicit $\text{Ca}^{2+}$ efflux from the yeast vacuole lumen

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**C** $\text{a}^{2+}$  transients trigger many SNARE-dependent membrane fusion events. The homotypic fusion of yeast vacuoles occurs after a release of luminal  $\text{Ca}^{2+}$ . Here, we show that trans-SNARE interactions promote the release of  $\text{Ca}^{2+}$  from the vacuole lumen. Ypt7p-GTP, the Sec1p/Munc18-protein Vps33p, and Rho GTPases, all of which function during docking, are required for  $\text{Ca}^{2+}$  release. Inhibitors of SNARE function prevent  $\text{Ca}^{2+}$  release. Recombinant Vam7p, a soluble Q-SNARE, stimulates  $\text{Ca}^{2+}$  release.

Vacuoles lacking either of two complementary SNAREs, Vam3p or Nyv1p, fail to release  $\text{Ca}^{2+}$  upon tethering. Mixing these two vacuole populations together allows Vam3p and Nyv1p to interact in trans and rescues  $\text{Ca}^{2+}$  release. Sec17/18p promote sustained  $\text{Ca}^{2+}$  release by recycling SNAREs (and perhaps other limiting factors), but are not required at the release step itself. We conclude that trans-SNARE assembly events during docking promote  $\text{Ca}^{2+}$  release from the vacuole lumen.

## Introduction

Conserved molecules govern membrane docking and fusion in the secretory and endocytic pathways of eukaryotic cells (Jahn et al., 2003). These molecules include Rab/Ypt GTPases, Sec17/18p (NSF/ $\alpha$ -SNAP) chaperones, Sec1p/Munc18 (SM) proteins, and SNARE proteins. SNAREs fold into tight core complexes containing four coiled  $\alpha$  helices that are contributed by three or four SNARE proteins (Sutton et al., 1998; Jahn et al., 2003). SNAREs are categorized as Q or R, based on whether they contribute a glutamine or arginine to the ionic (zero) layer of the SNARE core complex (Fasshauer et al., 1998). Core complexes typically contain three Q-SNARE coils and an R-SNARE coil. Complementary SNAREs must be present in trans on docked, apposed membranes for fusion to occur (Nichols et al., 1997). Trans-SNARE complexes are hypothesized to assume a "SNAREpin" structure (Weber et al., 1998; Jahn et al., 2003) similar to the tetrahelical SNARE core complex, but with the SNARE transmembrane anchors embedded in opposite docked membranes. The exact functions of trans-SNARE complexes are unresolved (Jahn et al., 2003).

Cytosolic  $\text{Ca}^{2+}$  is required for many membrane fusion events. In some cases, ambient  $[\text{Ca}^{2+}]$  is sufficient for fusion (Beckers and Balch, 1989; Baker et al., 1990); in other cases,

transient increases in  $[\text{Ca}^{2+}]$  are needed (Sullivan et al., 1993; Peters and Mayer, 1998; Chen et al., 1999; Pryor et al., 2000; Reddy et al., 2001; Rettig and Neher, 2002). In neurons, depolarization opens voltage-gated  $\text{Ca}^{2+}$  channels, triggering exocytosis (Rettig and Neher, 2002; Jahn et al., 2003). However, little is known about how  $\text{Ca}^{2+}$  regulates intracellular fusion events such as endosome-endosome fusion. Even less is known about how  $\text{Ca}^{2+}$  signals that regulate intracellular membrane fusion are themselves regulated.  $\text{Ca}^{2+}$  channels in the plasma membranes of mammalian cells physically and functionally interact with the Q-SNAREs syntaxin and SNAP-25 (Bennett et al., 1992; Yoshida et al., 1992; Sheng et al., 1994; Mochida et al., 1996; Wiser et al., 1996; Rettig et al., 1997).  $\text{Ca}^{2+}$  channels also interact with the exocytic machinery via the Sec6/8 complex and Rab3-interacting molecule binding proteins (Shin et al., 2000; Hibino et al., 2002). On the yeast vacuole, the R-SNARE Nyv1p inhibits the vacuolar  $\text{Ca}^{2+}$  ATPase Pmc1p (Takita et al., 2001). It is unclear how such interactions are integrated into cycles of membrane docking and fusion.

Trans interactions between SNAREs on opposite membranes have been proposed to facilitate or trigger  $\text{Ca}^{2+}$  signals in response to docking (Bezprozvanny et al., 1995; Wiser et al., 1996; Schekman, 1998), but technical obstacles have precluded direct tests of this hypothesis. The yeast vacuole offers powerful genetic and biochemical tools that

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Key words: membrane; docking; fusion; Rab GTPase; SM-protein

Abbreviations used in this paper: GDI, GDP dissociation inhibitor; RDI, Rho GDI; rVam7p, recombinant Vam7p; SM, Sec1p/Munc18.

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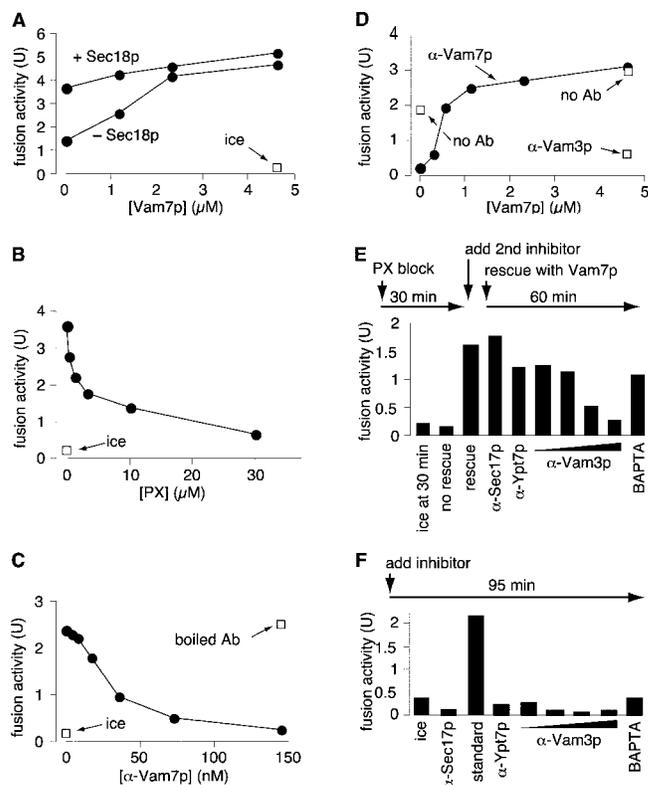
allow trans-SNARE interactions to be manipulated in a physiologically relevant *in vitro* docking and fusion reaction. Vacuole fusion begins with priming. In this step, Sec17/18p (yeast  $\alpha$ -SNAP/NSF) consumes ATP to disassemble cis-SNARE complexes (Mayer et al., 1996; Ungermann et al., 1998a). In the next stage, docking, the Rab GTPase Ypt7p promotes membrane tethering (Mayer and Wickner, 1997; Ungermann et al., 1998b), and specialized subdomains called vertex sites assemble at the docking junction (Wang et al., 2002, 2003). During Ypt7p-mediated docking,  $\text{Ca}^{2+}$  is released from the vacuole lumen (Peters and Mayer, 1998; Eitzen et al., 2000). This  $\text{Ca}^{2+}$  signal is necessary for fusion because depletion of luminal  $\text{Ca}^{2+}$  makes fusion dependent on added  $\text{Ca}^{2+}$ , and chelation of extraluminal  $\text{Ca}^{2+}$  with BAPTA prevents fusion (Peters and Mayer, 1998). We now show that  $\text{Ca}^{2+}$  release during docking occurs in response to trans-SNARE interactions.

## Results

### Vam7p stimulates fusion and reduces its BAPTA sensitivity

We were led to explore the roles of SNAREs in  $\text{Ca}^{2+}$  flux by experiments with the soluble Q-SNARE Vam7p. During priming, Sec17/18p disassembles cis-SNARE complexes. Vam7p, having neither a hydrophobic transmembrane anchor nor a covalently attached lipid anchor, is released from the membrane during priming (Ungermann and Wickner, 1998; Ungermann et al., 1998a; Boeddinghaus et al., 2002). For fusion to occur, Vam7p must reassociate with the vacuole membrane via its amino-terminal PX domain, which binds to phosphatidylinositol-3-phosphate (Cheever et al., 2001; Boeddinghaus et al., 2002). We found that recombinant Vam7p (rVam7p) promoted fusion *in vitro* (Fig. 1 A). This stimulation was more pronounced when recombinant Sec18p was not added to the reaction (Fig. 1 A), probably because added Sec18p promotes the release of endogenous Vam7p from SNARE complexes on the vacuole. Recombinant PX domain prevented Vam7p reassociation (Boeddinghaus et al., 2002) and hence fusion (Fig. 1 B). Anti-Vam7p antibody (Fig. 1 C) also prevented fusion (Ungermann and Wickner, 1998; Boeddinghaus et al., 2002). rVam7p reversed the PX and anti-Vam7p fusion blocks when added either at the beginning of the reaction (Fig. 1 D; PX, not depicted) or 30 min into the reaction, after priming and during docking (Fig. 1 E; anti-Vam7p, not depicted). rVam7p did not rescue fusion in reactions inhibited by antibodies against the SNARE Vam3p (Fig. 1, D and E), verifying that rVam7p specifically reverses blockades of Vam7p function.

Next, we asked whether various inhibitors of fusion (Fig. 1 F) acted before, during, or after the step(s) prevented by PX domain (Fig. 1 E). Fusion reactions were initiated in the presence of PX domain and incubated for 30 min to allow priming and docking, then the PX block was reversed with rVam7p. During the PX blockade, the reaction passed through the priming stage, as shown by the acquisition of resistance to anti-Sec17p antibody (Fig. 1 E). Anti-Vam3p antibody, which in kinetic experiments inhibits at docking (Nichols et al., 1997), inhibited the reaction at high but not low concentrations (Fig. 1 E). This may indicate that Vam3p



**Figure 1. Characterization of rVam7p and Vam7p antagonists.**

(A) Stimulation of fusion by rVam7p. Standard fusion reactions (see Materials and methods) were initiated in the presence of rVam7p at the indicated concentrations, with or without 10 nM of Sec18p. "Ice" indicates fusion signal in a control reaction incubated on ice instead of at 27°C. (B) Inhibition of fusion by rVam7p PX domain. (C) Inhibition of fusion by affinity-purified anti-Vam7p antibody. "Boiled Ab" indicates a sample containing anti-Vam7p heat denatured by incubation for 5 min at 100°C. (D) rVam7p reverses a fusion block imposed by 150 nM of affinity-purified anti-Vam7p antibody. "No Ab" indicates control samples without antibody. (E and F) Reversal of a PX block by rVam7p and characterization of the block. In E, reactions were initiated in the presence of 20  $\mu\text{M}$  of PX domain and incubated for 30 min at 27°C, and the indicated inhibitors were added. 4 min later, 10  $\mu\text{M}$  of rVam7p was added to reverse the PX block; the reactions were incubated at 27°C for an additional 60 min. Reactions moved to ice before rescue (ice), or not rescued by rVam7p (no rescue), were strongly inhibited by PX domain. In F, the indicated inhibitors were added from the beginning of the fusion reaction. Affinity-purified anti-Vam3p antibody was added at 58, 115, 230, or 460 nM.

is in an altered conformational or oligomeric state at the end of the PX block. Strikingly, partial resistance to the  $\text{Ca}^{2+}$  chelator BAPTA was also observed (Fig. 1 E). This BAPTA resistance was surprising for two reasons. First, although BAPTA inhibits a late stage in homotypic vacuole fusion, Vam7p was previously assigned to the intermediate docking stage in kinetic assays (Ungermann and Wickner, 1998; Boeddinghaus et al., 2002). Second, staging experiments had shown that the vacuole fusion reaction reaches a PX, or anti-Vam7p, resistant state in the presence of BAPTA, suggesting that Vam7p function is completed before  $\text{Ca}^{2+}$  action, not after (Boeddinghaus et al., 2002). Two possible explanations for these apparently divergent results are that the added rVam7p increases the number or sensitivity of  $\text{Ca}^{2+}$  sensors, or that it in-

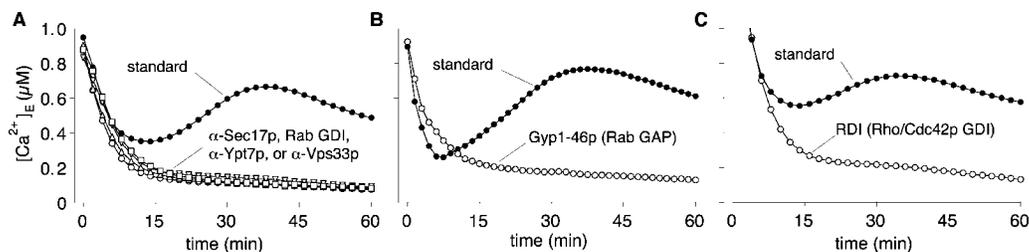


Figure 2. **Docking-dependent Ca<sup>2+</sup> release depends on Ypt7p-GTP, Rho GTPases, and the SM-protein Vps33p.** Standard Ca<sup>2+</sup> release reactions (see Materials and methods) were initiated in the absence of inhibitors ("standard"; closed circles in each plot) or in the presence of the indicated reagents: (A) 380 nM of anti-Sec17p IgG, 2.5  $\mu$ M of GDI, or 100 nM of affinity-purified anti-Vps33p; (B) 50  $\mu$ M of Gyp1-46p; (C) 15  $\mu$ M of RDI. (A–C) Closed circles denote standard reaction conditions. In A, open squares denote anti-Sec17p; open triangles denote Rab GDI; open inverted triangles denote anti-Ypt7p; and open circles denote anti-Vps33p. In B, open circles denote Gyp1-46p treatment. In C, open circles denote Rho GDI (RDI) treatment.

increases the frequency or amplitude of Ca<sup>2+</sup> efflux events, resulting in reduced BAPTA sensitivity.

### Docking factors and SNAREs promote Ca<sup>2+</sup> release

To see if Vam7p influences Ca<sup>2+</sup> efflux from the vacuole lumen, we used the photoprotein aequorin to monitor the extralumenal Ca<sup>2+</sup> concentration [Ca<sup>2+</sup>]<sub>E</sub> during in vitro docking and fusion (Peters and Mayer, 1998). We began by evaluating the importance of proteins that function during docking.

Vacuoles actively sequester extralumenal Ca<sup>2+</sup> during the first minutes of incubation with ATP (Fig. 2 A, standard). Experiments with the slow Ca<sup>2+</sup> chelator EGTA indicate that the relatively high initial [Ca<sup>2+</sup>]<sub>E</sub> is due to ambient Ca<sup>2+</sup> present in buffer solutions, and is not necessary for docking or fusion (Peters and Mayer, 1998; unpublished data; Margolis, N., personal communication). During docking, Ca<sup>2+</sup> is released from the vacuole lumen into the extralumenal space, with [Ca<sup>2+</sup>]<sub>E</sub> typically peaking at 30–40 min (Fig. 2 A). As reported previously (Peters and Mayer, 1998), this Ca<sup>2+</sup> release requires both Sec17/18p-mediated priming and Ypt7p-mediated docking. Ca<sup>2+</sup> was sequestered in the vacuole lumen but not released in the presence of anti-Sec17p antibody (Fig. 1 A). Recombinant Gdi1p (GDI; GDP dissociation inhibitor [GDI]) or affinity-purified anti-Ypt7p antibody, both inhibitors of Ypt7p function, also prevented Ca<sup>2+</sup> release (Fig. 2 A).

To determine whether the GTP-bound form of Ypt7p is needed for Ca<sup>2+</sup> release, we used Gyp1-46p and Gyp7-47p, GTPase activating proteins (GAPs) that promote the hydrolysis of Ypt7p-bound GTP (Vollmer et al., 1999; Eitzen et al., 2000; Wang et al., 2003). These GAPs prevent SNAREs and other fusion factors from accumulating at "vertex" membrane subdomains during docking and prevent fusion (Eitzen et al., 2000; Wang et al., 2003). Both Gyp1-46p (Fig. 2 B) and Gyp7-47p (not depicted) prevented Ca<sup>2+</sup> release, indicating a requirement for Ypt7p-GTP. The Rho GTPases Cdc42p and Rho1p also function during docking (Eitzen et al., 2001; Muller et al., 2001), possibly by promoting actin remodeling on the vacuole membrane (Eitzen et al., 2002). Rho GDI (RDI) extracts Rho GTPases from membranes and prevents fusion (Eitzen et al., 2001). RDI prevented Ca<sup>2+</sup> release during docking (Fig. 2 C). The SM protein Vps33p, a component of the HOPS-class C docking complex (Seals et al., 2000), functions during docking and regulates SNARE complex formation (Price et al., 2000; Sato et al., 2000). Anti-Vps33p antibody prevented Ca<sup>2+</sup> efflux (Fig. 2 A). Thus docking-dependent Ca<sup>2+</sup> release from the vacuole lumen requires Ypt7p-GTP, vacuolar Rho GTPases, and the SM protein Vps33p.

When rVam7p was added to a standard fusion assay, it caused an increase in docking-dependent Ca<sup>2+</sup> efflux (Fig. 3 A). Ca<sup>2+</sup> release was blocked by affinity-purified anti-Vam7p antibody or PX domain (Fig. 3, A and B). rVam7p

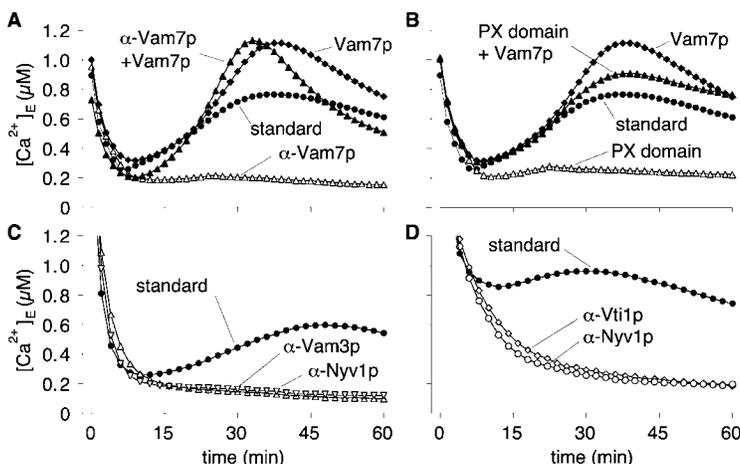
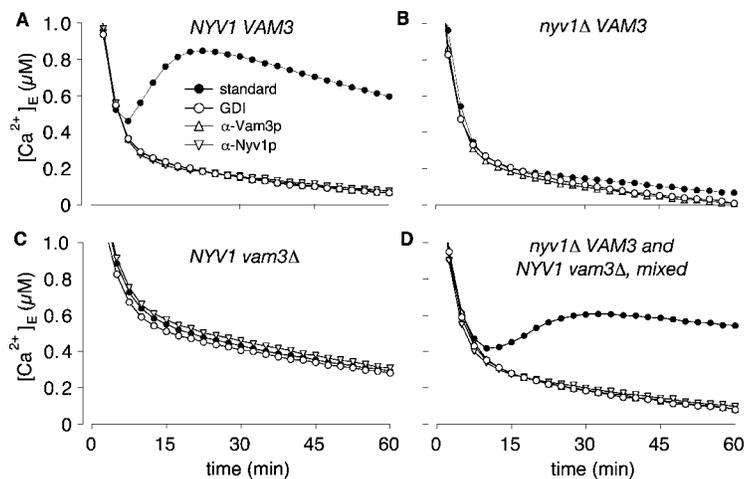


Figure 3. **SNAREs promote docking-dependent Ca<sup>2+</sup> release.** Standard reactions contained: (A) 150 nM of affinity-purified anti-Vam7p and/or 10  $\mu$ M of Vam7p; (B) 20  $\mu$ M of PX domain and/or 10  $\mu$ M of Vam7p; (C) 120 nM of affinity-purified anti-Nyv1p or 200 nM of affinity-purified anti-Vam3p. (D) 1.6  $\mu$ M of affinity-purified anti-Vti1p or 120 nM of affinity-purified anti-Nyv1p.

**Figure 4. Trans-SNARE interactions elicit docking-dependent  $\text{Ca}^{2+}$  release.** The data plotted in A–D are from the same assay. Standard reactions contained: (A) 10  $\mu\text{g}$  (protein content) of vacuoles derived from the parental strain BJ3505; (B) 10  $\mu\text{g}$  of vacuoles from an *nyv1* $\Delta$  derivative of BJ3505; (C) 10  $\mu\text{g}$  of vacuoles from a *vam3* $\Delta$  derivative of BJ3505; (D) a mixture of 5  $\mu\text{g}$  each of vacuoles from the *vam3* $\Delta$  and *nyv1* $\Delta$  strains. Symbols: closed circles, standard reaction conditions; open circles, reactions with recombinant GDI; triangles, reactions with anti-Vam3p antibody; inverted triangles, reactions with anti-Nyv1p antibody. Inhibitor concentrations are as in Fig. 1. With Nyv1p-deficient vacuoles, a small but reproducible decrease in  $[\text{Ca}^{2+}]_E$  was observed in the presence of docking inhibitors or anti-SNARE antibodies. This residual docking-dependent  $\text{Ca}^{2+}$  release (<5% of that seen with vacuoles from wild-type cells) may be mediated by R-SNAREs other than Nyv1p.



reversed the PX and anti-Vam7p blocks (Fig. 3, A and B).  $\text{Ca}^{2+}$  release was also prevented by antibodies against the Q-SNAREs Vam3p (Fig. 3 C) or Vti1p (Fig. 3 D), or against the R-SNARE Nyv1p (Fig. 3, C and D). Control IgG, heat-denatured antibodies, and antibodies against the vacuolar SNARE Ykt6p had no detectable effect on  $\text{Ca}^{2+}$  release under these conditions (unpublished data). Thus the SNAREs Vam3p, Vti1p, Vam7p, and Nyv1p regulate docking-dependent  $\text{Ca}^{2+}$  signaling.

#### Trans-SNARE interactions elicit $\text{Ca}^{2+}$ release

Next, we asked whether vacuoles lacking either of two SNARE proteins would exhibit  $\text{Ca}^{2+}$  efflux upon Ypt7p-mediated tethering. Vacuoles obtained from cells bearing *nyv1* $\Delta$  or *vam3* $\Delta$  null mutations (Fig. 4, B and C) took up  $\text{Ca}^{2+}$  from the extralumenal space but failed to release it. In reactions containing vacuoles that lacked either Nyv1p or Vam3p,  $[\text{Ca}^{2+}]_E$  was unresponsive to recombinant Gdi1p (Fig. 4, B and C) or anti-Ypt7p antibody (not depicted). Similarly, these SNARE-deficient vacuoles were unresponsive to antibodies against Vam3p, Nyv1p (Fig. 4, B and C) or Vam7p (not depicted). Both Nyv1p and Vam3p-deficient vacuoles undergo Ypt7p-dependent tethering (Ungermann et al., 1998b; Wang et al., 2003). The severe  $\text{Ca}^{2+}$  release defects exhibited by these vacuoles shows that membrane tethering, without a full complement of SNAREs, is not sufficient to trigger  $\text{Ca}^{2+}$  release.

Vam3p and Nyv1p can promote fusion from opposite membranes, i.e., in trans (Nichols et al., 1997; Ungermann et al., 1998b). Vacuoles lacking Vam3p do not fuse with vacuoles lacking Vam3p, and vacuoles lacking Nyv1p do not fuse with vacuoles lacking Nyv1p. However, vacuoles lacking Vam3p do fuse with vacuoles lacking Nyv1p, indicating that these Q- and R-SNAREs functionally interact in trans (Nichols et al., 1997; Ungermann et al., 1998b). We therefore asked whether docking-dependent  $\text{Ca}^{2+}$  flux is also restored by the mixture of Vam3p- and Nyv1p-deficient vacuoles. As shown in Fig. 4 D, this was indeed the case.  $\text{Ca}^{2+}$  release by the vacuole mixture was docking-dependent because it was prevented by GDI blockade of Ypt7p function.  $\text{Ca}^{2+}$  release by mixtures of vacuoles from *vam3* $\Delta$  and *nyv1* $\Delta$  cells is blocked by antibodies against Vam3p or

Nyv1p (Fig. 4 D). Thus, functional trans interactions between Q- and R-SNAREs trigger docking-dependent  $\text{Ca}^{2+}$  release from the vacuole lumen.

#### Cycles of trans-SNARE complex assembly promote $\text{Ca}^{2+}$ release

Sec17/18p disassemble complexed SNARE proteins. Recombinant Sec17p and Sec18p, and antibodies against these proteins, have provided useful tools for the manipulation of SNARE assembly dynamics. To further test whether the oligomeric state of SNARE proteins regulates  $\text{Ca}^{2+}$  release, we tested the effect of recombinant Sec18p (Fig. 5 A). Sec18p stimulated  $\text{Ca}^{2+}$  release at up to 300 nM; at higher concentrations this stimulation declined (see Discussion). The stimulatory effect of rVam7p and Sec18p (Fig. 5 A) combined was greater than with either reagent alone.

Next, we asked whether the effect of Sec17/18p was confined to the early, priming phase of the reaction. Standard reactions were started, and various reagents were added to the reactions at  $\sim 18$  min, after priming (Mayer et al., 1996; Ungermann et al., 1998a) and during the normal docking-dependent  $\text{Ca}^{2+}$  efflux (Fig. 5 B). Recombinant Sec18p caused an immediate increase in the amplitude of  $\text{Ca}^{2+}$  efflux (compare high Sec18p to standard), suggesting that Sec18p regulates the amplitude of  $\text{Ca}^{2+}$  release during docking. Anti-Sec17p antibodies added either alone or immediately before recombinant Sec18p caused a rapid attenuation of  $\text{Ca}^{2+}$  release (Fig. 5 B). Therefore, the stimulatory effect of recombinant Sec18p depends on endogenous Sec17p. When added in excess over endogenous Sec18p, recombinant Sec17p inhibits fusion, by recapturing unpaired SNAREs into cis complexes (Wang et al., 2000). This inhibition of fusion can be reversed by the addition of stoichiometric amounts of Sec18p. Excess Sec17p added at  $\sim 18$  min inhibited  $\text{Ca}^{2+}$  release (Fig. 5 B), and recombinant Sec18p reversed this inhibition. Sec18p-mediated stimulation of  $\text{Ca}^{2+}$  efflux is abolished by anti-Vam3p antibody (unpublished data), indicating a SNARE requirement. Previous work in our laboratory (Mayer et al., 1996; Ungermann et al., 1998a) showed that anti-Sec17p or anti-Sec18p antibodies added after  $\sim 15$  min have little effect on fusion, indicating that Sec17/18p function early (during priming)

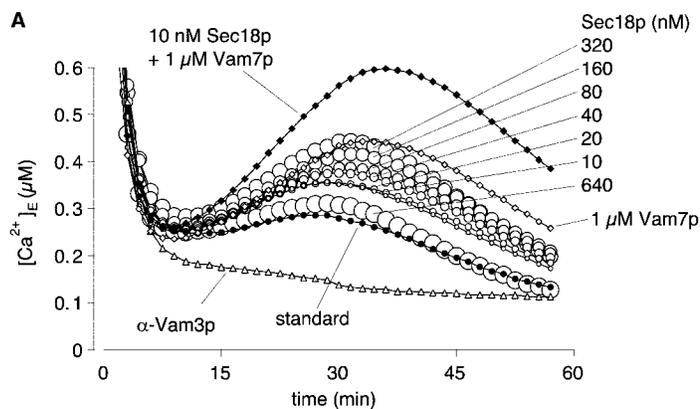
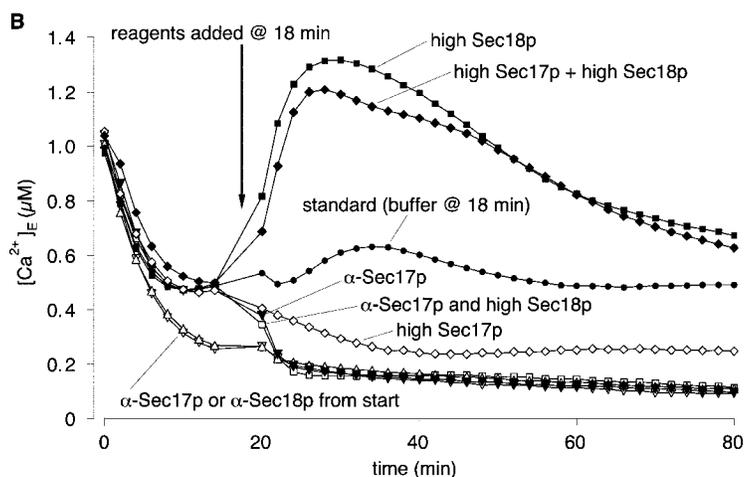


Figure 5. **Docking-dependent Ca<sup>2+</sup> release is stimulated by Sec17/18p.** (A) Simulation of Ca<sup>2+</sup> release by Sec18p. (B) Sec17/18p promote ongoing Ca<sup>2+</sup> release. Standard reactions were initiated, and at 18 min, the microplate was removed from the luminometer, and additional reagents were added: 200 nM of high Sec18p; 150 nM of high Sec17p; and 380 nM of anti-Sec17p or anti-Sec18p IgG. Anti-Sec17/18p antibodies were also added from the reaction start to two samples, as indicated on the plot.



in a standard fusion reaction. Under the conditions used to measure Ca<sup>2+</sup> release similar results were obtained, with fusion inhibited by less than 30% when antibodies were added at 15–18 min. The observation that Sec17/18p regulate Ca<sup>2+</sup> release even after 15 min (during docking) raised the question of whether Sec17/18p act during the Ca<sup>2+</sup> release step itself or act indirectly, by promoting the availability of unpaired SNAREs or SNARE associated factors such as the HOPS–class C complex.

To see if Sec17/18p action and Ca<sup>2+</sup> release could be uncoupled, we used an antipeptide antibody that permits reversible inhibition of the Ypt7p GTPase. Ypt7p block-reversal can be used to allow Sec17/18p-mediated priming while accumulating the vacuoles at an early, Ypt7p-dependent stage of docking. Relief of the anti-Ypt7p block by adding the antigenic peptide allows the vacuoles to proceed through docking and fusion in a relatively synchronous manner (Eitzen et al., 2001, 2002). Reactions were initiated either

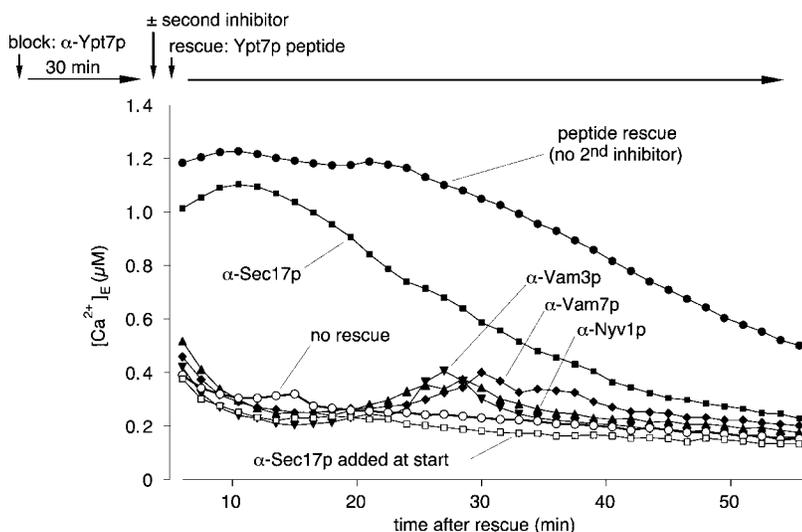
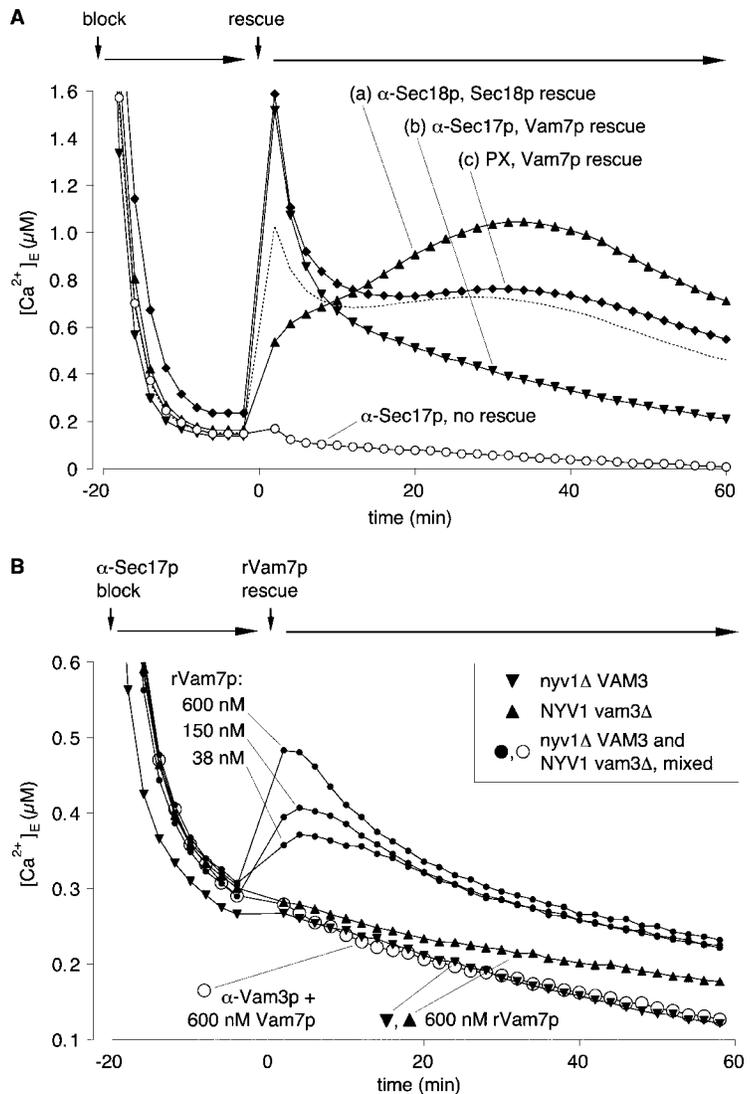


Figure 6. **Docking-dependent Ca<sup>2+</sup> release without ongoing Sec17/18p action.** Standard reactions were initiated in the presence of affinity-purified anti-Ypt7p peptide antibody and incubated at 26°C. At 30 min, the reactions were moved to ice, second inhibitors (antibodies against SNAREs or Sec17p) were added as indicated on the plot, and the anti-Ypt7p block was reversed by addition of excess Ypt7p peptide. No peptide was added to the “no rescue” sample. The samples were then assayed for Ca<sup>2+</sup> release at 26°C.

**Figure 7. Sec17/18p promote sustained  $\text{Ca}^{2+}$  efflux.** (A) Kinetics of  $\text{Ca}^{2+}$  release in three block/rescue protocols. Blocks were imposed in standard reactions for 20 min before rescue: trace a, anti-Sec18p block/Sec18p reversal; trace b, anti-Sec17p block/rVam7p reversal; trace c, PX block/rVam7p reversal. IgG against Sec17p and Sec18p were used at 380 nM, PX was used at 20  $\mu\text{M}$ , and rVam7p was used at 10  $\mu\text{M}$ . The dotted line shows the arithmetic mean of traces a and b. (B) Reactions containing SNARE-deficient vacuoles were initiated in the presence of anti-Sec17p, incubated for 20 min, and rescued with the indicated amounts of rVam7p. Control reactions not rescued with rVam7p (not depicted) did not exhibit  $\text{Ca}^{2+}$  efflux, similar to reactions containing both anti-Vam3p antibody and rVam7p.

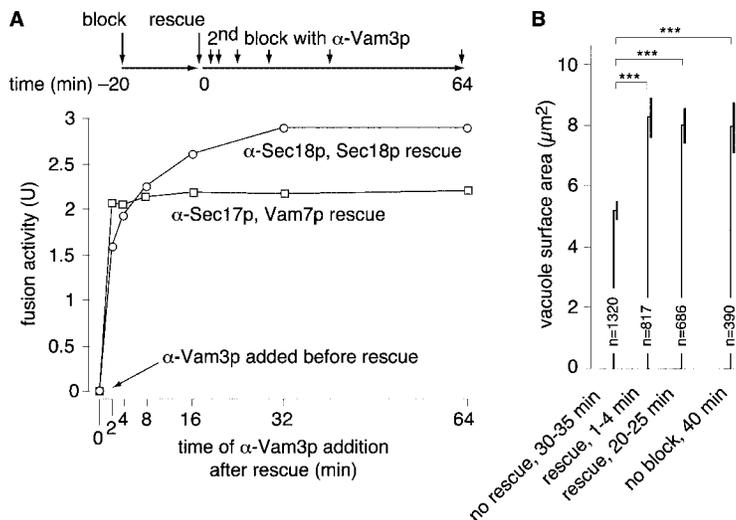


without or with the Ypt7p antibody (Fig. 6). In reactions blocked with anti-Ypt7p for 35 min and then reversed (Fig. 6), anti-SNARE antibodies added 5 min before reversal were still inhibitory, indicating that SNARE function is still needed after priming, and during or after Ypt7p function. In marked contrast, anti-Sec17p antibodies did not prevent  $\text{Ca}^{2+}$  release upon peptide rescue. In a control reaction, anti-Sec17p, when added from the start of a reaction without anti-Ypt7p, prevented  $\text{Ca}^{2+}$  release. Thus, Sec17/18p function is not required during  $\text{Ca}^{2+}$  release step itself.

We observed consistently that  $\text{Ca}^{2+}$  release upon reversal of a Ypt7p block decayed more rapidly in the presence than in the absence of anti-Sec17p antibody (Fig. 6). This result, along with the results shown in Fig. 5 B, suggests that Sec17/18p promotes sustained  $\text{Ca}^{2+}$  signaling by promoting cycles of SNARE complex disassembly and trans-complex formation. To test the idea that Sec17/18p can promote sustained  $\text{Ca}^{2+}$  release by stimulating SNARE complex turnover and thereby increasing the frequency of trans-SNARE interactions, we used three different block-reversal protocols to examine the kinetics of  $\text{Ca}^{2+}$  efflux (Fig. 7 A). In each case, the block was maintained for 20 min before reversal.

First, priming was blocked with anti-Sec18p antibody and reversed with recombinant Sec18p (Fig. 7 A, trace a).  $\text{Ca}^{2+}$  efflux was observed with kinetics similar to those seen in the standard reaction, with an additional delay corresponding to the block.

Second, we took advantage of recent observations that, under appropriate conditions, rVam7p can bypass the requirement for Sec17/18p-mediated priming (unpublished data; Thorngren, N., personal communication). It appears that the only essential event mediated by Sec17/18p *in vitro* is the liberation of Vam7p from cis-SNARE complexes; sufficient quantities of unpaired integral-membrane SNAREs are present on unprimed vacuoles to catalyze fusion when the reaction is supplemented with rVam7p. When rVam7p was added to an anti-Sec17p-blocked reaction (Fig. 7 A, trace b), a fast spike of  $\text{Ca}^{2+}$  release was observed and this spike rapidly decayed. Similar results were obtained when rVam7p was used to bypass an anti-Sec18p block (unpublished data). We also asked if rVam7p could interact with Vam3p and Nyv1p located in trans to one another to promote efflux. For this purpose, a complementation experiment similar to the one shown in Fig. 3 was used. We



**Figure 8. Fusion triggered by rVam7p.** (A) Kinetic window of anti-Vam3p sensitivity. Standard reactions were initiated in the presence of anti-Sec17p or anti-Sec18p IgG, incubated for 20 min at 27°C, and rescued with either Sec18p or Vam7p. At the indicated times after reversal anti-Vam3p antibody was added to the reactions. Fusion signals are normalized by subtracting the corresponding no-rescue signals. (B) Fusion occurs promptly upon Vam7p rescue of an anti-Sec17p block. Standard reactions were blocked or not blocked with anti-Sec17p as in A. Blocked reactions were rescued with Vam7p. Vacuoles were photographed at the times indicated after rescue, and membrane surface areas were calculated from vacuole diameters (see Materials and methods). Bars indicate geometric mean surface areas with 95% confidence intervals. Asterisks denote pairs of treatments that differ at  $P < 0.001$  by the Wilcoxon test. B summarizes data pooled from two independent experiments that yielded similar results when analyzed separately.

found that rVam7p elicited transient Ca<sup>2+</sup> efflux from anti-Sec17p-blocked mixtures of vacuoles derived from *vam3Δ* and *nyv1Δ* cells (Fig. 7 B), but it did not elicit Ca<sup>2+</sup> efflux from either population alone. Anti-Vam3p antibody completely suppressed rVam7p rescue (Fig. 7 B). rVam7p bypass of an anti-Sec17p block thus requires Vam3p and Nyv1p, even when these SNAREs are available only in trans and not in cis. These results suggest that on pretethered vacuoles, rVam7 promotes near-synchronous formation of many trans-SNARE complexes and rapid Ca<sup>2+</sup> efflux. In the absence of Sec17/18p activity, rVam7p-elicited efflux is short-lived relative to the sustained release promoted by Sec17/18p (Fig. 7 A, compare trace a with trace b).

In the third block-reversal protocol, PX domain was used to block Vam7p reassociation, and rVam7p was used to reverse the PX block as in Fig. 1 F. Under a PX block, priming and Ypt7p-dependent docking occur, but vertex assembly is incomplete and trans-SNARE pairing is prevented (Boeddinghaus et al., 2002; Wang et al., 2003). When the PX block was reversed, an immediate Ca<sup>2+</sup> spike was observed (Fig. 7 A, trace c), and this spike was followed by prolonged Ca<sup>2+</sup> release. When we plot the average values of the immediate spike from the anti-Sec17p/rVam7p reversal (Fig. 7 A, trace b) and the sustained but delayed spike from the anti-Sec18p/Sec18p reversal (Fig. 7 A, trace a), we obtain a curve (Fig. 7 A, dashed line) that closely matches the rescue curve for PX/rVam7p reversal. In addition, when the PX block was reversed by rVam7p in the presence of anti-Sec17p, the sustained component of the Ca<sup>2+</sup> flux was absent and only the spike was observed as in trace b (unpublished data). Together, these experiments show that the reversal of inhibitor blocks by rVam7p can be used to synchronize Ca<sup>2+</sup> release, and confirm that Sec17/18p promotes sustained cycles of Ca<sup>2+</sup> release but need not operate during a single Ca<sup>2+</sup> release event. We conclude that Sec17/18p disassembles SNARE complexes, producing unpaired SNAREs, which in turn enter new trans complexes that elicit Ca<sup>2+</sup> release.

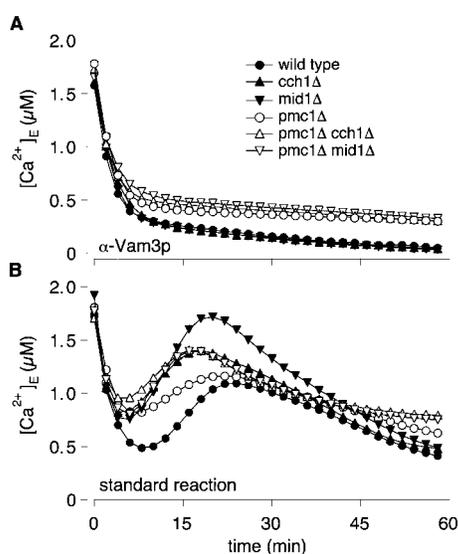
Under standard in vitro conditions (Conradt et al., 1994; Wang et al., 2002), two to three rounds of fusion occur in 60–90 min (unpublished data). Because of the prompt onset and decay of Ca<sup>2+</sup> release when rVam7p was

used to reverse an anti-Sec17p block, we asked if fusion was accelerated under these conditions. Three approaches were used. First, we used anti-Vam3p antibody to block docking at various times after reversal (Fig. 8 A). Within 2 min after reversal, the shortest time tested, fusion had become completely resistant to anti-Vam3p. In contrast, when anti-Vam3p was added immediately before rVam7p, no fusion was observed. Thus, Vam3p is required at the time of rVam7p addition, but it is needed (or its functional determinants are antibody accessible) for <2 min after rVam7p addition. To examine fusion more directly, we measured vacuole size (surface area) under four conditions: at 1–4 min after Vam7p reversal of anti-Sec17p; at 20–25 min after reversal; in reactions that were blocked but not reversed; and in reactions not blocked with anti-Sec17p (Fig. 8 B). We found that the geometric mean surface area of the vacuoles increased by 54% within 4 min after rVam7p reversal of the anti-Sec17 block, but no additional size increase was detected at later times. This methodology underestimates the actual amount of fusion because some limiting membrane is lost into the lumen in most fusion events (Wang et al., 2002), and because vacuoles too small to resolve (<250-nm diam) likely fuse to form small vacuoles that are then resolved, lowering the population's measured size. Thus, at least 0.5 round of fusion occurred in the experiment shown, a result consistent with the amount of alkaline phosphatase maturation observed in our standard fusion assay (unpublished data). In the third approach, we observed fusion in real time during the 1–4 min interval (Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200310105/DC1>). During this interval, we observed fusion among ~15% of the vacuoles. This measurement also is likely to underestimate fusion because recording was initiated ~1 min after reversal of the block by rVam7p, and because events out of the focal plane could not be clearly resolved. Thus, docking (assayed by sensitivity to anti-Vam3p), Ca<sup>2+</sup> efflux, and fusion occur promptly upon rVam7p-mediated bypass of the priming block. Together, our data show that the forward pathway of trans-SNARE complex assembly leads to Ca<sup>2+</sup> release and fusion. Sec17/18p are not needed during Ca<sup>2+</sup> efflux

but promote sustained  $\text{Ca}^{2+}$  efflux by catalyzing SNARE complex turnover and making unpaired SNAREs available for additional cycles of trans-complex assembly.

### Docking-dependent $\text{Ca}^{2+}$ release occurs through a novel pathway

To further characterize the nature of docking-dependent  $\text{Ca}^{2+}$  release, we tested vacuoles derived from several mutants with defects in  $\text{Ca}^{2+}$  transport. Deletion of the genes encoding each of the three known  $\text{Ca}^{2+}$  channel subunits in budding yeast, *Cch1p*, *Mid1p* (Fig. 9, A and B), and *Yvc1p* (not depicted), did not substantially change docking-dependent  $\text{Ca}^{2+}$  release. *Pmc1p* encodes a vacuolar  $\text{Ca}^{2+}$  ATPase that binds and is regulated by the R-SNARE *Nyv1p* (Takita et al., 2001), suggesting that it might be involved in SNARE-mediated  $\text{Ca}^{2+}$  fluxes. Vacuoles from *pmc1Δ* cells indeed sequestered  $\text{Ca}^{2+}$  slowly, resulting in a “resting”  $\text{Ca}^{2+}$  level elevated by  $\sim 200$  nM when docking was prevented (Fig. 9 A). However, when docking was allowed to proceed a docking-dependent  $\text{Ca}^{2+}$  efflux of normal amplitude was observed (Fig. 9 B). These results suggest that *Pmc1p* is not needed for docking-dependent  $\text{Ca}^{2+}$  efflux. However, it is possible that the docking-dependent flux occurs both through SNARE-mediated inactivation of *Pmc1p* and opening of a channel. Therefore, we constructed double mutants containing the *pmc1Δ* allele in combination with *cch1Δ* or *mid1Δ* alleles. Vacuoles from each of these double mutant strains exhibited  $\text{Ca}^{2+}$  sequestration and efflux phenotypes similar to the *pmc1Δ* single mutant (Fig. 9, A and B). These results suggest that docking-dependent  $\text{Ca}^{2+}$  release from the vacuole occurs through a novel pathway.



**Figure 9. SNARE-regulated  $\text{Ca}^{2+}$  release from vacuoles lacking  $\text{Ca}^{2+}$  transporters.** Vacuoles isolated from wild-type strain BY4742 or the indicated isogenic mutant strains (see Materials and methods) were assayed for  $\text{Ca}^{2+}$  release in the presence (A) or absence (B) of anti-Vam3p IgG. Reactions were under standard conditions (see Materials and methods) except that 250 nM rVam7p was added in all cases. The variability among the traces in B is comparable to that seen from day to day among independent vacuole preparations from a single strain.

## Discussion

### SNARE dynamics and $\text{Ca}^{2+}$ release

A working model of SNARE dynamics and  $\text{Ca}^{2+}$  function is shown in Fig. 10. Under standard in vitro fusion conditions, Sec18/18p-mediated priming (Fig. 10 A) disassembles cis-SNARE complexes, yielding unpaired SNAREs on the membrane and causing the release of endogenous Vam7p. Docking (Fig. 10 B) entails several steps. Membrane tethering is regulated by the GTPase *Ypt7p* (Mayer and Wickner, 1997), which also marks the locations where vertex subdomains will be assembled. Vertex assembly is a hierarchical process that results in the local enrichment of many fusion-related factors, phosphoinositides and ergosterol (unpublished data; Fratti, R., personal communication), actin, the HOPS-tethering complex, which includes the SM-protein *Vps33p*, and SNAREs (Wang et al., 2002, 2003). Vertex assembly may promote trans-SNARE interactions, which directly or indirectly trigger transient  $\text{Ca}^{2+}$  release events. Increases in  $[\text{Ca}^{2+}]_E$  in turn promote downstream events (Fig. 10 C) that lead to fusion. Fusion initiates at vertex sites (Wang et al., 2002); we speculate that both trans-SNARE pairing and focal  $\text{Ca}^{2+}$  release occur at the vertex. rVam7p bypasses the requirement for in vitro Sec17/18p-mediated priming. When added to tethered vacuoles, Vam7p causes a rapid, transient spike of  $\text{Ca}^{2+}$  release and fusion. Sustained  $\text{Ca}^{2+}$  release and fusion require ongoing Sec17/18p function for a continuing supply of unpaired SNAREs.

### Trans-SNARE interactions promote $\text{Ca}^{2+}$ release

Our experiments with antibodies, rVam7, PX domain, and vacuoles isolated from SNARE-deficient mutants implicate four vacuolar SNARE proteins in docking-dependent  $\text{Ca}^{2+}$  release: the Q-SNAREs Vam7p, Vam3p, and *Vti1p*; and the R-SNARE, *Nyv1p*. Antibodies against *Ykt6p*, a fifth SNARE found in vacuolar cis-complexes (Ungermann et al., 1999), did not inhibit  $\text{Ca}^{2+}$  release. As each of these SNAREs could contribute one coil to a tetrahelical “core complex” (Sutton et al., 1998), the requirement for all four is consistent with the hypothesis that tetrahelical trans-SNARE complexes regulate  $\text{Ca}^{2+}$  release.

Trans-SNARE interactions can occur only during docking. If  $\text{Ca}^{2+}$  release is triggered by trans-SNARE interactions, then docking-independent SNARE complex assembly and disassembly should not cause  $\text{Ca}^{2+}$  release. Consistent with this prediction, staging experiments show that SNAREs are needed for  $\text{Ca}^{2+}$  release at a post-priming step of the reaction (Figs. 6 and 7). Moreover, the addition of excess recombinant Sec17p, which inhibits fusion by promoting the reformation of cis-SNARE complexes from unpaired SNAREs, attenuates  $\text{Ca}^{2+}$  release (Fig. 4 C), indicating that cis-SNARE complex reformation does not trigger  $\text{Ca}^{2+}$  release. Together, these results indicate that neither cis-SNARE complex disassembly nor cis-complex reformation trigger  $\text{Ca}^{2+}$  release.

*Ypt7p* is required for tethering, vertex assembly, and trans-SNARE pairing (Mayer and Wickner, 1997; Ungermann et al., 1998b; Wang et al., 2002, 2003). As shown in Fig. 5, SNAREs are needed for  $\text{Ca}^{2+}$  release at a stage during or after *Ypt7p* function, for example, during docking. In ad-

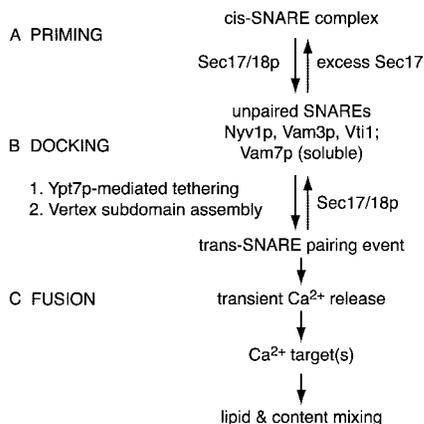


Figure 10. **Current working model of SNARE and Ca<sup>2+</sup> dynamics on the yeast vacuole.** See text for discussion.

dition, Rho GTPases and the SM-protein Vps33p act at docking, regulate SNARE complex formation (Sato et al., 2000; Eitzen et al., 2001; Muller et al., 2001), and are needed for Ca<sup>2+</sup> release.

The most direct evidence that trans-SNARE interactions trigger Ca<sup>2+</sup> release is shown in Fig. 4: in vitro complementation between populations of vacuoles lacking either Vam3p or Nyv1p indicates that these SNAREs can act from opposite membranes to trigger docking-dependent Ca<sup>2+</sup> release. Moreover, rVam7p stimulation of Ca<sup>2+</sup> release requires both Vam3p and Nyv1p, even when these SNAREs are located in trans to one another (Fig. 7 B).

### Role of Sec17/18p

Two types of staging experiments, Ypt7p block release and Vam7p bypass of Sec17/18p function, indicate that Sec17/18p need not function during the Ca<sup>2+</sup> release event itself. Instead, Sec17/18p are required for sustained cycles of Ca<sup>2+</sup> release, as shown in Fig. 5 B, Fig. 6 B, and Fig. 7 A. Sec17/18p catalyze the disassembly of SNARE complexes, liberating unpaired SNAREs which can participate in new trans interactions. Recombinant Sec18p promotes Ca<sup>2+</sup> release with maximal stimulation at ~300 nM (Fig. 5 A), close to the in vivo level of ~200 nM (Ghaemmaghami et al., 2003). At higher levels of Sec18p (>400 nM), the stimulation of Ca<sup>2+</sup> release is attenuated, possibly due to the disassembly of trans-SNARE complexes (Ungermann et al., 1998b) before they can complete their stimulation of Ca<sup>2+</sup> efflux. When Sec17/18p function is prevented, Ca<sup>2+</sup> release decays rapidly. These observations suggest that each trans-SNARE interaction event triggers only a short-lived transient of Ca<sup>2+</sup> release, and that repeated cycles of trans-SNARE interactions are needed for sustained Ca<sup>2+</sup> release.

### The Ca<sup>2+</sup> release pathway

Our data show that docking-dependent trans-SNARE interactions promote Ca<sup>2+</sup> release. However, we emphasize that the functional trans-SNARE interactions defined in this paper might not be identical to SNARE core complexes or trans-SNARE complexes isolated from vacuole detergent extracts (Ungermann et al., 1998b); additional work is needed

to understand SNARE association cycles and dynamics. Docking-dependent Ca<sup>2+</sup> release may be triggered directly by SNARE interactions with a Ca<sup>2+</sup>-releasing channel, or release may result from sequential interactions of SNAREs on apposed membranes with unidentified intermediary molecules.

It is unlikely that the observed Ca<sup>2+</sup> release occurs as an indirect consequence of fusion events. Mayer's group recently reported that vacuoles isolated from *vph1Δ* cells dock and release Ca<sup>2+</sup> in a Ypt7p-dependent manner, but do not go on to fuse (Bayer et al., 2003). In addition, manipulation of Sec17/18p function after priming can dramatically raise or lower the amplitude of Ca<sup>2+</sup> release with only modest effects on fusion. These observations, together with the Ca<sup>2+</sup>-dependence of fusion itself (Peters and Mayer, 1998), strongly suggest that the Ca<sup>2+</sup> release events studied here occur during docking, not after fusion.

A channel or transporter required for docking-dependent Ca<sup>2+</sup> release from the vacuole has not yet been identified, despite our efforts (Fig. 8) and studies of vacuolar Ca<sup>2+</sup> homeostasis in other laboratories (Takita et al., 2001; Bayer et al., 2003; Zhou et al., 2003). A number of uncharacterized ORFs in *S. cerevisiae* appear to encode ion transporters, and one of these could be responsible for docking-dependent Ca<sup>2+</sup> release. However, redundancy among more than one transporter might frustrate efforts to identify the relevant proteins through analyses of single knockouts.

Docking-dependent Ca<sup>2+</sup> release might occur through less conventional mechanisms. In most models of fusion, lipids at the fusion site transiently assume nonbilayer morphologies. Simulations (Muller et al., 2003) indicate that these rearrangements may form transient pores between cytoplasmic and noncytoplasmic compartments, and careful measurements of fusion events mediated by the influenza hemagglutinin protein confirm that transient leakage currents can accompany fusion (Frolov et al., 2003). Trans-SNARE complex formation might directly promote ion flux by perturbing bilayer structure.

### Implications of SNARE-dependent Ca<sup>2+</sup> release during docking

The physical proximity of Ca<sup>2+</sup> channels to the fusion machinery is suggested by experiments in which fusion is prevented by fast, but not slow, Ca<sup>2+</sup> chelators (Sullivan et al., 1993; Neher, 1998; Peters and Mayer, 1998; Pryor et al., 2000), and by the brief interval (≤200 μs) between channel gating and exocytosis in neurons (Llinas et al., 1981). Furthermore, many reports document physical and regulatory interactions between Ca<sup>2+</sup> channels and SNARE proteins in animal cells (Bennett et al., 1992; Yoshida et al., 1992; Sheng et al., 1994; Mochida et al., 1996; Wisner et al., 1996; Rettig et al., 1997). Ca<sup>2+</sup> channels associate with other fusion factors, including Rab3-interactor binding proteins (Hibino et al., 2002) and the synaptic Ca<sup>2+</sup> sensor synaptotagmin (Sudhof, 2002). SNAREs are also implicated in store-operated Ca<sup>2+</sup> entry, which may require membrane docking (Yao et al., 1999). Interactions between Ca<sup>2+</sup> signaling proteins and docking and fusion factors could have two functions: to allow channels to monitor the functional

status of docking over time, and to ensure that the fusion machinery and regions of peak  $\text{Ca}^{2+}$  flux coincide in space (Neher, 1998). For intracellular fusion events, these interactions may trigger  $\text{Ca}^{2+}$  flux in response to successful docking. In synapses, where voltage-gated  $\text{Ca}^{2+}$  channels respond to membrane depolarization, similar mechanisms might bias  $\text{Ca}^{2+}$  flux toward channels associated with primed and docked vesicles. Our experiments with vacuoles suggest that trans-SNARE complex formation is a checkpoint that controls progression to fusion. In this view, trans-SNARE interactions signify that docked membranes reside within a certain minimum distance and verify that specific biochemical events have transpired (e.g., priming and vertex subdomain assembly), triggering  $\text{Ca}^{2+}$  release and downstream events leading to fusion.

## Materials and methods

### Yeast strains

The standard strains used in our assays are BJ3505 (*MAT $\alpha$  pep4::HIS3 prb1- $\Delta$ 1.6R his3-200 lys2-801 trp1- $\Delta$ 101 [gal3] ura3-52 gal2 can1*; Jones, 2002) and DKY6281 (*MAT $\alpha$  leu2-3 leu2-112 ura3-52 his3- $\Delta$ 200 trp1- $\Delta$ 901 lys2-801*; Haas et al., 1994). *vam3 $\Delta$*  and *nyv1 $\Delta$*  derivatives of BJ3505 and DKY 6281 were prepared as described previously (Nichols et al., 1997). BY4742 (*MAT $\alpha$  his3 $\Delta$ 1 leu2 $\Delta$ 0 lys2 $\Delta$ 0 ura3 $\Delta$ 0*; Brachmann et al., 1998), BY4742 *ycv1 $\Delta$ ::neo*, BY4742 *cch1 $\Delta$ ::neo*, and BY4742 *mid1 $\Delta$ ::neo* were obtained from Research Genetics. BY4742 and its derivatives were used to generate AMY10 (= BY4742 *pmc1 $\Delta$ ::LEU2*), AMY11 (= BY4742 *cch1 $\Delta$ ::neo pmc1 $\Delta$ ::LEU2*), and AMY12 (= BY4742 *mid1 $\Delta$ ::neo pmc1 $\Delta$ ::LEU2*) by transformation with HindIII-cut pKC52 (Cunningham and Fink, 1994) and selection on CSM lacking Trp. The double mutant strains AMY11 and AMY12 grew well on YPD medium (Difco) and had similar growth defects as the *pmc1 $\Delta$*  single mutant AMY10 on YPD medium with 0.2 M  $\text{Ca}^{2+}$ .

### Reagents

rVam7p (residues 2–316) and Vam7p PX domain (residues 2–123) were expressed as GST fusions from the pGEX-KT vector (Hakes and Dixon, 1992) in BL21-pRP cells (Stratagene). VAM7 sequences were amplified from BJ3505 DNA using a forward primer with an engineered BamHI site (5'-cgcCGATCCGCAgctaattctgtagg-3') and reverse primers with engineered EcoRI sites (5'-cgGAATTCTCAgcaactgtttaaagtctagc-3' for rVam7p, and 5'-cgGAATTCACCTTgacaactgcaggaagac-3' for PX). Cells were grown in TB medium (Maniatis et al., 2001), 200 mg/l ampicillin, and 34 mg/l chloramphenicol to  $\text{OD}_{600} = 2.3$ , and expression was induced with 0.5 mM IPTG for 4 h at 26°C. Cell pellets (10,000 g, 20°C, 5 min) were resuspended in two pellet volumes of PBS with 2 mM EGTA, 1 mM EDTA, 1 $\times$  protease inhibitor cocktail (Haas, 1995), 1 mM PMSF, and 0.01% 2-mercaptoethanol. The cell suspension was frozen dropwise in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$ . Cells were thawed, lysed in a French press, mixed with Triton X-100 (0.5% final), and rocked for 20 min at 4°C before clearing (20,000 g, 4°C, 30 min). Glutathione-agarose (1 ml of slurry per 10 ml of lysate; Amersham Biosciences) was added to cleared lysate and rocked overnight at 4°C. The beads were washed with PBS supplemented as above (20 bed volumes), then with PBS alone (20 bed volumes), and finally with cleavage buffer (50 mM TrisCl, pH 8.0, 100 mM NaCl, 2 mM  $\text{CaCl}_2$ , 0.1% 2-mercaptoethanol; 10 bed volumes). Proteins were cleaved from the support with thrombin ( $\sim 10$  U/mg fusion protein, in two to three bed volumes of cleavage buffer; Sigma-Aldrich) for 3–4 h at 23°C. Thrombin was removed by flowing the eluted cleavage products directly over a second column containing 1 ml p-aminobenzamidine-agarose (Sigma-Aldrich). Proteins were exchanged into reaction buffer (20 mM PIPES/KOH, pH 6.8, 125 mM KCl, 5 mM  $\text{MgCl}_2$ , 200 mM sorbitol) on G-25 resin (Amersham Biosciences). Aliquots were frozen in liquid  $\text{N}_2$ , stored at  $-80^\circ\text{C}$ , and thawed just before use.

Antibodies were raised against peptides from Ypt7p (Eitzen et al., 2001), Vps33p (for Vps33p,  $\text{NH}_2$ -CLEDETEQWQKDGFDLNSKKT, and  $\text{NH}_2$ -CIE-DEHAADKITNENDDFSEA); against rVam7p and Sec17p (Haas and Wickner, 1996); and against recombinant cytoplasmic domains of Vam3p (Nichols et al., 1997), Nyv1p (Ungermann et al., 1998a), and Vti1p (Ungermann et al., 1999). IgG or affinity-purified antibodies were prepared as

described previously (Harlow and Lane, 1999). For anti-Ypt7p reversal (Eitzen et al., 2001) peptide was used at 20  $\mu\text{g}/\text{ml}$  final. Antibodies were stored in PS buffer (20 mM Pipes/KOH, 200 mM sorbitol, pH 6.8) at 4°C or  $-80^\circ\text{C}$  after buffer exchange on Sepharose G-25 resin (Amersham Biosciences) or dialysis. Some antibody preparations were concentrated in Microcon-30 ultrafiltration devices (Millipore).

### Fusion

30  $\mu\text{l}$  of standard fusion reactions contained a 1:1 mixture of vacuoles (Haas, 1995) from strains BJ3505 and DKY6281 (6  $\mu\text{g}$  total protein by Bradford assay; premixed in PS buffer) added to a mixture containing: 20 mM Pipes/KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM  $\text{MgCl}_2$ , 10  $\mu\text{M}$  coenzyme A, 4.5  $\mu\text{M}$  recombinant Pbi2p (IB2), 10 nM recombinant His<sub>6</sub>-Sec18p (except as noted), 1% (wt/vol) defatted BSA (Sigma-Aldrich), and 0.03 $\times$  protease inhibitor cocktail (Haas, 1995). ATP-regenerating system (Haas, 1995) was added from a 10 $\times$  stock to 1 $\times$  final (0.67 $\times$  for  $\text{Ca}^{2+}$  efflux assays). Fusion assays were incubated for 80 min at 27°C or as noted in figure legends, and fusion was measured by determining the amount of active alkaline phosphatase as described previously (Haas, 1995) except that phosphatase assay buffer was supplemented with 10 mM  $\text{CaCl}_2$ .  $\text{Ca}^{2+}$  efflux assays were performed with 10  $\mu\text{g}$  rather than 6  $\mu\text{g}$  of vacuoles per 30  $\mu\text{l}$  vol. For experiments using vacuoles from mutant cells, highly pure oxalyticase (Enzogenetics) was used to prepare spheroplasts for vacuole isolation.

### $\text{Ca}^{2+}$ assay

Aequorin luminescence assays were performed as described previously (Peters and Mayer, 1998; Muller et al., 2002) with modifications. Samples were analyzed in 96-well, low protein binding, conical bottom plates (Nunc) in a luminometer (Molecular Devices). IGOR Pro 4 (WaveMetrics) and JMP 5 (SAS Institute) were used for data analysis. Calibration was done using buffered  $\text{Ca}^{2+}$  EGTA standard solutions containing reaction salts including the ATP regenerating system, prepared as described previously (Tsien and Pozzan, 1989), and checked with MAX Chelator (Bers et al., 1994) version WEBMAXC 10.14.2002 (<http://www.stanford.edu/~cpatton>). Commercial  $\text{Ca}^{2+}$  standards gave similar results. A curve was fit to the luminance for each  $\text{Ca}^{2+}$  standard divided by peak luminance at saturating  $\text{Ca}^{2+}$  ( $L/L_{\text{max}}$ ), using a two-state model with three  $\text{Ca}^{2+}$ -binding sites (Allen et al., 1977):  $L/L_{\text{max}} = [(1 + K_R [\text{Ca}^{2+}]) / (1 + K_{\text{TR}} + K_R [\text{Ca}^{2+}])]^3$ .

We obtained  $K_R = 3.88 \pm 0.88 \times 10^6 \text{ M}^{-1}$  and  $K_{\text{TR}} = 1.45 \pm 0.29 \times 10^2$  (mean  $\pm$  SD) similar to values reported by Allen et al. (1977).  $K_R$  and  $K_{\text{TR}}$  were used to calculate  $[\text{Ca}^{2+}]$  in experimental samples. In addition to  $\text{Ca}^{2+}$ -dependent luminance, aequorin undergoes irreversible,  $\text{Ca}^{2+}$ -dependent inactivation. At constant-free  $[\text{Ca}^{2+}]$  (i.e., in buffered standard solutions), the apparent  $[\text{Ca}^{2+}]$  gradually decays exponentially over time. At 640 nM of free  $[\text{Ca}^{2+}]$ , the decay had a time constant  $\tau = 95 \pm 4$  min (mean  $r^2 > 0.97$ ). The traces shown are not adjusted for aequorin inactivation.

### Microscopy

Images were obtained on a microscope (model BX51/61; Olympus) with a Hg arc lamp, a 60 $\times$  1.4 NA Plan Apochromat objective, and a camera (model Sencisam QE; Cooke). The camera and microscope are controlled by IP Lab (Scanalytics). Vacuole sizes were measured by photographing wet mounts of reactions containing 2  $\mu\text{M}$  MDY-64, a fluorescent lipid probe (Molecular Probes). Vacuoles were measured using ImageJ (<http://rsb.info.nih.gov/ij/>) by manually circumscribing vacuoles with best-fit ellipses. The mean of the major and minor diameters of each ellipse was used to estimate vacuole surface area, by approximating vacuoles as spheres of radius  $\text{diam}/2$ . Surface areas were distributed approximately log normally, so these data were log transformed before statistical analysis (in JMP 5; SAS Institute) and geometric means were reported.

### Online supplemental material

Video 1. Video microscopy of fusion occurring after rVam7p rescue of an anti-Sec17p block. Video microscopy images were acquired with a 60 $\times$  1.4 NA Plan Apochromat objective and a camera (model Sencisam QE; Cooke) mounted on a hybrid BX51/61 platform (Olympus). The camera and microscope were controlled by IP Lab (Scanalytics) running under Mac OS X. Illumination was provided by an Hg arc lamp (Olympus). Time-lapse observations were made under illumination attenuated by >98% using neutral density filters (Olympus) and an infrared-blocking filter (Schott), and data was acquired with 2  $\times$  2 pixel binning. Images were acquired at ambient temperature, measured at 23–25°C with a thermocouple on the microscope stage. For time-lapse sequences a sharpening filter

(5 × 5 hat) was applied to each frame. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200310105/DC1>.

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