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A soluble SNARE drives rapid docking, bypassing ATP and Sec17/18p for vacuole fusion

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Membrane fusion requires priming, the disassembly of *cis*-SNARE complexes by the ATP-driven chaperones Sec18/17p. Yeast vacuole priming releases Vam7p, a soluble SNARE. Vam7p reassociation during docking allows *trans*-SNARE pairing and fusion. We now report that recombinant Vam7p (rVam7p) enters into complex with other SNAREs *in vitro* and bypasses the need for Sec17p, Sec18p, and ATP. Thus, the sole essential function of vacuole priming *in vitro* is the release of Vam7p from *cis*-SNARE complexes. In 'bypass fusion', without ATP but with added rVam7p, there are sufficient unpaired vacuolar SNAREs Vam3p, Vti1p, and Nyv1p to interact with Vam7p and support fusion. However, active SNARE proteins are not sufficient for bypass fusion. rVam7p does not bypass requirements for Rho GTPases,Vps33p, Vps39p, Vps41p, calmodulin, specific lipids, or Vph1p, a subunit of the V-ATPase. With excess rVam7p, reduced levels of PI(3)P or functional Ypt7p suffice for bypass fusion. High concentrations of rVam7p allow the R-SNARE Ykt6p to substitute for Nyv1p for fusion; this functional redundancy among vacuole SNAREs may explain why *nyv1*Δ strains lack the vacuole fragmentation seen with mutants in other fusion catalysts.

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Introduction

Regulated membrane fusion is catalyzed by proteins and lipids that are largely conserved among eukaryotes (Jahn *et al*, 2003). These include Ypt/Rab and Rho family GTPases and their effector proteins, the SNARE proteins and their chaperones of the SM (Sec1/Munc18), Sec18/NSF, and Sec17/α-SNAP families, phosphoinositides and sterols, calcium and calcium-binding proteins, and actin and actin-regulatory proteins. The Vo sector of the vacuolar proton pump has a role in vacuole fusion (Peters *et al*, 2001; Bayer *et al*, 2003), although its role in other intracellular fusion events is largely untested. Many of these factors are coupled in their action to ATP hydrolysis.

We study membrane fusion mechanisms through the homotypic fusion of yeast vacuoles (Wickner, 2002). Vacuole *in vitro* fusion occurs in three stages: priming, in which Sec18p, Sec17p, and ATP drive the disassembly of *cis*-SNARE complexes; docking, which includes the Ypt7p-dependent tethering of vacuoles and culminates in the formation of *trans*-SNARE complexes; and membrane fusion and lumenal compartment mixing. During priming, Vam7p, a soluble SNARE that lacks an apolar membrane anchor, is released from its association with other vacuolar SNAREs (Ungermann and Wickner, 1998). Vam7p association with the vacuole during docking requires Ypt7p (Ungermann *et al*, 2000) and 3-phosphoinositides (Cheever *et al*, 2001; Boeddinghaus *et al*, 2002) and permits *trans*-pairing of SNAREs (Merz and Wickner, 2004). During docking, spatial subdomains called vertex rings become selectively enriched with specific docking and fusion proteins and lipids, including Vam7p (Wang *et al*, 2002, 2003; Fratti *et al*, in preparation). The HOPS/VPS class C complex, comprising six subunits, is a Ypt7p effector (Seals *et al*, 2000), which also associates with SNARE complexes (Collins and Wickner, submitted). The HOPS subunit Vps39p is a nucleotide exchange factor for Ypt7p (Wurmser *et al*, 2000). The HOPS subunit Vps33p is an SM family protein that performs an essential but largely uncharacterized function during docking. *Trans*-association of SNAREs triggers a flux of calcium from the vacuole lumen (Merz and Wickner, 2004). Calmodulin (Cmd1p) associates with vacuoles and regulates fusion (Peters and Mayer, 1998). It has been suggested that the fusion stage of the reaction requires at least calcium and calmodulin (Peters and Mayer, 1998), *trans*-SNARE associations (Hanson *et al*, 1997; Nichols *et al*, 1997; Ungermann *et al*, 1998), the Vo sector of the V-ATPase (Peters *et al*, 2001), and actin (Eitzen *et al*, 2002). Several additional factors are also implicated in the fusion reaction, including the armadillo repeat protein Vac8p (Wang YX *et al*, 2001), regulators of actin assembly (Eitzen *et al*, 2002), and the Vtc protein complex (Muller *et al*, 2002).

We now report that the only essential function of ATP and ATP-driven vacuole priming *in vitro* is to release Vam7p, a soluble SNARE protein that associates peripherally with the vacuole membrane to permit *trans*-SNARE pairing. Recombinant Vam7p (rVam7p), added at physiological concentrations, permits fusion in the absence of ATP and Sec17/18p function. This bypass of priming does not bypass several other factors that are required for the standard *in vitro* fusion reaction, including Rho GTPases, HOPS subunits, and the Vo subunit Vph1p. Added Vam7p promotes docking and reduces the levels of Ypt7p, which are needed, and lowers (but may not eliminate) the requirement for 3-phosphoinositides. Elevated concentrations of Vam7p allow the R-SNARE

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Ykt6p to substitute for the R-SNARE Nyv1p in fusion, which may explain the intact vacuole structure seen in *nyv1*Δ mutants (Seeley *et al*, 2002).

Results

We assay vacuole fusion by isolating vacuoles from strains that either lack Pho8p (but have normal vacuolar proteases) or lack vacuolar proteases (and accumulate the catalytically inactive pro-Pho8p). *In vitro* fusion allows the proteases to gain access to pro-Pho8p and convert it to the catalytically active form, providing a coupled colorimetric assay of fusion (Haas *et al*, 1994). The availability of recombinant Vam7p (Merz and Wickner, 2004) has allowed us to re-evaluate the basic requirements of *in vitro* vacuole fusion reactions.

Under our standard reaction conditions, fusion of freshly purified vacuoles (Figure 1A) requires ATP (bar 2 versus 14) and incubation at 27°C. In some incubations, we employed an ATP-depleting system of glucose and hexokinase. Despite the presence of 1 mM ATP and an ATP-regenerating system, the addition of glucose and hexokinase caused some inhibition of fusion (Figure 1A, bar 3), demonstrating that this ATP-depletion system was active. Glucose or hexokinase alone had no effect on fusion (not shown), indicating that neither component is intrinsically inhibitory. In the presence of ATP, recombinant Vam7p (rVam7p), a SNARE that lacks a transmembrane anchor, caused a modest and variable increase in fusion (Figure 1A, bar 2 versus 6; Merz and Wickner, 2004). Surprisingly, however, fusion occurred when rVam7p was added in the absence of added ATP (Figure 1A, bar 10), even when hexokinase and glucose or 2-deoxyglucose were added as well (bars 11 and 12). The provision of free Vam7p thus appears to be the only essential function of ATP for vacuole fusion.

The amount of rVam7p added *in vitro* was compared to the amount of endogenous Vam7p on isolated vacuoles by immunoblot (Figure 1B). The vacuoles added to a standard 30 µl fusion reaction bear ~18 ng of endogenous Vam7p, resulting in a final concentration *in vitro* of ~16 nM. Because Vam7p exists in equilibrium between cytoplasm and membranes (Cheever *et al*, 2001; Boeddinghaus *et al*, 2002), our *in vitro* reaction with purified vacuoles will contain a lower concentration of Vam7p than that present in the cell. A recent study that quantified the per-cell abundance of many yeast proteins indicated that there are 2360 copies of Vam7p per cell (Ghaemmaghami *et al*, 2003). We estimate a cytoplasmic volume of ~40 fl per cell, and thus an *in vivo* Vam7p concentration of ~100 nM.

In early experiments, several µM rVam7p was required in the absence of ATP to obtain fusion signals that were comparable to those obtained in the standard, ATP-replete con-

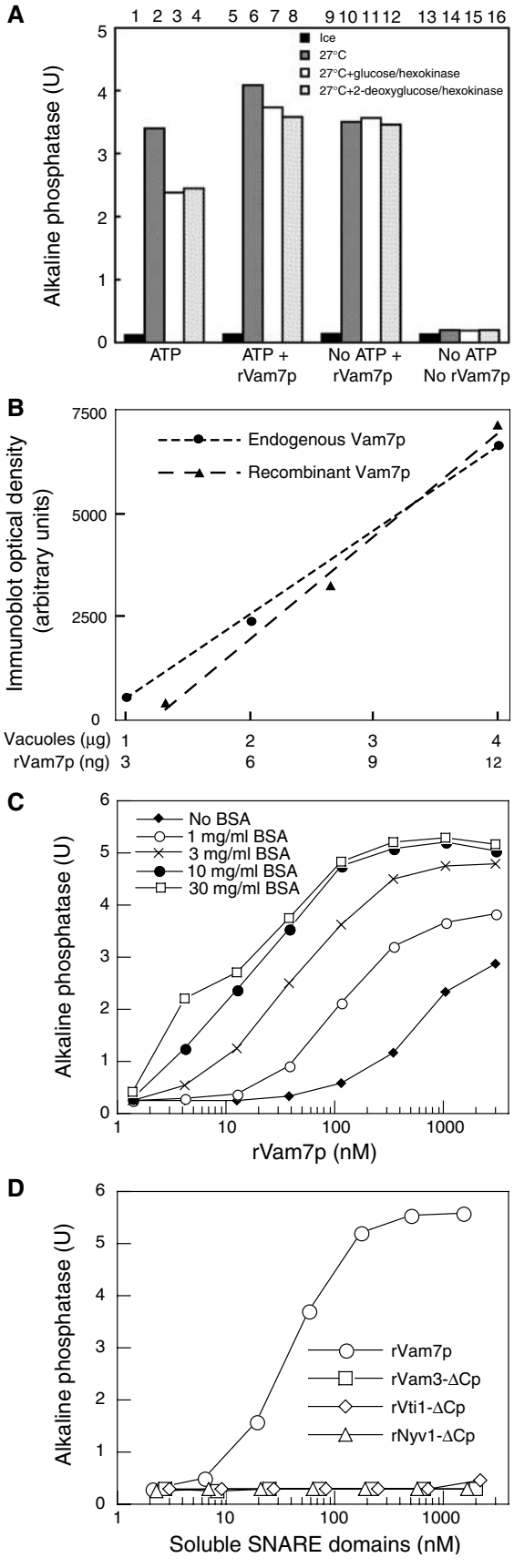

Figure 1 Bypass fusion. (**A**) Recombinant Vam7 protein stimulates vacuole fusion in the absence of ATP. Reactions were under standard conditions (Materials and methods). Where indicated, ATP and creatine kinase/creatine phosphate were omitted and reactions had 5 mM $MgCl_2$ instead of 6 mM $MgCl_2$. Recombinant Vam7p was added to a final concentration of 3.1 µM where indicated. (**B**) The indicated amounts of recombinant Vam7p (by Bradford assay, with albumin standard) and mixed BJ3505 and DKY6281 vacuoles were analyzed by SDS–PAGE and immunoblotting with anti-Vam7p antibodies (1:2000 dilution). The immunoblot was quantified by densitometry. (**C**) BSA promotes rVam7p-stimulated fusion. BSA and rVam7p were added at the indicated final concentrations to 'bypass' fusion reactions with 10 µM coenzyme A (CoA). (**D**) SNARE specificity of bypass fusion. Assays contained the indicated concentrations of either rVam7p or recombinant soluble domains of Vam3p, Vti1p, or Nyv1p.

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dition. However, this requirement for high concentrations of rVam7p only reflected a need for carrier protein, such as bovine serum albumin (BSA) or other proteins (data not shown), to avoid the loss of Vam7p activity. BSA was therefore included in all subsequent bypass fusion reactions. In the presence of sufficient carrier BSA (Figure 1C), half-maximal fusion without ATP was supported by ~20 nM rVam7p, an amount comparable to the amount of endogenous Vam7p (16 nM) in the *in vitro* reaction. Moreover, maximal fusion was supported by ~100–200 nM rVam7p, the concentration of endogenous Vam7p present *in vivo*. The recombinant soluble domains of the other vacuolar SNAREs did not support bypass fusion (Figure 1D).

Both bypass fusion (without ATP and with added rVam7p) and standard fusion, with or without supplementation with added rVam7p, yield mature Pho8p activity with comparable kinetics (data not shown). We also assayed the acquisition of resistance of the reaction to added antibody to Vam3p, a temporal landmark of docking (Ungermann and Wickner, 1998). Recombinant Vam7p accelerated the completion of docking in comparison to reactions without rVam7p (data not shown). The docking step of our standard *in vitro* fusion reactions may be slowed by the need for priming and by the low concentration of released Vam7p.

To survey the mechanistic relationship between standard and bypass fusion, we evaluated (Figure 2) the effects of

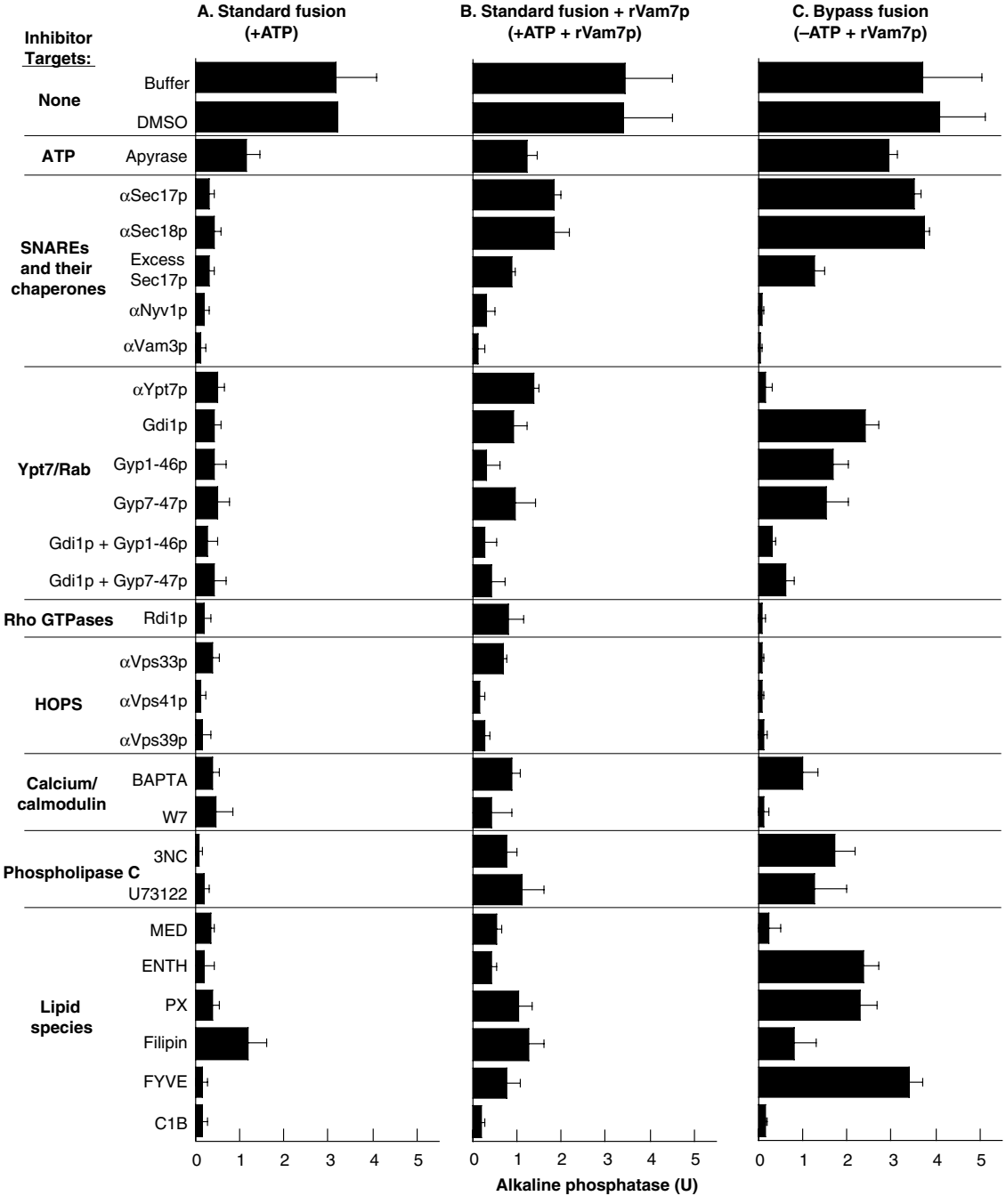

Figure 2 Sensitivities to fusion inhibitors. See Materials and methods for inhibitor concentrations. Reactions labeled '+ATP + Vam7p' had 170 nM rVam7p.

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various inhibitors under three conditions: (a) standard fusion, which contains ATP; (b) standard fusion with added rVam7p; and (c) bypass fusion, without ATP and with added Vam7p.

The no-ATP bypass reaction (Figure 2C) is unaffected by apyrase, which, like glucose/hexokinase, would further deplete any ATP, or by antibody to Sec17p or Sec18p, each of which blocks fusion under the standard reaction condition (Figure 2A). Bypass fusion therefore eliminates the requirement for the normal Sec17/18p- and ATP-dependent priming subreaction.

Bypass reactions lacking ATP and with rVam7p are sensitive to many of the same inhibitors as the standard ATP-replete reaction. rVam7p-mediated 'bypass' fusion remains fully sensitive to antibody against the SNAREs Vam3p and Nyv1p, suggesting that added rVam7p functionally interacts with active SNAREs that are not paired in *cis* on the isolated vacuoles. Bypass fusion is also sensitive to Rdi1p, which extracts the Rho family GTPases Cdc42p and Rho1p from membranes (Masuda *et al*, 1994; Eitzen *et al*, 2001), to antibody to Ypt7p or to the HOPS subunits Vps33p, Vps39p, and Vps41p, and to certain inhibitory lipid ligands such as MARCKS effector domain (MED), a ligand to phosphoinositides (Wang J *et al*, 2001), and C1b domain, a ligand to diacylglycerol (Johnson *et al*, 2000). Thus many central features of standard fusion are still required for the bypass fusion reaction.

Although the standard and bypass reactions employ many of the same pathways, there are clear differences. Bypass fusion shows altered sensitivity to calcium chelators (BAPTA) or inhibitors of calcium-dependent phospholipase C (U73122 and 3-nitrocoumarin; Thompson *et al*, 1991; Tisi *et al*, 2001), to certain Ypt7p inhibitors, and to phosphoinositide ligands (Figure 2). Several of these differences are further addressed below.

Vam7p associations during bypass fusion

The continued sensitivity of bypass fusion to antibodies to Vam3p or Nyv1p (Figure 2) suggests that rVam7p enters into SNARE complexes during bypass fusion. To assay this incorporation, we employed Vam7p with an N-terminal glutathione *S*-transferase (GST) 'tag'. GST-Vam7p supports bypass fusion (Figure 3A). Vacuoles were isolated from bypass fusion incubations with GST-Vam7p, solubilized in detergent, and subjected to immunoprecipitation with immobilized antibody to the Vam3p SNARE. Added GST-Vam7p entered into complexes with Vam3p without displacing the endogenous, untagged Vam7p (which is of lower M_r) from pre-existing SNARE complexes (Figure 3B). Isolation of the GST-Vam7p from the detergent extracts with glutathione beads confirmed that it had bound Vam3p (Figure 3C, middle panel, lane 1). Inhibition of fusion (Figure 3C, top panel) by antibody to Vps33p (lane 3) inhibited the association of GST-Vam7p with Vam3p (Figure 3C, middle panel), although these agents had little effect on the total vacuole-bound GST-Vam7p (bottom panel). Thus vacuole-bound GST-Vam7p enters a complex with Vam3p by a HOPS-dependent pathway.

Vacuoles synthesize PI(3)P during standard *in vitro* fusion incubations (Mayer *et al*, 2000), and Vam7p binds to PI(3)P through the Vam7p PX domain (Cheever *et al*, 2001; Boeddinghaus *et al*, 2002). How does Vam7p associate with vacuoles during bypass fusion? To determine the effect of ATP deprivation during bypass fusion on the levels of vacuolar PI(3)P, we assayed the vacuolar binding of recombinant FYVE domain, which had been derivatized with the Cy3 fluorophore. Vacuolar capacity for binding Cy3-FYVE, a measure of PI(3)P on the outer leaflet of the vacuole membrane, increased during the first 20 min of a standard fusion reaction (Figure 4A, filled circles), and this increase was blocked by the PI 3-kinase inhibitor LY294002 (Figure 4B). In the absence of ATP (Figure 4A, open circles), the level of accessible PI(3)P remained at its initial low level. This minimal Cy3-FYVE binding to freshly isolated vacuoles may even overestimate the levels of exposed PI(3)P, since excess nonfluorescent recombinant FYVE domain competed for the Cy3-FYVE-binding sites created during vacuole incubation with ATP but did not block the low level of Cy3-FYVE binding to freshly isolated vacuoles (data not shown). It might have been supposed that reduced levels of exposed PI(3)P would be

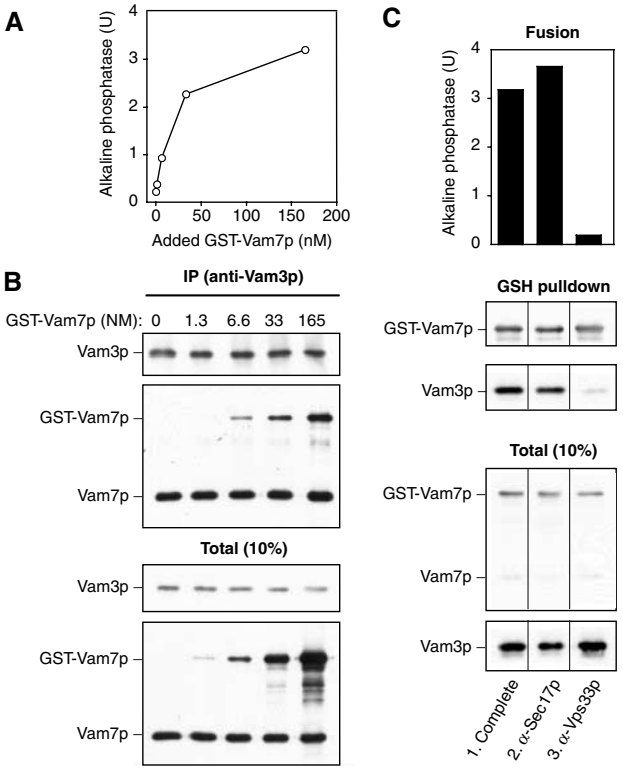

Figure 3 Added Vam7p enters SNARE complexes. (**A**) GST-Vam7p stimulates vacuole fusion and associates with Vam3p under 'Bypass' reaction conditions. Fusion reactions (180 μl) with indicated amounts of GST-Vam7p were incubated for 70 min at 27°C. Samples (30 μl) were withdrawn and assayed for vacuole fusion by alkaline phosphatase activity. (**B**) Vacuoles were reisolated from 5 × scale bypass fusion reactions with GST-Vam7p, solubilized, and Vam3p was immunoprecipitated from detergent extracts as described in Materials and methods. Samples representing 10% of the input (Total) and the α-Vam3p immunoprecipitated material (IP) were separated by SDS–PAGE and transferred to nitrocellulose membranes for immunoblot. (**C**) GST-Vam7p-driven bypass fusion and Vam7:Vam3 associations are sensitive to α-Vps33p. 'Bypass' fusion reactions (180 μl) containing 165 nM GST-Vam7p and the indicated inhibitors were incubated (27°C, 70 min) and mature alkaline phosphatase activity was assayed. Vacuoles from the remainder (150 μl) were reisolated, solubilized, and GST-Vam7p was retrieved by glutathione–Sepharose. Samples of the extracts (Total) and bead-bound material (GSH) were analyzed by SDS–PAGE and immunoblotting.

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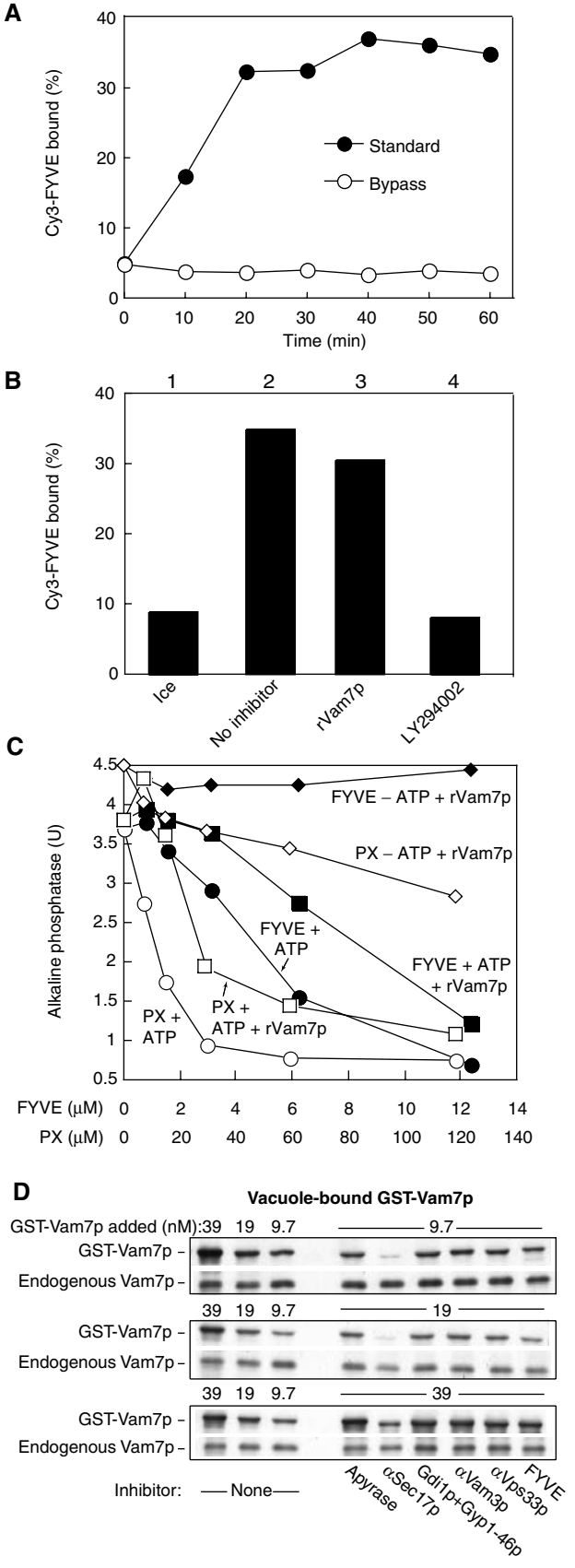
limiting for Vam7p binding and thereby render bypass fusion more sensitive than the PI(3)P-replete standard incubations to inhibition by recombinant PX or FYVE domain. However, bypass fusion at these lower levels of PI(3)P was far less sensitive to the addition of either PI(3)P ligand (Figure 4C, diamonds) than was the standard fusion reaction (circles). In accord with these findings, Vam7p binding to vacuoles under bypass fusion conditions was not inhibited by FYVE domain (Figure 4D). As noted (Boeddinghaus *et al*, 2002), the vacuole association of very low levels of Vam7p is inhibited by antibody to Sec17p (Figure 4D), but this inhibition is lost at the higher Vam7p concentrations employed in standard bypass fusion reactions. Although FYVE and PX depend on vacuolar PI(3)P in order to bind and inhibit standard fusion reactions, this inhibition may not simply reflect a blockade of Vam7p binding due to the sequestration of PI(3)P. We suggest that PI(3)P may have additional roles in fusion and that Vam7p may have additional vacuolar receptors. This is in accord with the complementation of *vam7*Δ phenotypes by the overexpression of Vam7p lacking its PX domain (Cheever *et al*, 2001). Bypass fusion may particularly require these other affinities for Vam7p binding to vacuoles, providing a pathway for Vam7p association with the other SNAREs which does not depend on its capacity to bind PI(3P).



Ypt7p and bypass fusion

Bypass fusion is fully inhibited by affinity-purified antibody to Ypt7p (Figure 2C). However, it is only partially sensitive to Gdi1p, which extracts Ypt7:GDP from the vacuole, or to Gyp1-46p or Gyp7-47p, which activates hydrolysis by Ypt7:GTP (Figure 2). To further explore the requirement for this Rab/Ypt GTPase, we mixed vacuoles with Gdi1p, Gyp1-46p, or Gdi1p + Gyp1-46p, to extract Ypt7p more rapidly and thoroughly (Eitzen *et al*, 2000). These vacuoles, and control vacuoles incubated without Gdi1p or Gyp1-46p, were assayed for bypass fusion with various concentrations of rVam7p. With addition of only 5 nM rVam7p, the bypass fusion reaction was completely inhibited by inactivation or extraction of Ypt7p (Figure 5A). Higher levels of rVam7p were needed for

Figure 4 PI(3)P and bypass fusion. (**A**) Quantitative analysis of vacuole surface PI(3)P. Vacuoles were incubated under standard or bypass conditions at 27°C for the indicated times, transferred to ice, and mixed with 3 μM Cy3-labeled $FVYE_2$. Samples were centrifuged (5 min, 4°C, 13 000 *g*) and vacuoles were resuspended in 30 μl PS buffer and solubilized with 100 μl 4 mM polydoconol detergent. Equal amounts of polydoconol were added to the supernatant fractions. Samples were analyzed using an ISS K2 fluorimeter (ISS Inc., Champaign, IL), exciting at 546 nm and recording emission at 570 nm. (**B**) Standard reactions were incubated for 30 min in the presence or absence of inhibitors and labeled as described above. To inhibit PI(3)P production, reactions were incubated at 4°C or 27°C in the presence of the phosphatidylinositol 3-kinase inhibitor LY294002 (200 μM). A separate sample was incubated at 27°C for 28 min in the absence of inhibitors and then mixed with Vam7p, hexokinase, and glucose. This sample was further incubated at 27°C for 2 min to allow Vam7p binding to PI(3)P prior to Cy3-FYVE labeling as described above. (**C**) $FYVE_2$ and PX were added to standard fusion reactions, standard reactions + 170 nM rVam7p, or bypass fusion reactions, and fusion was assayed after 90 min at 27°C. (**D**) Bypass fusion reactions of 90 μl (3 ×) bypass reactions were prepared with 39, 19, or 9.7 nM GST-Vam7p (substituted for the rVam7p) in the presence of the indicated inhibitors. Reactions also contained 550 nM IB2. Reactions were incubated for 90 min at 27°C, and then 60 μl of each reaction was transferred to a fresh tube and centrifuged at 20 000 *g*, 4°C, for 10 min. The supernatants were removed and pellets were resuspended in SDS sample loading buffer. Samples were electrophoresed on a 10% gel and immunoblotted using a biotinylated Vam7p antibody as primary and neutravidin–HRP as secondary.

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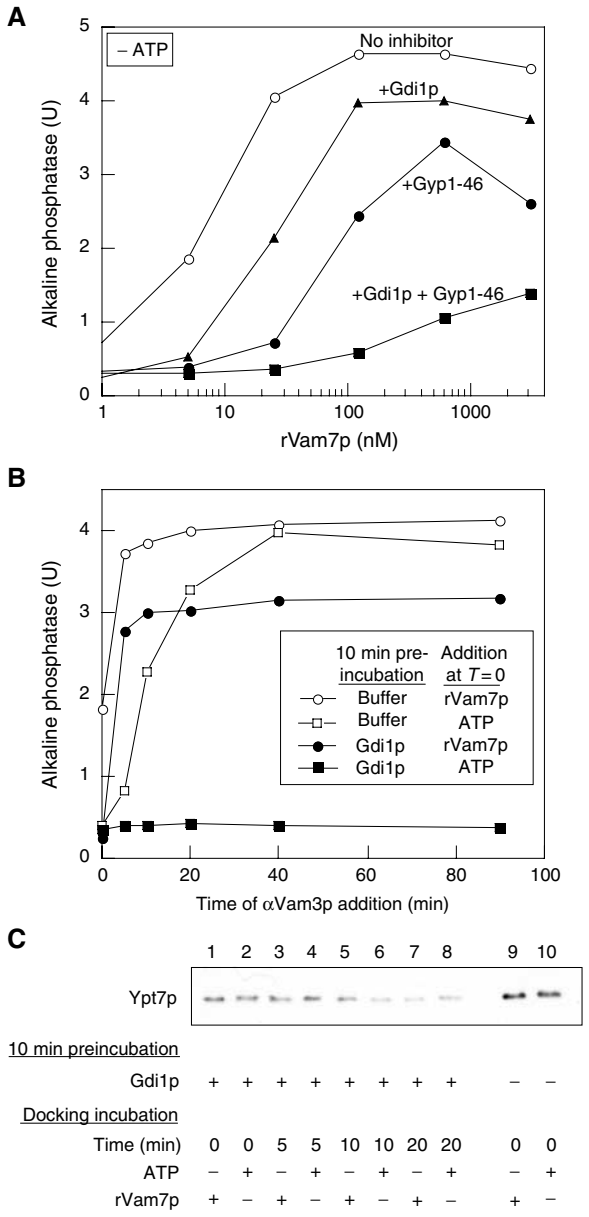

Figure 5 Recombinant Vam7p reduces the levels of Ypt7p needed for fusion. (**A**) Bypass fusion reactions without rVam7p were preincubated for 10 min on ice with either no inhibitor, Gdi1p, Gyp1-46p, or Gdi1p and Gyp1-46p (see Materials and methods for concentrations), and then mixed with the indicated concentrations of rVam7p. (**B, C**) Four large reactions (13 ×) containing vacuoles, reaction salts, BSA, and either glucose/hexokinase for the bypass reactions or coenzyme A for the standard reaction (see Materials and methods for concentrations of reagents) were preincubated with Gdi1p or PS buffer for 10 min at 27°C. Reactions were returned to ice and 30 μl aliquots were added to tubes containing mixtures of anti-Vam3 IgG with either 1.5 μM rVam7p or ATP and then incubated at 27°C for 90 min; these samples represent the 0-time addition of anti-Vam3p. Vam7p (1.5 μM final) or ATP was added to the remaining large reactions on ice and 30 μl of each reaction was removed for a 0-time immunoblot analysis of the pellet (13 000 rpm, 5 min, 4°C in a microfuge). The remaining four large reactions were then moved to 27°C. Aliquots were removed at 5, 10, 20, 40, and 90 min for immunoblot analysis of pellets (C) and other 30 μl aliquots were transferred to tubes containing anti-Vam3 IgG before returning to 27° for the remaining 90 min (B).

fusion of Gdi1p- or Gyp1-46p-treated vacuoles. Even after treatment with both Gdi1p and Gyp1-46p, which function together to better extract Ypt7p, vacuoles showed some limited fusion when supplemented with very high levels (3 μM) of rVam7p. These data are consistent with bypass fusion requiring less Ypt7p to support docking than that required for standard fusion reactions. To test this concept, vacuoles were preincubated with reaction buffer alone or with Gdi1p for 10 min and then mixed with either ATP or rVam7p to initiate the standard or bypass fusion reactions. To monitor docking, aliquots were withdrawn and mixed with antibody to Vam3p and then allowed to complete a full 90 min incubation. Over 40 min, standard fusion (Figure 5B, open squares) acquired gradual resistance to anti-Vam3p, indicative of the completion of docking. The presence of Gdi1p during the 10 min preincubation (filled squares) completely prevented docking, and hence fusion. A very different pattern was seen when aliquots from the same two vacuole samples, preincubated with buffer or Gdi1p, were used to initiate bypass fusion. Without Gdi1p pre-extraction, docking was rapid under bypass fusion conditions (open circles). Vacuoles that had been pre-extracted with Gdi1p completed docking more slowly, yet still showed full resistance to antibody to Vam3p after 5 min (filled circles). Immunoblot analysis (Figure 5C) showed substantial (although not complete) extraction of Ypt7p by Gdi1p during the 10 min preincubation (lanes 1 and 2 versus 9 and 10), and the little further extraction that occurred during the subsequent 5 min (lanes 3 and 4) was not dependent on whether the incubation was in the presence of added Vam7p or ATP. These data indicate that Vam7p accelerates docking and that a lower level of Ypt7p is sufficient for bypass fusion than that needed for standard fusion in the presence of ATP.

ATP and ion fluxes in vacuole fusion

ATP drives Ca^{2+} uptake into vacuoles through the Pmc1p and Vcx1p transporters (Cunningham and Fink, 1996). In either the presence or absence of ATP, rVam7p diminishes fusion inhibition by BAPTA, a calcium chelator that inhibits the standard fusion reaction (Figure 2). Added Vam7p promotes a docking-dependent release of intravacuolar Ca^{2+} (Merz and Wickner, 2004). Is this docking-dependent calcium flux (Peters and Mayer, 1998) still activated by rVam7p during fusion reactions without priming? To allow normal ATP-dependent formation of the transmembrane Ca^{2+} gradient, we blocked priming by adding anti-Sec17p antibody in the presence of ATP. In the presence of ATP and α-Sec17 antibody (Figure 6A), fusion required the addition of Vam7p and was still blocked by antibody to Vam3p. Bypass fusion reactions, whether in the absence of ATP (Figure 6B, filled circles) or with ATP but without Sec17p function (open squares), showed similar requirement for added Vam7p. Fusion reactions with ATP and α-Sec17 antibody were therefore examined for docking-dependent flux of Ca^{2+} from the vacuole lumen (Peters and Mayer, 1998; Merz and Wickner, 2004). Standard fusion reactions with ATP showed calcium removal from the vacuole suspension solution into the vacuole lumen during the first minutes of incubation and then release from the interior as docking occurs (Figure 6C, filled circles). This release, which is due to *trans*-SNARE interactions (Merz and Wickner, 2004), was stimulated by rVam7p (filled triangles) or Sec18p (filled inverted triangles) and was blocked by antibody to Sec17p (open triangles), as reported (Merz and Wickner, 2004). In the presence of antibody to Sec17p, rVam7p still promotes Ca^{2+} flux (filled diamonds). This

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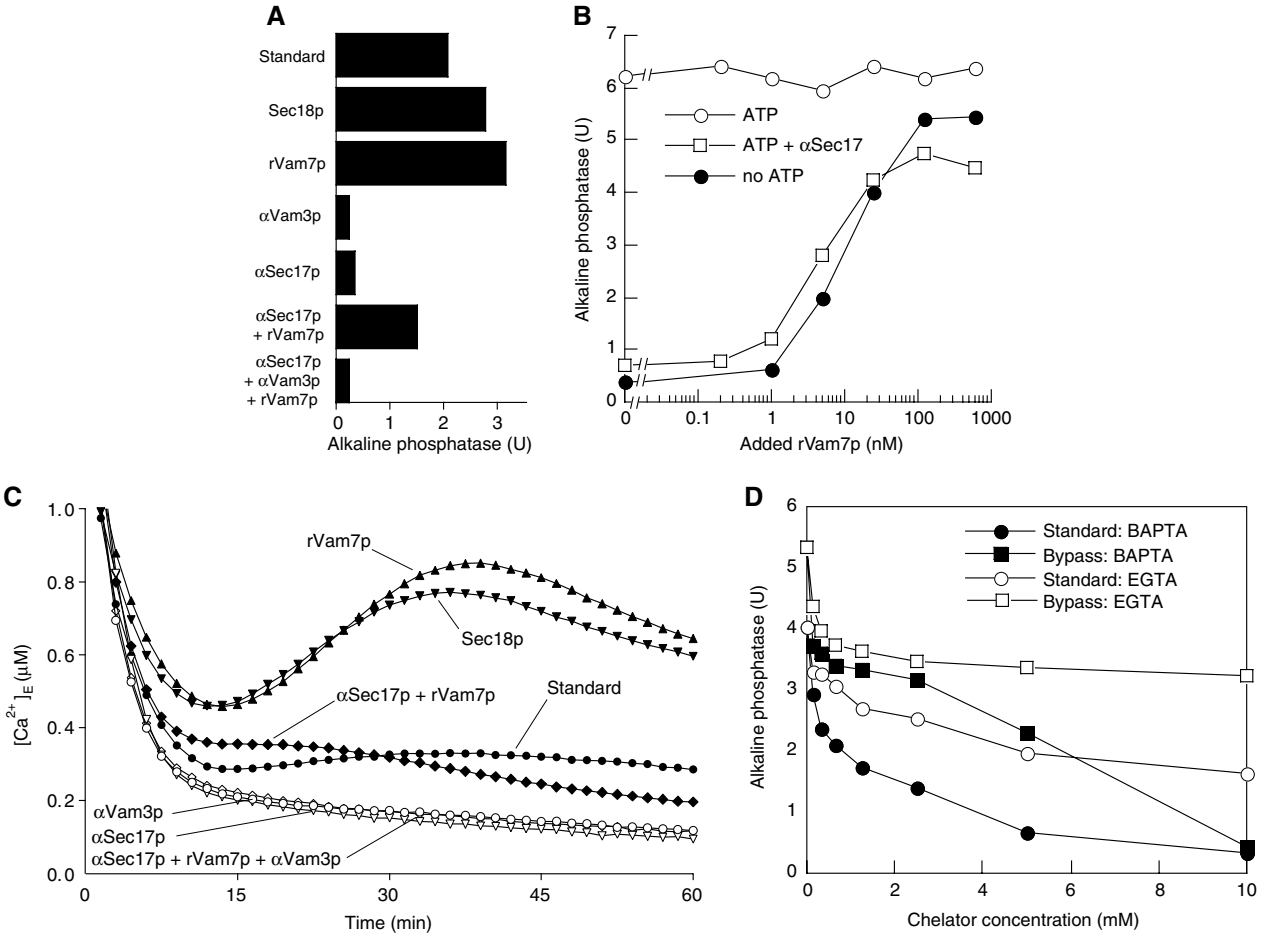

Figure 6 Recombinant Vam7p supports Ca^{2+} efflux and fusion in the absence of priming. (**A**) The indicated proteins were added to standard fusion reactions prior to incubation at 27°C. (**B**) Standard fusion reactions without inhibitor, with antibody to Sec17p, or bypass fusion reactions were performed with the indicated concentrations of added rVam7p. All reactions had 10 mg/ml BSA and 2% (v/v) dimethylsulfoxide. (**C**) Standard Ca^{2+} release reactions (Merz and Wickner, 2004) with ATP were initiated in the presence of the indicated reagents: 10 nM Sec18p, 1 μM rVam7p, 380 nM anti-Sec17p IgG, and 200 nM affinity-purified anti-Vam3p antibody. When used in combination, reagents were added in the sequence specified in the labels. (**D**) Standard or bypass fusion reactions were performed in the presence of the indicated Ca^{2+} chelator.

Ca^{2+} flux, like that seen without an anti-Sec17p blockade of priming, is fully sensitive to antibody against Vam3p (inverted open triangles), showing that Vam7p must act through a Vam3p-dependent pathway, presumably driving *trans*-SNARE complex formation from unpaired SNAREs. To further test whether the Ca^{2+} requirements for fusion were similar in standard fusion with ATP and in bypass fusion reactions without ATP, we compared the effects of two Ca^{2+} chelators, EGTA and BAPTA. Either chelator showed less inhibition of ATP-deficient bypass fusion (Figure 6D), in accord with the need for ATP to clear the extravacuolar calcium (Peters and Mayer, 1998). The greater inhibitory potency of BAPTA versus EGTA is largely rationalized by the eight-fold greater affinity of BAPTA for Ca^{2+} at the pH of our *in vitro* fusion reaction (Harrison and Bers, 1987, 1989).

In addition to Ca^{2+} gradients, proton gradients across the vacuole membrane may regulate vacuole fusion (Ungermann *et al*, 1999b). The Vo sector of the vacuolar ATPase is needed for vacuole fusion, *in vivo* and *in vitro* (Peters *et al*, 2001), and it has been suggested that the principal role of Vo in fusion is not through V-ATPase-driven proton pumping (Bayer *et al*, 2003). Nevertheless, vacuole fusion in these studies required ATP, which drives proton pumping by the vacuolar ATPase. We tested whether Vo is still needed for bypass fusion in the absence of ATP, when ATP-driven proton pumping cannot occur. Vph1p is a 100 kDa subunit of the Vo complex. Vph1p is required on both vacuoles for fusion in the absence of ATP (Figure 7), although Vph1p-deficient vacuoles have normal levels of all known fusion proteins (data not shown). There is a small but measurable fusion between vacuoles from *vph1*Δ cells and wild-type vacuoles in the presence of ATP (Figure 7, gray bars), and this fusion is blocked by antibody to Vam3p. Although further study will be required to establish the precise catalytic function of Vo in vacuole fusion, our studies provide an independent route of confirming that Vph1p/Vo is not simply needed for ATP-driven proton pumping.

R-SNARE function

Purified vacuoles have two R-SNAREs, Nyv1p and Ykt6p, and three Q-SNAREs, Vam3p, Vam7p, and Vti1p (Ungermann *et al*, 1999a). The N-terminal region of Ykt6p mediates the

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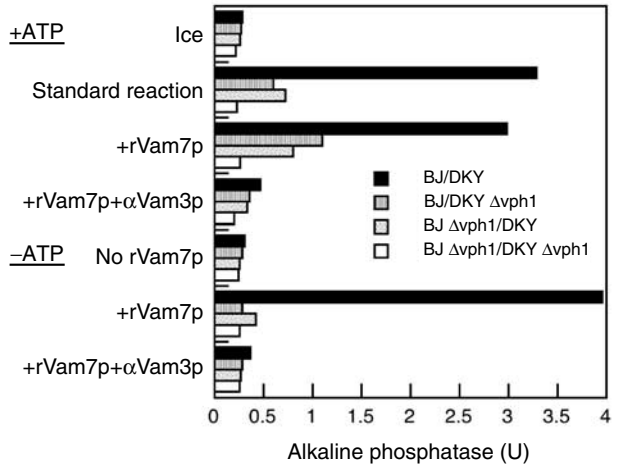

Figure 7 The Vo domain of the vacuolar ATPase is needed for vacuole fusion in the absence of ATP. Vacuoles were isolated from BJ3505 and DKY6281 strains and their vph1-Δ derivatives and assayed for fusion. Fusion reactions were performed under the standard or bypass fusion conditions (+ATP or −ATP, respectively) with or without 11 μM Vac8p and the following pure components (PCs): 12 nM His_6-Sec18p, 240 nM LMA1, and 6 μM calmodulin. Vacuoles were premixed in equal concentrations in the pairs BJ/DKY, BJ/DKY vph1-Δ, BJvph1-Δ/DKY, or BJvph1-Δ/DKYvph1-Δ and added to complete mixtures of the other reaction components on ice. Alkaline phosphatase activity was assayed after 90 min at 27°C.

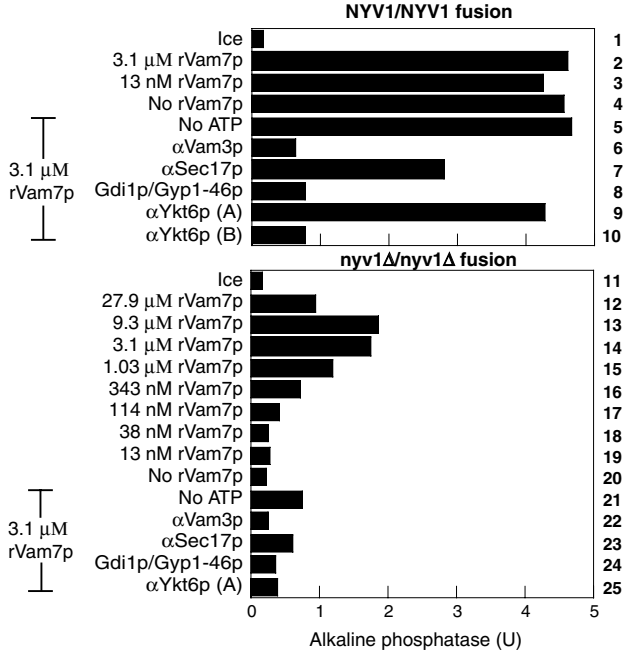

Figure 8 Recombinant Vam7p permits Ykt6p to substitute for Nyv1p in vacuole fusion. Vacuoles were isolated from BJ3505 and DKY6281 wild-type (NYV1) and nyv1-Δ strains and assayed for fusion. Assays contained 125 mM KCl, 6 mM $MgCl_2$, 2% EtOH, 10 mg/ml BSA, 15 μM coenzyme A, 12 nM His_6-Sec18p, 550 nM IB2, 1 mM ATP, 29 mM creatine phosphate, and 1 mg/ml creatine kinase. Recombinant Vam7p was added at the indicated concentrations. See Materials and methods for concentrations of inhibitory proteins. Affinity-purified antibody to His_6-Ykt6p (preparation A, lanes 9 and 25) was added at 330 nM. For affinity isolation of this antibody, MBP-Ykt6p was purified on amylose resin (NE Biolabs) and immobilized on Affigel-15 (Bio-Rad). An independent preparation of antibody to Ykt6p (preparation B, lane 10), purified on protein A–Sepharose and added at 4 μM, was the generous gift of Dr C Ungermann.

acylation of Vac8p (Dietrich *et al*, 2004). Deletion of the R-SNARE Nyv1p prevents vacuole fusion (Nichols *et al*, 1997; Ungermann *et al*, 1998b). Fusion can be partially restored to vacuoles from *nyv1*Δ cells by the addition of 3.1 μM rVam7p (Figure 8). Antibody to each of the five vacuolar SNAREs can inhibit fusion. Although antibodies that inhibit the acylation function of Ykt6p (Dietrich *et al*, 2004) inhibit vacuole fusion (Figure 8, bar 10), other antibody preparations (that may recognize the Ykt6p C-terminal domain) do not (bar 9). We employed such antibodies to determine whether elevated levels of Vam7p might be allowing the Ykt6p R-SNARE to replace Nyv1p functionally. Fusion of wild-type *NYV1* vacuoles is not sensitive to these antibodies to Ykt6p (bar 2 versus 9). However, the rVam7p-dependent fusion of *nyv1*Δ vacuoles is blocked by this antibody (bar 14 versus 25), suggesting that rVam7p permits the recruitment of Ykt6p as an alternate R-SNARE to form *trans*-SNARE complexes. The nonfragmented morphology of vacuoles in *nyv1*Δ strains (Nichols *et al*, 1997; Seeley *et al*, 2002) may reflect the capacity of Ykt6p to not only catalyze Vac8p acylation but also substitute for Nyv1p as the R-SNARE in the vacuole fusion reaction *in vivo*.

Discussion

ATP has been proposed to have several functions in vacuole fusion. These include driving Sec18p- and Sec17p-mediated disassembly of *cis*-SNARE complexes (Sollner *et al*, 1993; Ungermann *et al*, 1998a), phosphorylation of phosphatidylinositol (Mayer *et al*, 2000), ATP binding and perhaps hydrolysis by the Vps33p subunit of HOPS (Gerhardt *et al*, 1998), ATP binding and hydrolysis by actin (Pollard, 1986; Pollard *et al*, 2000; Eitzen *et al*, 2002), protein phosphorylation (Peters *et al*, 1999; Seeley *et al*, 2002), and the generation of proton and Ca^{2+} gradients across vacuole membranes (Ungermann *et al*, 1999b; Bonilla and Cunningham, 2002; Merz and Wickner, 2004). Although each of these may indeed promote or regulate vacuole fusion, the addition of exogenous rVam7p allows the *in vitro* fusion reaction to occur in the absence of ATP. In other fusion reactions, many of these roles of ATP may be indispensable. For example, PC12 cell exocytosis requires an ATP-dependent priming step of NSF action, phosphoinositide synthesis, and protein phosphorylation (Klenchin and Martin, 2000). However, the vacuole is unique among studied fusion systems in having a SNARE (Vam7p) without an apolar membrane anchor and a substantial cytoplasmic pool of this SNARE. The only essential role of ATP and Sec18p for *in vitro* vacuole fusion is to liberate Vam7p from *cis*-associations. This allows Vam7p to participate in docking, both for assembly of vertex domains, which are enriched in certain proteins (Wang *et al*, 2002) and lipids (Fratti *et al*, in preparation), and for *trans*-SNARE pairing (Ungermann and Wickner, 1998). While most SNAREs on purified vacuoles reside in *cis*-complexes, a small proportion of Vam3p, Nyv1p, and Ykt6p does not (Ungermann *et al*, 1999a), and we find that these unpaired SNAREs interact with added rVam7p during docking (Figure 3).

How are other potentially ATP-dependent functions bypassed for vacuole fusion? We have previously shown (Mayer *et al*, 1996; Xu *et al*, 1998) that ATP is only required during the early steps of the standard fusion reaction. While the standard fusion reaction requires protein phosphatase 1,

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which is found in association with calmodulin (Peters *et al*, 1999), the bypass fusion reaction (in the absence of ATP) is still W7 sensitive, that is, calmodulin-dependent. Calmodulin may have roles other than the delivery of protein phosphatase 1 to the vacuoles. Actin remodeling may also not require ATP, as ADP-actin can assemble into filaments (Pollard, 1986). ATP may not be essential for isolated vacuoles to maintain proton and calcium gradients. Vacuoles purified in the cold may retain lumenal stores of calcium, and efflux of this lumenal calcium in exchange for protons via Vcx1p (Cunningham and Fink, 1996) may acidify the vacuole without serious depletion of the vacuolar Ca^{2+} gradient. Although ATP is only required for priming, it may nonetheless participate in several other regulatory functions such as protein phosphorylation. Fusion with ATP and Vam7p may benefit from an optimal level of all four unpaired SNAREs, yet this might be balanced by some ATP-driven repression. In this regard, fusion with ATP and Vam7p still shows some inhibition by anti-Sec17p (Figures 2B and 6B), which is not seen during bypass fusion without ATP (Figure 2C).

Our studies are in accord with those of Lang *et al* (2002). They found that a substantial portion of the SNAREs on isolated plasma membrane sheets from PC12 cells were not complexed with other SNAREs, although their isolated membrane sheets had been preincubated with ATP. With a functional assay of fusion, we have shown that uncomplexed SNAREs are capable of participating in a bona fide fusion reaction that depends on complementary SNAREs, an SM-protein, Rab and Rho GTPases, and other factors as well. However, this is clearly a bypass reaction, in that *SEC18* and *SEC17* are essential genes and the encoded proteins are required for SNARE complex disruption and for SNAREs to participate in further cycles of pairing *in vivo*. We presume that the reason that there are uncomplexed SNAREs available for the formation of *trans*-complexes, in our studies of bypass fusion and in the studies of Lang *et al* (2002), is that Sec18p/NSF had catalyzed their disassembly from SNARE complexes *in vivo*, prior to organelle isolation. In addition, unpaired SNARE proteins may be selectively transported to the vacuole, as is the case for v-SNAREs involved in ER-to-Golgi traffic (Miller *et al*, 2003).

rVam7p, either with or without ATP, diminishes the inhibition of fusion by BAPTA or EGTA, Ca^{2+} chelators that inhibit the standard fusion reaction. During docking, *trans*-SNARE interactions promote the release of Ca^{2+} from the vacuole lumen (Merz and Wickner, 2004), and this Ca^{2+} release is strongly stimulated by rVam7p (Merz and Wickner, 2004). The levels of released Ca^{2+}, elevated by Vam7p stimulation, may partially reduce chelator inhibition of fusion in the presence of ATP. However, Ca^{2+} accumulation in the vacuole lumen requires ATP, to power the Pmc1p Ca^{2+} pump and, through V-ATPase-mediated lumenal acidification, to drive Ca^{2+} uptake through the Vcx1p H^+/Ca^{2+} antiporter. In the absence of ATP, there is a shallower Ca^{2+} gradient across the vacuole membrane (AJ Merz and W Wickner, unpublished). At 5 mM, BAPTA is expected to clamp extralumenal free $[Ca^{2+}]$ at <10 nM, far below Ca^{2+} levels required for fusion in the standard ATP-replete condition. How, then, can rVam7p allow partial BAPTA bypass in the absence of ATP? rVam7p may do this in two ways: it may increase the release of lumenal Ca^{2+} and it may reduce the Ca^{2+} requirement for fusion. Consistent with this idea, we find that Vam7p association with the vacuole is inhibited by BAPTA (RA Fratti and W Wickner, unpublished). The addition of excess rVam7p may compensate for this effect, allowing fusion at very low levels of ambient $[Ca^{2+}]$.

Characterization of the bypass fusion reaction illuminates several aspects of the standard vacuole fusion pathway. For example, rVam7p addition diminishes the amount of Ypt7p needed to complete docking. If Ypt7p-dependent tethering is reversible, then added rVam7p may allow a more efficient 'capture' of tethered vacuoles by rapid *trans*-SNARE association. The bypass fusion reaction also shows that Vph1p, a 100 kDa subunit of the V-ATPase Vo sector, is required for vacuole fusion in either the absence or presence of ATP. Vo therefore has a role in fusion that is distinct from its role in ATP-driven proton pumping. Each of these studies of bypass fusion illuminates the normal roles of ATP, Sec18/17p, Ypt7p, Vo, and Nyv1p in the physiological fusion pathway.

Deletion of genes required for vacuole fusion generally results in the fragmentation of vacuoles *in vivo*. In contrast, Nyv1p is needed for fusion under our normal *in vitro* conditions, but *nyv1*Δ mutant cells do not exhibit a vacuolar fragmentation phenotype and are free of vacuolar transport defects. We find that *in vitro* fusion can proceed in the total absence of the vacuolar R-SNARE Nyv1p when rVam7p is added. rVam7p-mediated fusion in the absence of Nyv1p may employ Ykt6p as the R-SNARE, explaining the apparent discrepancy between earlier *in vivo* and *in vitro* observations. These findings are in accord with those of Fukuda *et al* (2000), who report that recombinant Ykt6p and Nyv1p compete for association with Vam3p, Vti1p, and Vam7p. A similar situation exists in ER-to-Golgi transport, where Ykt6p can substitute for the R-SNARE Sec22p (Liu and Barlowe, 2002). In addition, overexpression of either vacuolar Vam3p or the endosomal Q-SNARE Pep12p can substitute for the loss of the other (Darsow *et al*, 1997), and this rescue depends on Vam7p (Gerrard *et al*, 2000). However, rVam7p addition to vacuoles lacking the Q-SNARE Vam3p does not rescue either Ca^{2+} release or fusion under any condition so far tested (AJ Merz and W Wickner, unpublished results). Taken together, these results demonstrate that there is significant plasticity in SNARE specificity at multiple transport steps. This plasticity may in part explain the need for the additional layers of specificity imposed by Rab GTPases, Rab effectors, and other docking factors.

Even in bypass fusion, SNAREs are not the only essential proteins. The sensitivity of the bypass fusion reaction to Rho GDI (Rdi1p) and to antibodies to HOPS subunits, as well as to lipid ligands, suggests that the underlying mechanisms are complex. Fusion has been proposed to consist of first bringing membranes into close apposition and then straining the bilayer to catalyze fusion. While SNAREs (Weber *et al*, 1998), Ca^{2+} (Wilschut *et al*, 1980), and NSF(Sec18p)/SNAP(Sec17p) (Otter-Nilsson *et al*, 1999; Brugger *et al*, 2000) are each capable of fusing model membranes, recent studies have provided convincing evidence that SNAREs, in conjunction with Ca^{2+} and Ca^{2+}-binding proteins such as synaptotagmin, form the core machinery for membrane fusion (McNew *et al*, 2000; Tucker *et al*, 2004). Several additional proteins, including Vo (Peters *et al*, 2001; Bayer *et al*, 2003) and actin (Eitzen *et al*, 2002), may also participate in fusion at docking junctions enriched in specific lipids and proteins.

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Materials and methods

Yeast strains

BJ3505 (*Matα ura3-52 trp1-Δ101 his3-Δ200 lys2-801 gal2* (*gal3*) *can1 prb1-Δ1.6R pep4::HIS3*) (Jones, 2002) and DKY6281 (*Matα ura3-52 leu2-3,112 trp1-Δ901 his3-Δ200 lys2-801 suc2-Δ9 pho8::TRP1*) (Haas *et al*, 1994) were used throughout the study. Their nyv1Δ or vph1Δ derivatives (Nichols *et al*, 1997; Bayer *et al*, 2003) were employed where indicated; the latter were a kind gift of Professor Andreas Mayer.

Reagents

All biochemicals were equilibrated in PS buffer (20 mM Pipes–KOH, pH 6.8, 200 mM sorbitol) unless otherwise indicated. Protein concentrations were assayed (Bradford, 1976) with BSA standards. Antibody inhibitors were added at the following final concentrations: αSec18p IgG (378 nM), αSec17p IgG (378 nM), αVam3p IgG (353 nM), affinity-purified αVti1p IgG (447 nM), affinity-purified αNyv1p IgG (267 nM), affinity-purified αVps33p IgG (32 nM), αVps39p IgG (21 μM), αVps41p IgG (2.8 μM), and affinity-purified αYpt7p IgG (133 nM). Protein inhibitors were added at the following final concentrations: Gdi1p (2.8 μM), His_6-Gyp7-47p (681 nM), His_6-Gyp1-46p (11.4 μM), GST-Rdi1p (24 μM), C1b domain from PKC (Johnson *et al*, 2000) (41 μM), ENTH domain (29 μM), Marks effector domain (MED; Wang J *et al*, 2001) synthesized by WM Keck Biotechnology Resource Center (New Haven) (10 μM), and His_6-Sec17p (299 nM). Chemical inhibitors were added at the following concentrations: U73122 (Calbiochem) dissolved in DMSO (80 μM), 3-nitrocoumarin (a kind gift of E Martegani, Milano, Italy) dissolved in DMSO (524 μM), Filipin III (Sigma) dissolved in DMSO (30 nM), BAPTA (Sigma) (5 mM), W7 (Calbiochem) dissolved in DMSO (833 μM), and apyrase (Sigma) (33.3 U/ml).

Protein and antibody purification

IgG fractions were prepared from several antisera (αSec17p, αSec18p, αVam3p, αVps39p, and αVps41p) by ammonium sulfate precipitation, desalting, and passage through a column of DE52 (Harlow and Lane, 1999). Antibodies were affinity purified on peptide Sulfolink affinity columns using the cognate peptide for αYpt7p (Eitzen *et al*, 2001) and αVps33p (N-terminal peptide) (Seals *et al*, 2000). Rabbit αVti1p was raised against GST-Vti1 and purified using this protein bound to Sulfolink resin (Pierce). αNyv1p was raised against His_6-Nyv1 (Ungermann *et al*, 1999a) and purified using GST-Nyv1p bound to Sulfolink resin. rVam7p (Merz and Wickner, 2004), Gdi1p (Garrett, 1995), His_6-Gyp7-47p (Eitzen *et al*, 2000), GST-Rdi1p (Eitzen *et al*, 2001), His_6-Gyp1-46p (Wang *et al*, 2003), and His_6-Sec17p (Haas and Wickner, 1996) were purified as described. His_6-Sec18p was purified according to Haas and Wickner (1996) with an additional purification step of gel filtration on an S300 HR column (Pharmacia) in 20 mM Pipes–KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, and 10% glycerol. Recombinant LMA1 was purified by a modified method of Xu *et al* (1998). Calmodulin was purified according to Brockerhoff *et al* (1992). IB2 was purified according to Slusarewicz *et al* (1997).

Fusion assay

Vacuoles were isolated as described (Haas, 1995). Standard fusion assays (+ATP) in 30 μl contained 125 mM KCl, 6 mM $MgCl_2$, 1 mM ATP (Pharmacia), 1 mg/ml creatine kinase (Roche), 29 mM creatine phosphate (Roche), 10 μM coenzyme A (Sigma), 20 mM Pipes–KOH, pH 6.8, 200 mM sorbitol, and 3 μg each of *pep4*Δ and *pho8*Δ vacuoles, premixed in equal concentrations, from BJ3505 and DKY6281. Bypass fusion assays (−ATP) contained 125 mM KCl, 5 mM $MgCl_2$, 10 mg/ml defatted BSA (Sigma), 20 mM glucose, 33.3 U/ml hexokinase (Sigma), 170 nM recombinant Vam7 protein (Merz and Wickner, 2004) in PS buffer, and vacuoles (as above). Components for each fusion reaction were mixed on ice, with premixed vacuoles added last unless otherwise noted. Reactions were incubated at 27°C for 90 min and then assayed for alkaline phosphatase activity (Haas, 1995) at 30°C for 5 min.

Immunoprecipitations and glutathione pulldowns

Sedimented vacuoles (11 000 *g*, 10 min, 4°C) were suspended in solubilization buffer (20 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 2 mM EDTA, 0.5% Triton X-100 (Anatrace), 20% glycerol, 1 × PIC (0.46 μg/ml leupeptin, 3.5 μg/ml pepstatin, and 2.4 μg/ml pefabloc-SC), and 1 mM PMSF). Extracts were incubated on ice (20 min), and insoluble material was removed by ultracentrifugation (100 000 *g*, 11 min, 4°C). Clarified detergent extracts were incubated (3.5 h, 4°C) with affinity-purified anti-Vam3p antibodies covalently coupled as described (Harlow and Lane, 1999) to protein A–Sepharose (100 μg antibody per ml packed bed of CL-4B beads, Amersham Biosciences) or to glutathione–Sepharose (CL-4B beads, Amersham Biosciences). Beads were reisolated by centrifugation (3000 *g*, 2 min, 4°C). Bound complexes were washed 4–5 times with solubilization buffer and eluted by boiling in reducing SDS sample buffer.

Recombinant SNARE protein production

The cytoplasmic domains of Vam3p and Vti1p fused to GST have been described previously (von Mollard *et al*, 1997; Dulubova *et al*, 2001). Cytoplasmic domain sequences of NYV1 (residues 2–231) and YKT6 (residues 2–195) were amplified by PCR from yeast genomic DNA using oligonucleotide primers containing flanking *Bam*HI or *Xho*I sequences (NYV1-FWD: CCG GAT CCG AAA CGC TTT AAT GTA AGT TAT GTG GAA GTT ATA AAA; NYV1-REV: CGG CCT CGA GGC TTA ATT TTT GAC CTT CTG CCA CCA; YKT6-FWD: CCG GAT CCG AGA ATC TAC ATC GGT GTA TTT CGC; YKT6-REV: CGG CCT CGA GGC TTA CGA ATT GGA TTT TTT AGC TTG C). Digested PCR products were ligated into parallel vectors (Sheffield *et al*, 1999) for fusion to GST (GST-Nyv1) or MBP (MBP-Ykt6) and expression in *Escherichia coli*. Expression was induced with 1 mM IPTG (4 h, 37°C). The cells were harvested, resuspended in PBS (Harlow and Lane, 1999), and frozen dropwise in liquid nitrogen. Frozen cells were thawed and broken in a French pressure cell. Lysates were cleared by centrifugation (23 000 *g*, 25 min, 4°C), and soluble extracts were incubated with PBS-equilibrated affinity resin (4 h, 4°C). GST-Vam3p, -Vti1p, and -Nyv1p were eluted from glutathione–Sepharose (50 mM Tris, pH 8.0, 10 mM reduced glutathione, and 1 mM DTT). Eluted protein was either dialyzed into PS buffer for vacuole fusion analysis or immobilized on beads for the isolation of affinity-purified antibodies. These GST-SNARE proteins (≈10 mg each) were coupled to Sulfolink resin (Pierce) following the instructions from the manufacturer. MBP-Ykt6p was eluted from amylose resin with PBS containing 10 mM maltose, and this protein (≈30 mg) was coupled to Affigel-15 (Bio-Rad).

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References

Bayer MJ, Reese C, Buhler S, Peters C, Mayer A (2003) Vacuole membrane fusion: Vo functions after *trans*-SNARE pairing and is coupled to the Ca^{2+}-releasing channel. *J Cell Biol* **162:** 211–222

Boeddinghaus C, Merz AJ, Laage R, Ungermann C (2002) A cycle of Vam7p release from and PtdIns-3P-dependent rebinding to the yeast vacuole is required for homotypic vacuole fusion. *J Cell Biol* **157:** 79–89

Bonilla M, Cunningham KW (2002) Calcium influx and release in yeast: TRPC and VGCC rule another kingdom. *Sci STKE* **127:** PE17

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Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72:** 248–254

Brockerhoff SL, Edmonds CG, Davis TN (1992) Structural analysis of wild-type and mutant yeast calmodulins by limited proteolysis and electrospray mass spectrometry. *Protein Sci* **1:** 504–516

Brugger B, Nickel W, Weber T, Parlati F, McNew JA, Rothman JE, Sollner T (2000) Putative fusogenic activity of NSF is restricted to a lipid mixture whose coalescence is also triggered by other factors. *EMBO J* **19:** 1272–1278

Cheever ML, Sato TK, de Beer T, Kutateladze TG, Emr SD, Overduin M (2001) Phox domain interaction with PtdIns(3)P targets the Vam7 t-SNARE to vacuole membranes. *Nat Cell Biol* **3:** 613–618

Cunningham KW, Fink GR (1996) Calcineurin inhibits VCX1-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in *Saccharomyces cerevisiae*. *Mol Cell Biol* **16:** 2226–2237

Darsow T, Rieder SE, Emr SD (1997) A multispecificity syntaxin homolog, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. *J Cell Biol* **138:** 517–529

Dietrich LEP, Gurezka R, Veit M, Ungermann C (2004) The SNARE Ykt6 mediates protein palmitoylation during an early stage of homotypic vacuole fusion. *EMBO J* **23:** 45–53

Dulubova I, Yamaguchi T, Wang Y, Sudhof TC, Rizo J (2001) Vam3p structure reveals conserved and divergent properties of syntaxins. *Nat Struct Biol* **8:** 258–264

Eitzen G, Thorngren N, Wickner W (2001) Rho1p and Cdc42p act after Ypt7p to regulate vacuole docking. *EMBO J* **20:** 5650–5656

Eitzen G, Wang L, Thorngren N, Wickner W (2002) Remodeling of organelle-bound actin is required for yeast vacuole docking and fusion. *J Cell Biol* **158:** 669–679

Eitzen G, Will E, Gallwitz D, Haas A, Wickner W (2000) Sequential action of two GTPases to promote vacuole docking and fusion. *EMBO J* **19:** 6713–6720

Fukuda R, McNew JA, Weber T, Parlati F, Engel T, Nickel W, Rothman JE, Sollner TH (2000) Functional architecture of an intracellular membrane t-SNARE. *Nature* **407:** 198–202

Garrett MD, Novick PJ (1995) Expression, purification, and assays of Gdilp from recombinant *Escherichia coli*. *Methods Enzymol* **257:** 232–240

Gerhardt B, Kordas TJ, Thompson CM, Patel P, Vida T (1998) The vesicle transport protein Vps33p is an ATP-binding protein that localizes to the cytosol in an energy-dependent manner. *J Biol Chem* **273:** 15818–15829

Gerrard SR, Mecklem AB, Stevens TH (2000) The yeast endosomal t-SNARE, Pep12p, functions in the absence of its transmembrane domain. *Traffic* **1:** 45–55

Ghaemmaghami S, Huh WK, Bower K, Howson RW, Belle A, Dephoure N, O'Shea EK, Weissman JS (2003) Global analysis of protein expression in yeast. *Nature* **425:** 737–741

Haas A (1995) A quantitative assay to measure homotypic vacuole fusion *in vitro*. *Methods Cell Sci* **17:** 283–294

Haas A, Conradt B, Wickner W (1994) G-protein ligands inhibit *in vitro* reactions of vacuole inheritance. *J Cell Biol* **126:** 87–97

Haas A, Wickner W (1996) Homotypic vacuole fusion requires Sec17p (yeast α-SNAP) and Sec18p (yeast NSF). *EMBO J* **15:** 3296–3305

Hanson PI, Roth R, Morisaki H, Jahn R, Heuser JE (1997) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep etch electron microscopy. *Cell* **90:** 523–535

Harlow E, Lane D (1999) *Using Antibodies, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Harrison SM, Bers DM (1987) The effect of temperature and ionic strength on the apparent Ca-affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. *Biochim Biophys Acta* **925:** 133–143

Harrison SM, Bers DM (1989) Correction of absolute stability constants of EGTA for temperature and ionic strength. *Am J Physiol* **256:** 1250–1256

Jahn R, Lang T, Sudhof TC (2003) Membrane fusion. *Cell* **112:** 519–533

Johnson JE, Giorgione J, Newton AC (2000) The C1 and C2 domains of protein kinase C are independent membrane targeting modules, with specificity for phosphatidylserine conferred by the C1 domain. *Biochemistry* **39:** 11360–11369

Jones EW (2002) Vacuolar proteases and proteolytic artifacts in *Saccharomyces cerevisiae*. *Methods Enzymol* **351:** 127–150

Klenchin VA, Martin TFJ (2000) Priming in exocytosis: attaining fusion-competence after vesicle docking. *Biochemie* **82:** 399–407

Lang T, Margittai M, Holzler H, Jahn R (2002) SNAREs in native plasma membranes are active and readily form core complexes with endogenous and exogenous SNAREs. *J Cell Biol* **158:** 751–760

Liu Y, Barlowe C (2002) Analysis of Sec22p in endoplasmic reticulum/Golgi transport reveals cellular redundancy in SNARE protein function. *Mol Biol Cell* **13:** 3314–3324

Masuda T, Tanaka K, Nonaka H, Yamochi W, Maeda A, Takai Y (1994) Molecular cloning and characterization of yeast rho GDP dissociation inhibitor. *J Biol Chem* **269:** 19713–19718

Mayer A, Scheglmann D, Dove S, Glatz A, Wickner W, Haas A (2000) Phosphatidylinositol-(4,5)-bisphosphate regulates two steps of homotypic vacuole fusion. *Mol Biol Cell* **11:** 807–817

Mayer A, Wickner W, Haas A (1996) Sec18p (NSF)-driven release of Sec17p (alpha-SNAP) can precede docking and fusion of yeast vacuoles. *Cell* **85:** 83–94

McNew JA, Parlati F, Fukuda R, Johnston RJ, Paz K, Paumet F, Sollner TH, Rothman JE (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* **407:** 153–159

Merz AJ, Wickner W (2004) *Trans*-SNARE interactions elicit Ca^{2+} efflux from the yeast vacuole lumen. *J Cell Biol* **164:** 195–206

Miller EA, Beilharz TH, Malkus PN, Lee MC, Hamamoto S, Orci L, Schelman R (2003) Multiple cargo binding sites on the COPII Subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. *Cell* **114:** 497–509

Muller O, Bayer MJ, Peters C, Andersen JS, Mann M, Mayer A (2002) The Vtc proteins in vacuole fusion: coupling NSF activity to Vo *trans*-complex formation. *EMBO J* **21:** 259–269

Nichols BJ, Ungermann C, Pelham HRB, Wickner WT, Haas A (1997) Homotypic vacuole fusion is mediated by t- and v-SNAREs. *Nature* **387:** 199–202

Otter-Nilsson M, Hendriks R, Pecheur-Huet E-I, Hoekstra D, Nilsson T (1999) Cytosolic ATPases, p97 and NSF, are sufficient to mediate rapid membrane fusion. *EMBO J* **18:** 2074–2083

Peters C, Andrews PD, Stark MJR, Cesaro-Tadic S, Glatz A, Podtelejnikov A, Mann M, Mayer A (1999) Control of the terminal step of intracellular membrane fusion by protein phosphatase 1. *Nature* **285:** 1084–1088

Peters C, Bayer MJ, Buhler S, Andersen JS, Mann M, Mayer A (2001) *Trans*-complex formation by proteolipid channels in the terminal phase of membrane fusion. *Nature* **409:** 581–587

Peters C, Mayer A (1998) Ca^{2+}/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. *Nature* **396:** 575–580

Pollard T, Blanchoin L, Mullins RD (2000) Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Ann Rev Biophys Biomol Struct* **29:** 545–576

Pollard TD (1986) Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J Cell Biol* **103:** 2747–2754

Seals DF, Eitzen G, Margolis N, Wickner WT, Price A (2000) A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. *Proc Natl Acad Sci USA* **97:** 9402–9407

Seeley ES, Kato M, Margolis N, Wickner W, Eitzen G (2002) Genomic analysis of homotypic vacuole fusion. *Mol Biol Cell* **13:** 782–794

Sheffield P, Garrard S, Derewenda Z (1999) Overcoming expressio and purification problems of RhoGDI using a family of 'parallel' expression vectors. *Protein Expres Purif* **15:** 34–39

Slusarewicz P, Xu Z, Seefeld K, Haas A, Wickner W (1997) IB2 is a small cytosolic protein that participates in vacuole fusion. *Proc Natl Acad Sci USA* **94:** 5582–5587

Sollner T, Bennet MK, Whiteheart SW, Scheller RH, Rothman JE (1993) A protein assembly–disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* **75:** 409–418

Thompson AK, Mostafapour SP, Denlinger LC, Bleasdale JE, Fisher SK (1991) The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J Biol Chem* **266:** 23856–23862

Tisi R, Coccetti P, Banfi S, Martegani E (2001) 3-Nitrocoumarin is an efficient inhibitor of budding yeast phospholipase C. *Cell Biochem Funct* **19:** 229–235

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Tucker WC, Weber T, Chapman ER (2004) Reconstitution of Ca^{2+}-regulated membrane fusion by synaptotagmin and SNAREs. *Science* **304:** 435–438

Ungermann C, Fischer von Mollard G, Jensen ON, Margolis N, Stevens TH, Wickner W (1999a) Three v-SNAREs and two t-SNAREs present in a pentameric *cis*-SNARE complex on isolated vacuoles, are essential for homotypic fusion. *J Cell Biol* **145:** 1435–1442

Ungermann C, Nichols BJ, Pelham HRB, Wickner W (1998a) A vacuolar v-t-SNARE complex, the predominant form *in vivo* and on isolated vacuoles, is disassembled and activated for docking and fusion. *J Cell Biol* **140:** 61–69

Ungermann C, Price A, Wickner W (2000) A new role for a SNARE protein as a regulator of the Ypt7/Rab-dependent stage of docking. *Proc Natl Acad Sci USA* **16:** 8889–8891

Ungermann C, Sato K, Wickner W (1998b) Defining the functions of *trans*-SNARE pairs. *Nature* **396:** 543–548

Ungermann C, Wickner W (1998) Vam7p, a vacuolar SNAP-25 homolog, is required for SNARE complex integrity and vacuole docking and fusion. *EMBO J* **17:** 3269–3276

Ungermann C, Wickner W, Xu Z (1999b) Vacuole acidification is required for *trans*-SNARE pairing, LMA1 release, and homotypic fusion. *Proc Natl Acad Sci USA* **96:** 11194–11199

von Mollard GF, Nothwehr SF, Stevens TH (1997) The yeast v-SNARE Vti1p mediates two vesicle transport pathways through interactions with the t-SNAREs Sed5p and Pep12p. *J Cell Biol* **137:** 1511–1524

Wang J, Arbuzova A, Hangyas-Mihalyne G, McLaughlin S (2001) The effector domain of myristoylated alanine-rich C kinase substrate binds strongly to phosphatidylinositol 4,5-bisphosphate. *J Biol Chem* **276:** 5012–5019

Wang L, Merz AJ, Collins K, Wickner W (2003) Hierarchy of protein assembly at the vertex ring domain for yeast vacuole docking and fusion. *J Cell Biol* **160:** 365–374

Wang L, Seeley ES, Wickner W, Merz AJ (2002) Vacuole fusion at a ring of vertex docking sites leaves membrane fragments within the organelle. *Cell* **108:** 357–369

Wang Y-X, Kauffman EJ, Deux JE, Weisman LS (2001) Fusion of docked membranes requires the armadillo repeat protein Vac8p. *J Biol Chem* **276:** 35133–35140

Weber T, Zemelman BV, McNew JA, Westermann B, Bmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. *Cell* **92:** 759–772

Wickner W (2002) Yeast vacuoles and membrane fusion pathways. *EMBO J* **21:** 1241–1247

Wilschut J, Duzgumes N, Fraley R, Papahadjopoulos D (1980) Kinetics of calcium ion induced fusion of phosphatidylserine vesicles followed by a new assay of mixing of aqueous vesicle contents. *Biochemistry* **19:** 6011–6121

Wurmser AE, Sato TK, Emr SD (2000) New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. *J Cell Biol* **151:** 551–562

Xu Z, Sato K, Wickner W (1998) LMA1 binds to vacuoles at Sec18p (NSF), transfers upon ATP hydrolysis to a t-SNARE (Vam3p) complex, and is released during fusion. *Cell* **93:** 1125–1134

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