Stimulation of Actin Polymerization by Vacuoles via Cdc42p-dependent Signaling*"1

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We have previously shown that actin ligands inhibit the fusion of yeast vacuoles in vitro, which suggests that actin remodeling is a subreaction of membrane fusion. Here, we demonstrate the presence of vacuole-associated actin polymerization activity, and its dependence on Cdc42p and Vrp1p. Using a sensitive in vitro pyrene-actin polymerization assay, we found that vacuole membranes stimulated polymerization, and this activity increased when vacuoles were preincubated under conditions that support membrane fusion. Vacuoles purified from a VRP1 gene deletion strain showed reduced polymerization activity, which could be recovered when reconstituted with excess Vrp1p. Cdc42p regulates this activity because overexpression of dominant-negative Cdc42p significantly reduced vacuole-associated polymerization activity, while dominant-active Cdc42p increased activity. We also used size-exclusion chromatography to directly examine changes in yeast actin induced by vacuole fusion. This assay confirmed that actin undergoes polymerization in a process requiring ATP. To further confirm the need for actin polymerization during vacuole fusion, an actin polymerization-deficient mutant strain was examined. This strain showed in vivo defects in vacuole fusion, and actin purified from this strain inhibited in vitro vacuole fusion. Affinity isolation of vacuole-associated actin and in vitro binding assays revealed a polymerization-dependent interaction between actin and the SNARE Ykt6p. Our results suggest that actin polymerization is a subreaction of vacuole membrane fusion governed by Cdc42p signal transduction.

The cytoskeleton plays an important role in regulating many cellular processes including cell division, motility, polarization, endocytosis, and exocytosis (1–4). Two general classification of actin regulatory functions have been established; those that depend on stable actin filaments, such as polarized organelle transport (5–7), and those that depend on dynamic actin remodeling, such as cell motility (2). There is a growing body of evidence that supports the need for dynamic actin monomer-polymer transitions during exocytosis and membrane fusion (8, 9). Traditionally, cortical actin has been viewed as a barrier structure that would only require disassembly to allow membrane contact prior to exocytosis (10–12). This would seemingly be the case for neurotransmitter release at the synapse, where actin remodeling is controlled by scinderin, which severs actin in a calcium-dependent manner to promote exocytosis (13). Interestingly, scinderin is also reported to negatively regulate exocytosis via actin nucleating activity (14, 15). However, it remains feasible that both activities are needed for distinct subreactions of exocytosis. Indeed, several studies implicate a dual role for F-actin as both a barrier that requires disassembly and a positive regulator to provide direction and force (16–19). We have proposed that F-actin stabilizes docked vesicle and provides localized force, which would be needed to overcome the energy barrier of membrane fusion (20).

Rho GTPases are well known for their role in governing actin polymerization (21). Cdc42p triggers actin polymerization via direct (22, 23) or indirect (24, 25) activation of the WASp-WIP complex, which subsequently activates actin polymerization via Arp2/3 (26). We have shown that membrane fusion is inhibited by antibodies to Las17p (yeast WASp ortholog) (16) or deletion of VRP1 (yeast WIP ortholog) (27), using an established in vitro assay of vacuole membrane fusion (28). Cdc42p is located on the vacuole membrane and is needed for homotypic vacuole fusion (29, 30), which suggests that actin polymerization may be a subreaction of the fusion mechanism. Indeed, both of the actin ligands jasplakinolide and latrunculin, which stabilize and destabilize F-actin, respectively, inhibit vacuole fusion implicating the need for cycles of actin polymerization and depolymerization. However, final membrane bilayer mixing is only inhibited by F-actin destabilization via latrunculin, suggesting actin polymerization is the final process needed (16).

Here, we used two assays to demonstrate that purified vacuole membranes stimulate actin polymerization in vitro. We used a pyrene-actin polymerization assay to show that vacuole membranes stimulate actin polymerization via a Cdc42p-de-
pendent signaling mechanism. We used an actin-sizing assay to show that F-actin is formed during incubations of vacuoles in conditions that support in vitro membrane fusion. We also show that polymerization-deficient actin is an inhibitor of vacuole fusion, and F-actin directly interacts with SNAREs that have longin domains. Our results suggest that membrane-stimulated actin polymerization is a subreaction of the membrane fusion mechanism.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Sample Preparation**—Yeast strains used in this study are listed in Table 1. Rho overexpression strains were prepared using Drop&Drag cloning (31) as described under supplemental data. Strains were grown at 28 °C in YPD (1% yeast extract, 2% peptone, 2% dextrose), or YPDGk (1% yeast extract, 2% peptone, 1% dextrose, 1% galactose, 30 µg/ml kana-mycin) when growing Rho overexpression strains. Cytosol was prepared from strain K91-1A grown to ~4 A600 by vortexing cells for 10 min at 4 °C with glass beads using a ratio of 1 vol of glass beads to 2 vol of cells, resuspended at 1/100th the original culture volume in lysis buffer (20 mM Pipes-KOH, pH 6.8, 200 mM sorbitol, 1 mM Mg-ATP, 1 mM dithiothreitol, 2× PIC). Lysates were cleared by centrifugation at 25,000 × g for 30 min at 4 °C. Typical cytosol protein concentrations were 20 mg/ml. Actin was purified from cytosol by DNasel affinity chromatography followed by DEAE ion exchange chromatography as previously described (32). Actin was concentrated to ~4 mg/ml using a Centricron-10 (Millipore).

**Biochemical Reagents**—Reagents were purchased from Sigma unless otherwise indicated; the actin binding drugs jasplakinolide (Invitrogen) and latrunculin B (Biomol) were dissolved in Me2SO at 1 mM; phalloidin was dissolved in methanol and U73122 and U73343 were dissolved in ethanol at 10 mM; apyrase VII was dissolved in PS-buffer (20 mM Pipes-KOH, pH 6.8, 200 mM sorbitol) at 1 unit/µl. Vrp1p, Rd1p, and Gdi1p were prepared and used as previously described (27, 29). Antibodies against Cdc42p, Rho1p, and Vrp1p were purified as IgG from rabbit sera as previously described (16, 29). 1× ATPreg (ATP-regenerating system) was made as a 10× stock solution in PS-buffer (10× ATPreg = 5 mM ATP, 5 mM MgCl2, 200 mM creatine phosphate, 2 mg/ml creatine kinase). Protease inhibitor mixture (PIC) was made as a 60× stock solution (60× PIC = 10 µg/ml leupeptin, 20 µg/ml pepstatin, 25 mM o-phenanthroline, 5 mM Pefabloc SC).

| Strain        | Genotype                        | Source |
|---------------|---------------------------------|--------|
| K91-1A        | MatA, pho8::Al134, pho13::PH113, his3, ura3, lys2 | This study |
| BJY38         | Mato, pep7::kanMX, prb1::LEU2, his3, leu2, ura3 | This study |
| KTY1          | Mato, pep7::kanMX, prb1::LEU2, his3, leu2, ura3 | This study |
| KTY2          | Mato, pep7::kanMX, his3, leu2, ura3, lys2 | This study |
| KTY5          | Mato, vrp1::kanMX, pep7::URA3, prb1::LEU2, his3, leu2, ura3, lys2 | This study |
| DBY6945       | Mato, his3, leu2, ura3, tub2, act1::LEU2, pRb668(pRA3)::ACT1 | This study |
| IDY318        | Mato, his3, leu2, ura3, tub2, act1::LEU2, idp318(URA3)::A3-29/C374A | This study |
| LJYC42wt      | Mato, pep7-3, prb1-1122, prc1-407, leu2, ura3, pGREG555:CDC42 | This study |
| LJYC42G       | Mato, pep7-3, prb1-1122, prc1-407, leu2, ura3, pGREG555:CDC42-G12V | This study |
| LJYC42T       | Mato, pep7-3, prb1-1122, prc1-407, leu2, ura3, pGREG555:CDC42-T17N | This study |
| LJYR1wt       | Mato, pep7-3, prb1-1122, prc1-407, leu2, ura3, pGREG555:ROHI1 | This study |
| LJYR1G        | Mato, pep7-3, prb1-1122, prc1-407, leu2, ura3, pGREG555:ROHI1-G19V | This study |
| LJYR1T        | Mato, pep7-3, prb1-1122, prc1-407, leu2, ura3, pGREG555:ROHI1-T24N | This study |

**Vacuole Isolation, Fusion, and Pretreatment Reactions**—Vacuoles were isolated from yeast by floatation on Ficoll gradients as previously described (33). Vacuole fusion was assayed essentially as previously described (33). Briefly, fusion reactions (30 µl) contained 3.5 µg of vacuoles from each of KTY1 (pro-ALP fusion reporter) and KTY2 (protease donor to cleave pro-ALP upon fusion) in fusion reaction buffer (F.R.B. = 20 mM PIPES-KOH, pH 6.8, 125 mM KCl, 5 mM MgCl2, 200 mM sorbitol, 10 µM CoA, 1× PIC), 1× ATPreg, and 0.5 mg/ml cytosol. To assay the stimulation of actin polymerization, vacuoles were preincubated at 30 °C in buffers normally used for in vitro vacuole fusion. Reactions contained 0.3 mg/ml of vacuoles in F.R.B. + 1× ATPreg: 0.5 mg/ml of cytosol was included where indicated. Vacuoles were re-isolated (centrifugation at 18,000 × g, 4 °C, 5 min) from preincubation reactions after 2-fold dilution in cold PS-buffer and then added to polymerization assays.

**Pyrene-actin Polymerization Assay**—To assay actin polymerization activity we used an established pyrene-actin polymerization assay (34). Briefly, 70 µl of a sample dissolved in G-buf/ NP/Mg (5 mM Tris-Cl, pH 8, 0.2 mM CaCl2, 0.2 mM ATP, 0.17% Nonidet P-40, 0.35 mM MgCl2) was mixed with 50 µl of 6–12 µM actin polymerization stock mixture containing 35% pyrene-labeled actin in G-buffer (5 mM Tris-Cl, pH 8, 0.2 mM CaCl2, 0.2 mM ATP) (Cytoskeleton Inc.). Actin polymerization buffer (10× A.P.B. = 50 mM Tris-Cl, pH 8, 500 mM KCl, 20 mM MgCl2, 10 mM ATP) was added to some reactions, which initiates spontaneous polymerization because of a lowering of the critical concentration for polymerization (34). Fluorescence intensity readings were taken every 18 s using a QM-4SE spectrofluorimeter (Ex360/Em407, 10-nm bandwidth, 2-s integration), with a four position heated sample holder set to 30 °C (Photon Technologies Inc.). Baseline fluorescence of the pyrene-actin stock mixture was taken for ~5 min, test samples were then added, and measurements continued for 1 h. Actin polymerization activity (A.P.A) was calculated from polymerization curves by determining the average rate of fluorescence intensity increase for 3000 s of reaction time (i.e. after sample addition, multiple slopes were calculated over the course of a reaction and averaged), divided by the micrograms of test sample protein (ΔFI/µg).

**Actin Sizing Assay**—To examine the dynamics of yeast actin assembly on intact vacuoles, reactions devoid of cytosol, but containing 0.1 µM purified yeast actin were analyzed for
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![Graphs A, B, C, D](image)

**FIGURE 1. Determination of actin polymerization activity.** The pyrene-actin polymerization assay and its components are described under “Experimental Procedures.” A, stimulation of actin polymerization by yeast cytosol (cyto). Polymerization reactions contained 3.5 μg pyrene-actin and 0.1 mg/ml cytosol. 10 μM jasplakinolide, which stabilizes F-actin, enhanced cytosol-stimulated polymerization (cyto + JP), whereas 10 μM latrunculin B, which destabilizes F-actin, inhibited polymerization (cyto + Lat B). B, stimulation of actin polymerization by purified yeast vacuoles. Polymerization reactions contained 3.5 μg pyrene-actin and 0.25 mg/ml vacuoles isolated from wild-type (KTY1) or vrp1Δ (KTY5) strains. Actin polymerization buffer (1/40th vol.) was added near the middle of experiments (A.P.B. added) to test the polymerization capacity of reactions. C, actual actin polymerization activities (A.P.A.) calculated from curves in A and B. D, Vrp1p recovered vacuole-stimulated polymerization activity in vacuoles from the vrp1Δ strain. Vacuoles were either preincubated in FRB (left bars), or FRB + 25 μg/ml semi-purified Vrp1p (right bars) for 20 min at 4 °C, reisolated and assayed for polymerization activity as in C. A.P.A. values (± S.E.) were normalized to wild-type vacuoles-no addition for each of three independent experiments. Typical polymerization curves and actual A.P.A. for Vrp1p add-back experiments are shown in the supplemental data (Fig. S1, A and B).

Changes in the size of actin complexes by size-exclusion chromatography. For this assay 150-μl reactions containing 0.3 mg/ml vacuoles in F.R.B. + ATPreg were incubated at 30 °C for the specified times and conditions. Reactions were stopped by transferring to ice and addition of 15 μl of 10× F-actin stabilization buffer (1 mM phalloidin, 5 mM MgCl2, 5 mM EGTA, and 5% Triton X-100). Membranes were dissolved by gentle mixing and incubation on ice for 15 min. 150 μl of the sample was applied to a Superose 6 HR 10/30 column (GE Healthcare) run at 0.5 ml/min in GF-running buffer (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 2 mM MgCl2, 0.5 mM EGTA, and 0.5% Triton X-100). 0.5-ml fractions were collected, and the elution profile of actin was analyzed by immunoblotting of equal fractions.

Immunoprecipitation of Actin-Vacuole Protein Complexes and Direct Binding Assays—150-μl reactions containing 0.3 mg/ml vacuoles in F.R.B. + ATPreg plus cytosol were incubated for the indicated times and conditions. Membranes were then washed by 2-fold dilution in PS-buffer, re-isolated by centrifugation, and dissolved in 400 μl of A-buffer (20 mM HEPES-KOH, pH 7.5, 80 mM KCl, 2 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM EGTA, 0.5 mM EDTA, 0.2 mM ATP, 5% (v/v) glycerol, 1.25% (wt/v) β-octylglucoside, 2× PIC). Actin-associated complexes were immunoprecipitated by addition of 100 μl of anti-actin antibodies cross-linked to protein A-agarose (20% slurry in A-buffer). Non-specific binding was determined in parallel using preimmune serum cross-linked beads. Binding reactions were incubated for 2 h at 4 °C with rotation. Beads were then washed three times in A-buffer, eluted with 500 μl of 100 mM glycine-HCl, 0.5% Triton X-100, pH 2.5, followed by trichloroacetic acid precipitation and immunoblot analysis. Production of GST and MBP-tagged proteins was performed as previously described (36). Direct binding of F-actin and SNAREs was examined by incubating 10 μM of immobilized GST-Vam3p, GST-Vam7p, GST-Vti1p, GST-Nyv1p, GST, or 10 μM MBP-Ykt6p or MBP with 30 μM yeast G-actin in 1× A.P.B. 0.2% Nonidet P-40 for 1 h at 4 °C, or at 27 °C to allow for actin polymerization. Actin and SNARE interactions were determined by immunoblotting for actin after washing beads in HS-buffer (20 mM Tris-HCl, pH 8, 500 mM NaCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.2% Nonidet P-40).

**Microscopy and in Vivo Analyses—**Vacuoles were stained with FM4-64 (27), and vacuole morphology was either observed while cells were still growing in YPD media (A600 ~ 1) after incubation for 1 min in hypotonic conditions (1:5 dilution in water). This hypotonic shock has previously been shown to induce vacuole fusion in vivo (37). Images were acquired using a Axioskop2 with a 100×/1.4 NA plan apochromat oil immersion lens (Zeiss), a CoolSnap HQ camera (Photometrics) and ImagePro Plus software (Media Cybernetics).

**RESULTS**

We have recently shown that actin ligands, which block either F-actin assembly or disassembly, inhibit vacuole membrane fusion (16). These results suggested that both actin depolymerization and repolymerization (i.e. actin remodeling) was needed for membrane fusion. However, this does not agree with the traditional view that actin is a barrier requiring only depolymerization. Our approach here was to directly examine vacuole-associated actin polymerization activity. To determine the capacity of a sample to stimulate actin polymerization we first
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A, actin polymerization activity was determined for wild-type vacuoles (BJ2168) that were preincubated for 0–80 min in either PS-buffer (black bars), F.R.B. + 1 × ATPreg, or F.R.B. + 1 × ATPreg and 0.5 mg/ml cytosol (hatched bars). At the specified times vacuoles were washed in PS-buffer, reisolated and added to pyrene-actin polymerization mixtures. Polymerization reactions contained 5 μM pyrene-actin and 0.33 mg/ml vacuoles. Shown are average A.P.A. values (± S.E.) from at least three experiments normalized to unincubated samples. Typical polymerization curves and A.P.A. values are shown in supplemental data (supplemental Fig. S1, A–D). Actin polymerization activities from pyrene-actin titrations containing 0.33 mg/ml vacuoles preincubated as in A and B. Polymerization curves for actin titration are shown in supplemental data (supplemental Fig. S1, C and D).

We next performed a vacuole titration to determine the optimal concentration needed to stimulate actin polymerization. We found that polymerization increased with increasing vacuole concentration with peak specific activity obtained at vacuole concentrations of 0.33 mg/ml (Fig. 2, A and C). The titration was repeated with vacuoles that were preincubated for 30 min in a reaction buffer typically used for in vitro membrane fusion (33). This showed a similar optimal concentration; however, A.P.A. values were significantly increased compared with unincubated vacuoles (Fig. 2, B and C). Pyrene-actin was also titrated which showed optimal actin concentration were ~5 μM for our assay conditions (Fig. 2D).

Because optimization experiments revealed that preincubation of vacuoles increased polymerization activity (Fig. 2, C and D), compare 0 min bars to 30 min bars, we further tested the effect of vacuole preincubation on their capacity to stimulate actin polymerization. Vacuoles were incubated for 0–80 min in F.R.B., reisolated and assayed for stimulation of actin polymerization. Initially, activity levels decreased; however, after 40 min of incubation, activity levels rose 1.6-fold higher than unincubated samples (Fig. 3A, - cytosol). Including cytosol in preincubations stimulated a 2.4-fold increase in activity, which was also used pyrene-labeled actin, which undergoes a fluorescence intensity increase when incorporated into F-actin (33). As expected, yeast cytosol, which stimulates vacuole membrane fusion (16, 27), also stimulated the polymerization of pyrene-
Regulation of Vacuole-associated Actin Polymerization

A

B

C

FIGURE 4. Analysis of endogenous vacuole-stimulated actin dynamics. The remodeling of unlabeled yeast actin by intact vacuoles was examined by actin-sizing assay as described under "Experimental Procedures." A, wild-type vacuoles (KT1) were incubated in F.R.B. + 1× ATPreg and 0.1 µM yeast actin. At the indicated times, reactions were stopped by placing on ice; F-actin was stabilized, and membranes were dissolved by addition of F-actin stabilization buffer. Samples were passed over a Superose 6 HR 10/30 column for size separation and eluted fractions were probe for actin by immunoblot. B, actin-sizing assays as in A, except that vacuoles were incubated for 60 min in the presence of 0.05 units/µl apyrase (−ATP), or were isolated from a vrp1Δ strain (KT5). C, elution profile of other vacuolar proteins from actin-sizing assays as in A, incubated for 60 min. Load = 5% of the starting sample; HMW, high molecular weight; LMW, low molecular weight.

more rapid with maximal activity levels obtained after only 20 min of preincubation (Fig. 3, + cytosol). These data suggest that purified vacuoles contain residual actin polymerization activity upon isolation, which is initially reduced during preincubation while longer incubations can regenerate activity. We tested this hypothesis by incubating vacuoles in the absence of reaction buffer, and these did not recover activity (Fig. 3, −rxn buffer). Hence, residual activity was "run-down" in the absence of an adequate reaction buffer. We also tested membrane fusion for these conditions and times (Fig. 3B). Although A.P.A. levels did not temporally coincide with membrane fusion levels, reactions condition that enhanced (+ cytosol) or reduced (−rxn buffer) membrane fusion had similar effects on A.P.A. levels.

Endogenous Actin Remodeling—Intact vacuoles could not be directly assayed using the pyrene-actin assay because of light scattering during fluorescence measurements. Therefore, we developed an actin-sizing assay based on gel filtration chromatography, which would examine the remodeling of unlabeled yeast actin (rather than pyrene-actin) by intact vacuoles. For this assay, vacuoles were incubated in membrane fusion reaction buffer and, at specific times, membranes were solubilized and actin filaments stabilized by the addition of 10× F-buffer. Samples were then applied to a Superose 6 column for size-fractionated and the elution profile of actin was determined by immunoblot analysis. Large complexes representing F-actin eluted near the column void volume (fractions 13–15) well separated from monomeric actin (fractions 31–35) and what likely represents dimer/trimer nucleating actin fragments (fractions 27–30) (see Ref. 35). Cytosol was not included in these reactions to limit our examination specifically to vacuole-stimulated changes in F-actin. This analysis showed that actin was initially monomeric with small amounts of F-actin, however, incubation triggered the formation of F-actin (Fig. 4A). This agrees well with our previous results which showed that vacuoles were more active in stimulating pyrene-actin polymerization after 20–40 min of preincubation (Fig. 3). Similar experiments were performed with vacuoles purified from the Vrp1p gene deletion strain, and these did not show significant F-actin formation (Fig. 4B, vrp1Δ). Reactions done in the presence of apyrase (i.e. the absence of ATP) showed reduced F-actin formation (Fig. 4B, −ATP). These results suggest that F-actin formation is an ATP-dependent subreaction of in vitro vacuole membrane fusion. Most vacuolar proteins did not localize to the high molecular weight fractions (Fig. 4C, Vam3p); similar distribution was observed for, Nyv1p, alkaline phosphatase and Vac8p).4 However, V-ATPase subunits migrated at intermediate and high molecular weight fractions (Fig. 4C, Vph1p). As well, small amounts of Ykt6p and Vrp1p co-localized with F-actin (Fig. 4C, Ykt6, Vrp1p).

Components of the Actin-remodeling Pathway—To further define conditions and components necessary for vacuole-associated actin polymerization activity, we determined A.P.A. values for vacuoles preincubated in a variety of conditions (i.e. ± salts/ATPreg/cytosol/inhibitors). We found that membrane-associated polymerization activity required incubation with physiological salt and was significantly enhanced when cytosol was included (Fig. 5A). However, polymerization activity was not significantly dependent on the presence of ATPreg because the highest A.P.A. levels were obtained when incubations contained only salt and cytosol (Fig. 5A, lane 6). This apparently differs from actin-sizing assay results which showed that apyrase inhibits vacuole-stimulated F-actin formation. However, this difference is likely due to ATP concentration, because ATPreg would provide continuously high levels of ATP, which has been previously shown to diminish polymerization activity (39). It is possible that ATP levels supplied by the addition of cytosol were sufficient for maximal polymerization activity. Preincubation of vacuoles in the presence of apyrase to eliminate ATP, reduced A.P.A. values (Fig. 5B, APY) which supports the need for an ATP-dependent mechanism to drive vacuole-stimulated polymerization. Phosphatidylinositol-(4,5)-diphosphate can be generated in the presence of ATP (17) and can be digested to inositol trisphosphate and diacylglycerol by phospholipase C (Plc1p); these lipid-signaling molecules activate

4 G. Eitzen, unpublished observations.
FIGURE 5. Components required for vacuole-associated actin polymerization activity. A.P.A. was calculated from polymerization reactions containing 5 μM pyrene-actin and 0.33 mg/ml wild-type vacuoles (KT1) preincubated for 30 min at 30 °C in F.R.B + ATPreg and 0.5 mg/ml cytosol, plus additional factors or conditions as indicated. A, actin polymerization activities of vacuoles preincubated in the presence of no inhibitor (none), 0.05 units/μl apyrase (APy), 5 μM RabGDI (GDI), 8 μM RhoGDI (RDI), or 0–90 μM of the Plc1p inhibitor U73122 or its inactive analog U73343. A set of reactions were performed without inhibitor, but were proteinase K-treated (PrK) following preincubations (10 min with 0.35 μM proteinase K); then 0.1 mM phenylmethylsulfonyl fluoride to inhibit proteinase K. The effect of inhibitor treatment on the level of vacuolar Rho and Rab GTases is shown in supplemental data, Fig. S3. D, actin polymerization activities of vacuoles preincubated in the presence of 0.1 mg/ml preimmune, Cdc42p, Rho1p, and Vrp1p IgG. Shown are average activities (± S.E.) calculated from three experiments normalized to control reactions (A, lane B; no inhibitor; C, no drug, D, proteinase K).

We also tested whether Rab and Rho GTases were required for vacuole-stimulated actin polymerization. Membrane extraction of the vacuolar Rab protein, Ypt7p, or the Rho proteins, Rho1p and Cdc42p, was achieved by preincubation with Gdi1p and GST-Rdi1p, respectively (supplemental data, Fig. S3). Polymerization activity was reduced only by GST-Rdi1p (Fig. 5B). Taken together, these results suggest that during membrane fusion an actin polymerization-promoting complex is formed on the vacuole membrane with components that can be recruited from the cytosol to enhance activity. As well, polymerization activity is likely regulated by Rho GTases because incubation with Rdi1p reduced activity.

role for Cdc42p in vacuole-stimulated actin polymerization. Paradoxically, expression of dominant-inactive Rho1p-T24N also caused an increase in vacuole-associated actin polymerization activity and, therefore, we cannot fully exclude a role for Rho1p.

Polimerization-deficient Actin Inhibits Vacuole Fusion—To directly test whether actin polymerization is needed for vacuole fusion we examined vacuole morphology in a polymerization-deficient actin mutant strain (Refs. 32, 42; see supplemental data, Fig. S5). Under normal growth conditions, this strain has fragmented vacuoles, which is indicative of a membrane fusion defect (Fig. 7A, normal). Furthermore, stimulation of in vivo vacuole fusion via hypotonic shock (37) triggered the fusion of wild-type vacuoles, but not vacuoles in the polymerization-deficient strain (Fig. 7A, hypotonic). We also added purified wild-type and polymerization-deficient actin into in vitro vacuole fusion reactions. Wild-type actin had little effect on fusion up to 1 μM; however, mutant actin inhibited fusion at all but the lowest concentration tested (Fig. 7B). These results support the need for actin polymerization during membrane fusion.
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Actin-Vacuolar Protein Interactions—Finally, we wanted to identify actin-vacuole protein interactions that occur over the course of fusion reactions. Actin antibodies cross-linked to protein A-agarose were used to affinity isolate complexes from vacuoles that were incubated for 0, 30, and 60 min in fusion reaction buffer. Control experiments included reactions done in the presence of 10 μM phalloidin to stabilize F-actin, or in the presence of 10 μM Lat B to destabilize F-actin. Many vacuolar proteins examined did not show interactions (Fig. 8A, Nyv1p; not shown, but tested include ALP, Vam3p, Vam7p, Vti1p, Vac8p, and Rho and Rab GTPases). However, the SNARE protein Ykt6p showed increased actin association over time when phalloidin treated, and reduced association when Lat protein Ykt6p showed increased actin association over time or Vac8p, and Rho and Rab GTPases). However, the SNARE proteins examined did not show interactions (Fig. 8A, Nyv1p; not shown, but tested include ALP, Vam3p, Vam7p, Vti1p, Vac8p, and Rho and Rab GTPases). However, the SNARE protein Ykt6p showed increased actin association over time when phalloidin treated, and reduced association when Lat B-treated (Fig. 8A). The vacuolar V1-ATPase subunit Vma2p also showed association, but levels were relatively unchanged by incubation time suggesting this association was not specifically generated through the reaction process. An association between the V1-ATPase subunit B (Vma2p homolog) and actin has been previously reported in osteoclasts, which may have a role in targeting of the V1-ATPase to membranes allowing for spatiotemporal regulation of acidification (43).

We also tested whether Ykt6p and other vacuolar SNAREs directly bind to actin. MBP-tagged Ykt6p and GST-tagged Vam3p, Vam7p, Vti1p, and Nyv1p were expressed in E. coli, immobilized on amylose or glutathione affinity beads and then incubated with purified yeast actin (we would have liked to use GST-Ykt6p but this was insoluble in our hands). Immunoblot of the bead-bound fraction clearly showed an association with Ykt6p, which increased after an incubation period that would stimulate actin polymerization (Fig. 8C, Ykt6p pull-down, compare ice and 27 °C). This agrees with the results from our immunoprecipitation experiment. GST-Vam3p (Fig. 8C), GST-Vam7p, GST-Vti1p, GST, and MBP (Fig. S6, supplemental data) all did not interact with actin. However, GST-Nyv1p did show an interaction-specifically with F-actin (Fig. 8C, Nyv1p 27 °C). Because Nyv1p was not identified under native conditions it is likely that actin interacts with SNAREs that are not part of a SNARE complex, and therefore the interaction with Ykt6p may be related its non-SNARE complex functions (44, 45). Interestingly, the structures of both Nyv1p and Ykt6p contain N-terminal longin domains extension, which are thought to fold into profilin-like domains (44, 46). Profilin is known to bind actin and stimulate cytoskeletal remodeling (47) and because both longin domain SNAREs have the potential to interact with actin, we speculate that this domain may have an important role in directing actin remodeling specifically needed for membrane fusion.
DISCUSSION

Studies of membrane fusion using yeast vacuoles have revealed a complex, multistep mechanism (28). We have previously shown that proteins involved in actin remodeling such as Las17p and Vrp1p, the WASp-WIP complex of yeast, are needed for vacuole fusion (16, 27), and drugs that block either actin polymerization or depolymerization inhibit vacuole fusion in vitro. Therefore, membranes undergoing fusion might stimulate actin polymerization if this was a key element of the fusion process. To test this we used a sensitive in vitro pyrene-actin polymerization assay (43) and showed that vacuoles indeed stimulate actin polymerization. Incubation of vacuoles in buffers that support membrane fusion also increased their capacity to stimulate polymerization (Fig. 3). This activity requires the common actin remodeling machinery of the WASp/WIP complex, which is associated with the vacuole membrane (16). Deletion of VRP1 (yeast WIP) resulted in a loss of polymerization activity (Fig. 1, B–D, vrp1Δ) and fusion activity (27). Taken together with results showing polymerization-deficient actin inhibits vacuole fusion (Fig. 7), we conclude that vacuoles actively catalyze actin polymerization, and that this activity is linked to the membrane fusion mechanism (see model, Fig. 9).

We examined numerous inhibitors of vacuole fusion to determine how vacuolar actin polymerization activity is connected to the vacuole fusion pathway. Inhibitors that block the tethering/docking stages of membrane fusion such as Rab/SNARE inhibitors (28) had little effect on polymerization activity (Fig. 5B). This suggests that the polymerization mechanism is driven parallel to the fusion mechanism at this stage of the reaction. Vacuole priming is needed prior to tethering/docking and requires the activity of the AAA-ATPase, Sec18p, and ATP (28). Vacuolar polymerization activity was also dependent on ATP priming (Figs. 4 and 5, A and B), however, this did not seem to be linked to Sec18p priming since anti-Sec18p antibod-
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ies did not inhibit polymerization activity. Another fusion factor requiring ATP is the generation of the phospholipid PI(4,5)P2 (40). More recently, diacylglycerol, the metabolic product of PI(4,5)P2 cleavage via PtdInsP2 has also been shown to be required for fusion (41). Although the exact role these lipid molecules play in fusion remain elusive, here we have shown that the PtdInsP2 inhibitor, U73122, enhanced vacuole-associated polymerization activity and therefore the stimulation of polymerization activity may be linked to reactions that generate PI(4,5)P2 during fusion.

We also showed that actin polymerization is regulated by Cdc42p. It was previously shown that Cdc42p has a role in vacuole fusion (29, 30), and then subsequently shown that actin remodeling is needed for membrane fusion (20). However, a direct link between actin remodeling and Cdc42p was not shown, and this was additionally uncertain since both Cdc42p and Rho1p are localized to the vacuole membrane and required for fusion (29). Here we provide two lines of evidence that directly link Cdc42p to the regulation of actin polymerization. First, this activity was reduced when vacuoles were isolated from a strain expressing dominant-inactive Cdc42p (Fig. 6), and, secondly, vacuoles incubated with Cdc42p antibodies show reduced activity (Fig. 5D). Our results are supported by previous in vivo studies in PC12 cells, which showed that exocytosis could be enhanced by expressing dominant-active Cdc42p (48). It was also shown that polymerization via WASp/WIP activation played a positive role in secretory pathways and that latrunculin treatment blocks secretion (48). We achieved limited enhancement of in vitro actin polymerization via dominant-active Cdc42p; however this may be caused by other limiting factors such as Cdc42p effector proteins. Interestingly, we observed enhanced polymerization when dominant-negative Rho1p was expressed. We speculate that Rho protein cross-talk may account for this unexpected result because there are many examples of such cross-talk in other systems (49, 50). We are currently investigating whether vacuolar Cdc42p and Rho1p cross-talk during the activation of actin polymerization and membrane fusion.

To provide direct evidence that actin polymerization is needed for membrane fusion we characterized a polymerization-deficient actin mutant strain. This strain showed vacuole fusion defects in vivo and purified polymerization-deficient actin inhibited vacuole fusion in vitro (Fig. 7). Wild-type actin also inhibited fusion, but only at very high concentrations, which suggest a critical balance is needed between the levels of actin needed to promote fusion as opposed to higher levels which inhibit fusion. This could function through a balanced association with factors involved in fusion such that high concentrations of actin would sequester this factor away from its desired function. Indeed, we found that two vacuole SNAREs, Ykt6p, and Nvy1p, directly bind F-actin in a purified system, although only Ykt6p binds actin during vacuole fusion (Fig. 8). These SNAREs, along with actin have been shown to assemble into a vertex ring structure on docked vacuoles, and it is at this site where membrane fusion occurs (20, 51, 52). Coordination of protein enrichment in the vertex ring (SNAREs for example) was shown to largely depend on actin such that drugs which affect G- to F-actin transitions blocked this enrichment (52). Interestingly, the two SNAREs that we found to interact with actin contain longin domains. Longin domains are ~100 amino acid extension that occur in a small subset of R-SNAREs (three in yeast: Nvy1p, Ykt6p, and Sec22p) as well as in several other proteins required for vesicle trafficking (53). The longin domain of Nvy1p has been shown to aid in its membrane targeting and thus facilitate protein sorting to the vacuole (46). Currently there are very few studies that report direct actin-SNARE interactions (54); however several studies report functional co-localization of actin and SNAREs (52, 55–57). Future experiments are aimed at determining whether longin domains are essential for actin interactions, and the role these interactions might have in the membrane fusion mechanism.

In conclusion, our results support the hypothesis that actin polymerization is a subreaction of the membrane fusion mechanism, which is regulated by membrane-bound Cdc42p, PI(4,5)P2, and Vrp1p (Fig. 9). We view F-actin formation as a positive effector of membrane fusion and, through interactions with docking proteins, may provide local structural elements to spatially restrict fusogenic complex assembly.

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