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Rho1p and Cdc42p act after Ypt7p to regulate vacuole docking

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Rho GTPases, which control polarized cell growth through cytoskeletal reorganization, have recently been implicated in the control of endo- and exocytosis. We now report that both Rho1p and Cdc42p have a direct role in mediating the docking stage of homotypic vacuole fusion. Vacuoles prepared from strains with temperature-sensitive alleles of either Rho1p or Cdc42p are thermolabile for fusion. RhoGDI (Rdi1p), which extracts Rho1p and Cdc42p from the vacuole membrane, blocks vacuole fusion. The Rho GTPases can not fulfill their function as long as priming and Ypt7p-dependent tethering are inhibited. However, reactions that are reversibly blocked after docking by the calcium chelator BAPTA have passed the point of sensitivity to Rdi1p. Extraction and removal of Ypt7p, Rho1p and Cdc42p from docked vacuoles (by Gdi1p, Gyp7p and Rdi1p) does not impede subsequent membrane fusion, which is still sensitive to GTPγS. Thus, multiple GTPases act in a defined sequence to regulate the docking steps of vacuole fusion.

Keywords: Cdc42p/GTPase/membrane fusion/Rho1p/*Saccharomyces cerevisiae*

Introduction

Small GTPases of the Rab, Arf and Rho families regulate several key steps of vesicle trafficking (Bourne *et al*., 1991; Matozaki *et al*., 2000). These 20–30 kDa monomeric proteins bind and hydrolyze GTP and bind membranes via lipid modifications. They act as molecular switches that cycle between active (GTP-bound) and inactive (GDP-bound) states. Effector complexes associate with the active state and are responsible for transducing downstream effects. The bound-nucleotide state is regulated by guanine-nucleotide exchange factors (GEFs), which exchange GTP for GDP, and the subsequent hydrolysis of GTP is accelerated by GTPase-activating proteins (GAPs). Membrane association is controlled by a guanine-dissociation inhibitor (GDI), which extracts the GDP-bound form of the protein and may aid in its recycling.

Each family of GTPases has a characteristic function in trafficking. Arf proteins regulate vesicle generation (Chavier and Goud, 1999). Vesicle budding requires Arf-GTP and coat complexes, while subsequent uncoating requires Arf-GTP hydrolysis. On uncoated membranes, activated Rab proteins associate with effector complexes to drive the early tethering stages of vesicle docking and help to establish docking specificity. The Rho family of GTPases transduce signals for cytoskeletal reorganizaton and cell polarity (Drubin and Nelson, 1996; Cabib *et al*., 1998; Hall, 1998). This is necessary for the spatial targeting of vesicles within cells (Govindan *et al*., 1995; Pruyne *et al*., 1998; Guo *et al*., 2001). Rho proteins are also implicated in the regulation of endo- and exocytosis. For example, addition of rhoGDI to permeabilized mast cells blocks stimulated secretion, suggesting that a crucial Rho protein has been extracted (Brown *et al*., 1998; Hong-Geller and Cerione, 2000). Activated Cdc42p regulates cytoskeletal assembly, which controls antigen endocytosis in dendritic cells and basolateral endocytosis in MDCK cells (Kroschewski *et al*., 1999; Garrett *et al*., 2000). Endosome dynamics is governed by RhoD, which catalyzes actin cytoskeleton disassembly (Murphy *et al*., 1996). Finally, membrane fusion can be stimulated by actin disassembly (Vitale *et al*., 1991; Muallem *et al*., 1995) or cycles of actin polymerization and depolymerization (Vitale *et al*., 1995; Bernstein *et al*., 1998; Lang *et al*., 2000), which may result in the transient removal of a physical actin barrier. Each of these reactions may be controlled by Rho proteins (Norman *et al*., 1994; Hall, 1998). Not all studies support the 'actin barrier' model, since actin polymerization or stabilizing agents act as positive effectors of membrane fusion in some systems (Koffer *et al*., 1990; Jahraus *et al*., 2001). Other effects of Rho proteins on vesicular traffic may be independent of cytoskeletal reorganization. For example, Rho3p interacts with the exocytosis machinery in yeast, and several subunits of the exocyst complex, including the rab GTPase Sec4, can act as multicopy suppressors of rho3Δ (Imai *et al*., 1996; Adamo *et al*., 1999; Robinson *et al*., 1999). However, for all of these trafficking events, it is not clear whether Rho proteins act on membrane fusion *per se* or merely regulate an actin barrier or the cytoskeletal 'tracks' for motor-driven vesicle movement to docking sites.

Homotypic fusion of yeast vacuoles can occur *in vitro* without added cytosol, allowing a detailed examination of those GTPase-regulated events that occur in the absence of cytoplasmic cytoskeletal meshwork. This reaction occurs in three sequential stages: priming, docking and membrane fusion (see Wickner and Haas, 2000). We now report that docking requires not only the Rab GTPase Ypt7p, but also two Rho GTPases, Rho1p and Cdc42p. These Rho proteins can only function after Ypt7p (Rab) function has been fulfilled, yet extraction of Ypt7p, Rho1p and Cdc42p from vacuoles that have completed docking does not impede subsequent fusion. This last stage of the reaction is still sensitive to the addition of GTPγS, although the molecular identity of its target is not yet established.

Results

Ypt7p is required for vacuole docking (Mayer and Wickner, 1997). Once docking is complete, the final stage of membrane fusion is not affected by the extraction of Ypt7p yet remains sensitive to GTPγS (Eitzen *et al*., 2000). Our current studies began with a search for a late acting GTPase. The yeast genome encodes five *RHO* genes and *CDC42*; antibodies to Rho1p inhibited fusion (Figure 1A, lane 7). To further test whether Rho1p is required for fusion, the alkaline phosphatase gene (*PHO8*) was deleted from strain NHY21, which contains a temperature-sensitive allele of the essential gene *RHO1*, and from the wild-type parental strain ONHY1 (Yamochi *et al*., 1994). Vacuoles isolated from these strains were fused with vacuoles isolated from a strain in which the proteinase A gene, *PEP4*, is deleted and which therefore contain the catalytically inactive pro-alkaline phosphatase. Fusion, as measured by maturation of the pro-alkaline phosphatase by proteinase A donated from rho1-ts or wild-type vacuoles, occurred normally when vacuoles were mixed and incubated at 27°C; however, pre-incubation of vacuoles bearing a temperature-sensitive Rho1p at 37°C inhibited their capacity for fusion (Figure 1B, solid squares) whereas vacuoles with wild-type Rho1p were not thermolabile (solid circles). Similarly, vacuoles from a strain with a cdc42-ts allele were also thermolabile for fusion (Figure 1C, solid squares). Vacuoles from strains with deletions in the non-essential *RHO* genes (*RHO2–5*) were not defective for fusion (Figure 1D). Therefore, the two essential Rho GTPases, Rho1p and Cdc42p, are required for *in vitro* vacuole fusion.

To corroborate the role of Cdc42p in vacuole fusion, we examined the vacuole morphology in cdc42-ts cells. Several large round vacuoles were seen in cells at the permissive temperature in cdc42-ts (Figure 1E, cdc42-1) and parental strains (Figure 1E, CDC42). However, after a brief incubation at the non-permissive temperature (37°C), there was increased vacuole fragmentation in cells with a cdc42-ts allele. The rho1-ts strain also has fragmented vacuoles when incubated at the non-permissive temperature (Figure 1F). Antibodies to a peptide fragment of Cdc42p also inhibit vacuole fusion (Müller *et al*., 2001).

Rdi1p extracts Rho1p and Cdc42p from vacuoles and blocks fusion

Rdi1p blocks vacuole fusion (Figure 2A) by extracting membrane-bound Rho proteins such as Rho1p and Cdc42p (Masuda *et al*., 1994). A soluble lumenal enzyme, proteinase A, is not released from vacuoles during fusion reactions with Rdi1p, demonstrating that inhibition is not due to vacuolar lysis (Figure 2B).

Both Rdi1p and Gdi1p extract lipid-anchored GTPases from membranes (Garrett *et al*., 1994; Masuda *et al*., 1994). Their actions are distinct, as Rdi1p extracts Rho1p and Cdc42p from the vacuole but does not extract Ypt7p, while Gdi1p extracts Ypt7p but not Rho1p or Cdc42p (Figure 3A, lanes 1–3). Pre-incubation of vacuoles with GTPγS may affect extraction by Rdi1p or Gdi1p since only

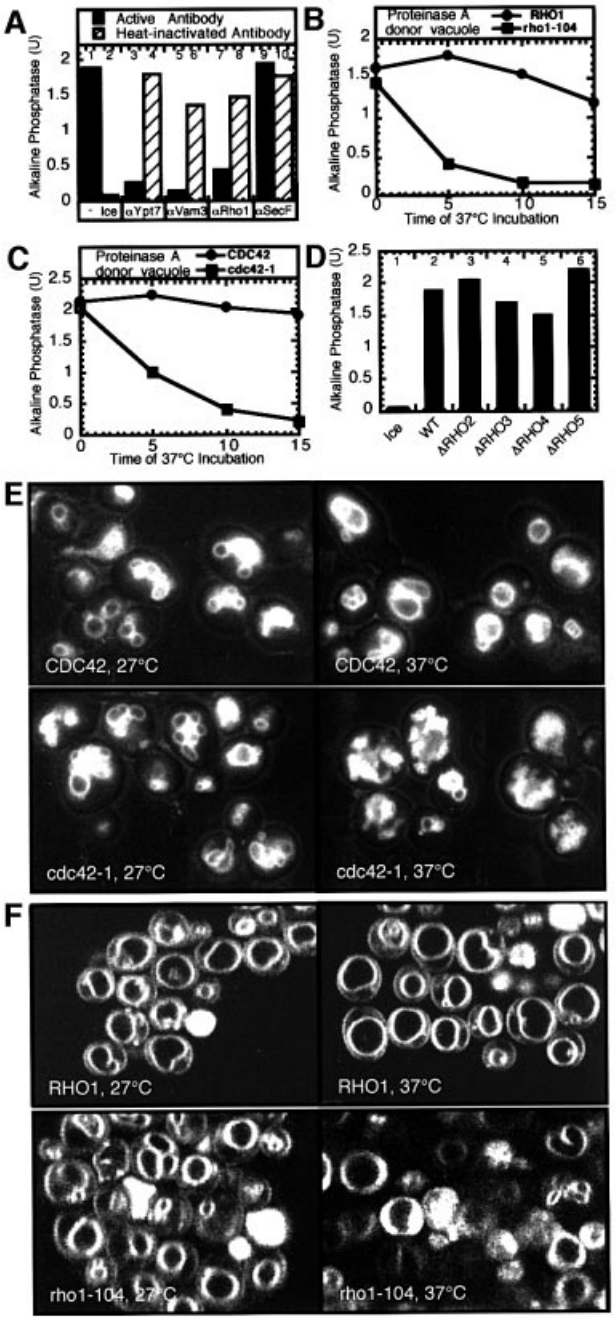

Fig. 1. Rho1p and Cdc42p are essential for vacuole fusion. (**A**) Anti-Rho1p antibodies inhibit vacuole fusion. Anti-Vam3p, anti-Rho1p or anti-SecF IgG fraction or affinity-purified anti-Ypt7p antibodies were added to standard fusion reactions. Aliquots were heated to 95°C for 10 min for heat inactivation. (**B** and **C**) Vacuole-containing rho1-ts or cdc42-ts proteins are thermolabile for fusion. Vacuoles isolated from strain (B) GEY6048 (*RHO1*) (circles) and GEY1658 (*rho1-104*) (squares) or (C) GEY6038 (*CDC42*) (circles) and GEY2298 (*cdc42-1*) (squares) were incubated at 37°C for the indicated times before adding to a fusion reaction with vacuoles from strain BJ3505. (**D**) Deletion of the *RHO2*, *3*, *4* and *5* genes does not affect vacuole fusion. Vacuoles isolated from strains GEY6028 (WT), GEY0908 (ΔRHO2), GEY2288 (ΔRHO3) and GEY1808 (ΔRHO5), which lack alkaline phosphatase, were tested for fusion with vacuoles from strain BJ3505. Vacuoles isolated from strain GEY0554 (ΔRHO4), which lacks proteinase A, were tested for fusion with vacuoles from strain DKY6281. (**E** and **F**) *In vivo* vacuole morphology in RHO mutant and parental strains. The vacuoles from strains TD4 (*CDC42*), DJTD2-16A (*cdc42-1*), ONHY1 (*RHO1*) and NHY21 (*rho1-104*) were stained with FM4-64. Cells were grown at 27°C to an OD_{600} ~ 1. An aliquot of each was removed and incubated at 37°C for 90 min prior to observation.

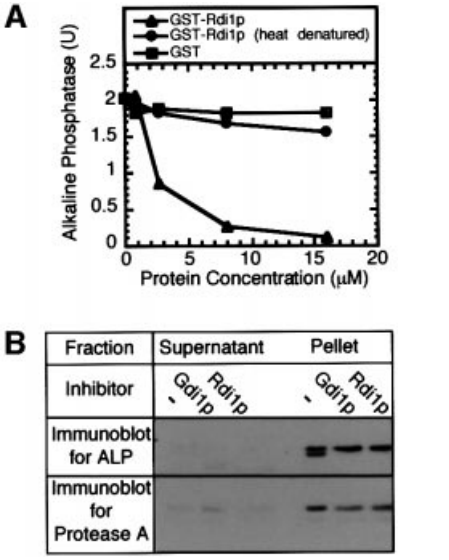

Fig. 2. Rdi1p (RhoGDI) blocks fusion. (**A**) Addition of GST-tagged Rdi1p (triangles), heat-denatured (95°C incubation for 10 min) GST–Rdi1p (circles) or GST (squares) to standard fusion reactions. (**B**) Inhibition by Rdi1p of pro-alkaline phosphatase processing is not due to vacuole lysis. Standard fusion reactions were incubated at 27°C for 90 min in the absence or presence of Gdi1p or Rdi1p. Reactions were centrifuged (5 min, 10 000 *g*), supernatants were removed from pelleted vacuoles, and equal fractions were analyzed by immunoblotting for proteinase A and alkaline phosphatase.

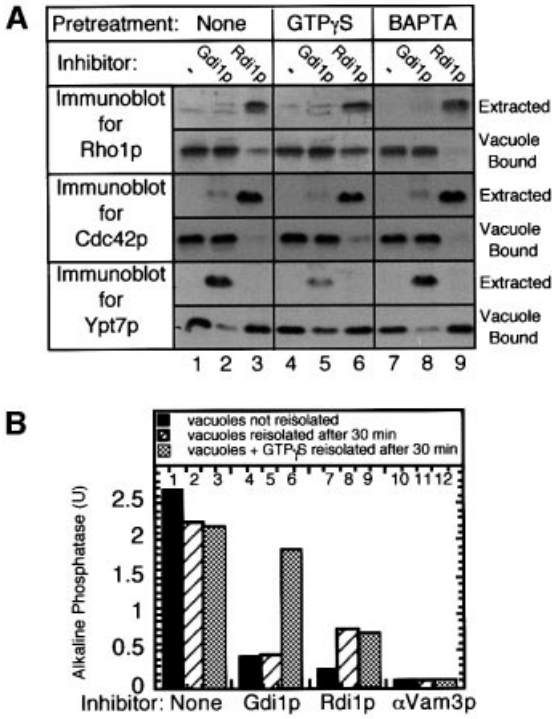

Fig. 3. Rdi1p extracts Rho1p and Cdc42p from vacuoles. (**A**) GTPase extractions. Standard fusion reactions (90 μl) with no inhibitor (None), 3 mM GTPγS or 3 mM BAPTA were incubated for 30 min at 27°C. Aliquots (30 μl) then received PS buffer (–), Gdi1p or Rdi1p and were further incubated for 60 min at 27°C. Reactions were centrifuged (5 min, 10 000 *g*) and equal fractions of supernatants and vacuole pellets were analyzed by immunoblotting for Rho1p, Cdc42p and Ypt7p. (**B**) GTPγS blocks Gdi1p but not Rdi1p inhibition. Fusion reactions were incubated with no inhibitor, Gdi1p, Rdi1p or anti-Vam3p antibodies in the absence (striped bars) or presence (gray bars) of GTPγS. After 30 min at 27°C, vacuoles were re-isolated, supplied with fresh reaction buffer without inhibitors and incubated for 60 min. Control samples without GTPγS (black bars) were not re-isolated.

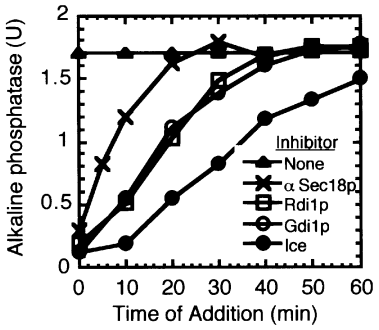

Fig. 4. Kinetic analysis of Rdi1p inhibition. Fusion reactions (270 μl) were incubated at 27°C. At the indicated times, aliquots (30 μl) were removed and added to tubes on ice or containing PS buffer, anti-Sec18p antibodies, Gdi1p or Rdi1p. Reactions were incubated for 90 min (total) and then assayed for alkaline phosphatase.

the GDP-bound form of each GTPase is extractable. The extraction of Ypt7p and Rho1p is inhibited when vacuoles are pre-incubated with GTPγS, although there is no change in the efficiency of Cdc42p extraction (Figure 3A, lanes 4–6). Pre-incubation with the calcium chelator BAPTA, another late stage inhibitor, does not affect GTPase extraction (Figure 3A, lanes 7–9), underscoring the specificity of Gdi1p and Rdi1p action. Pre-incubation of vacuoles with Rdi1p or Gdi1p irreversibly blocks fusion (Figure 3B, lanes 2, 5 and 8). Co-incubation of vacuoles with GTPγS and Gdi1p inhibits Ypt7p extraction (Figure 3A, lane 5) and thereby allows the reaction to resume when these inhibitors are removed after 30 min (Figure 3B, lane 6). Thus, the inhibitory effect of Gdi1p is overcome by locking Ypt7p in the GTP-bound form. However, incubation of vacuoles with GTPγS prevents neither the Rdi1p-mediated extraction of Cdc42p (Figure 3A, lane 9) nor the consequent irreversible loss of fusion (Figure 3B, lanes 7–9), underscoring the importance of Cdc42p for the fusion reaction.

Rho proteins act after Ypt/Rab proteins

In vitro vacuole fusion can be kinetically dissected into distinct stages of priming, docking and fusion. The stage affected by each inhibitor can be inferred from the time that it takes for the reaction to become insensitive to inhibitor addition. The fusion reaction becomes insensitive to the addition of Rdi1p after 30–40 min (Figure 4) with the same kinetics as for Gdi1p, an established inhibitor of docking (Mayer and Wickner, 1997; Eitzen *et al*., 2000). This curve of inhibition falls between priming, defined by the relative sensitivity to Sec18p antibodies, and the completion of fusion, measured by the mature, active alkaline phosphatase in samples transferred to ice at various times.

The *in vitro* reaction can also be blocked at specific stages by reversible inhibitors. We have employed this strategy, in three experiments, to show that the Rho GTPases can only act after Sec18p and Ypt7p and that these Rho GTPases have fulfilled their function before Ca^{2+}-triggered fusion. In one experiment, priming was

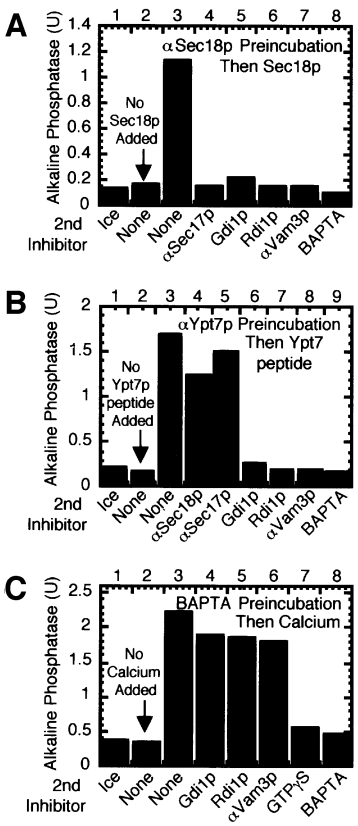

Fig. 5. Rho GTPases can only act after Sec18p and Ypt7p but vacuoles blocked for fusion with the calcium chelator BAPTA are past Rho function. Fusion reactions were incubated at 27°C in the presence of (**A**) anti-Sec18p antibodies, (**B**) anti-Ypt7 peptide antibodies or (**C**) BAPTA. After 30 min the inhibition was reversed by the addition of (A) His_6-Sec18p, (B) Ypt7p peptide or (C) calcium, in the presence of a second inhibitor or PS buffer (none) as indicated. Lane 2, no reversal of inhibition. Reactions were incubated for an additional 60 min before assaying for alkaline phosphatase.

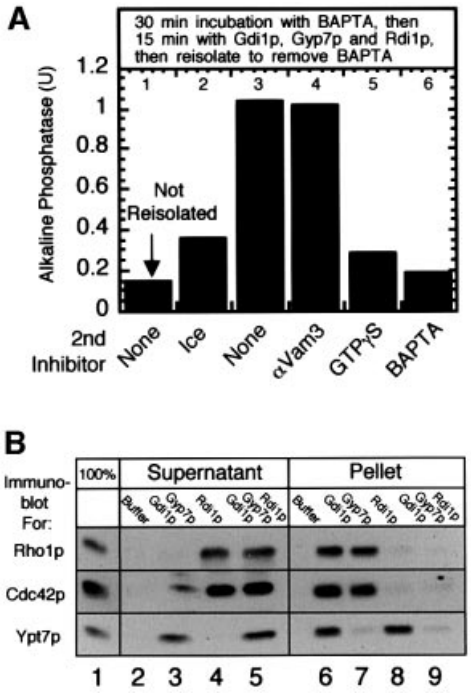

Fig. 6. Docked vacuoles, stripped of Ypt7p, Rho1p and Cdc42p, are still sensitive to GTPγS. (**A**) Docked vacuoles no longer require Ypt7p, Rho1p or Cdc42p yet are still sensitive to GTPγS. Fusion reactions (180 μl) were incubated at 27°C in the presence of BATPA for 30 min (docked vacuoles). Lane 1, 30 μl was removed and incubated for 60 min at 27°C. Twenty-five microliters of Gdi1p, 5 μl of Gyp7p and 30 μl of Rdi1p were added to the rest of the reaction and incubated for 15 min at 27°C. The reaction was centrifuged (5 min, 10 000 *g*) and the vacuole pellet was resuspended in 150 μl of PS buffer supplemented with 125 mM KCl, 5 mM $MgCl_2$, 30 μM Ca^{2+}, 10 μM CoA. Aliquots (30 μl) were either placed on ice or added to tubes containing PS buffer, anti-Vam3p IgG, GTPγS or BAPTA and incubated at 27°C for 60 min prior to assaying for alkaline phosphatase. (**B**) Ypt7p, Rho1p and Cdc42p are extracted from docked vacuoles by Gdi1p, Gyp7p and Rdi1p. Fusion reactions (120 μl) were incubated at 27°C in the presence of BAPTA for 30 min (docked vacuoles). Samples (30 μl) were added to tubes containing 6 μl of PS buffer, 5 μl of Gdi1p and 1 μl of Gyp7p, 6 μl of Rdi1p or 5 μl of Gdi1p, 1 μl of Gyp7p and 6 μl of Rdi1p, and incubated at 27°C for 15 min. Samples were centrifuged for 5 min at 10 000 *g* and equal portions of the supernatant and pellet were analyzed by immunoblot analysis.

blocked by incubation with Sec18p antibodies for 30 min. This block was reversed (Mayer *et al*., 1996) upon addition of active recombinant Sec18p (Figure 5A, compare lanes 2 and 3). Although uninhibited reactions are normally resistant to Rdi1p addition by 30 min (Figure 4), reactions blocked for 30 min by anti-Sec18p remain sensitive to docking and fusion inhibitors, including Rdi1p (lane 6), when the block is reversed by Sec18p addition. Priming must therefore be completed before the Rho proteins can complete their function. In a similar manner, the reaction is blocked by incubation with antibodies to a synthetic Ypt7p peptide (Figure 5B, lane 2). Inhibition can be reversed after 30 min by the addition of the cognate Ypt7p peptide (lane 3). These reactions are no longer sensitive to the addition of priming inhibitors (lanes 4 and 5) but remain sensitive to docking and fusion inhibitors including Rdi1p (lanes 6–9). The Ypt7p function must therefore also be completed prior to satisfying the Rho function. Finally, the fusion stage of the reaction can be blocked by the calcium chelator BAPTA (Figure 5C, lane 2). After 40 min this block can be reversed by the addition of calcium (lane 3). These reactions are no longer sensitive to priming or docking inhibitors, including Rdi1p (lanes 4–6). The Rho proteins therefore complete their function prior to the calcium-triggered initiation of the membrane fusion step.

When docked, BAPTA-blocked vacuoles are incubated with Rdi1p (to extract Rho1p and Cdc42p), Gdi1p and Gyp7p (to extract Ypt7p) or a mixture of Rdi1p, Gdi1p and Gyp7p (to extract all three GTPases) and then re-isolated, the fusion step remains sensitive to GTPγS (Figure 6A). Immunoblot analysis showed that these vacuoles were indeed depleted of Ypt7p, Rho1p and Cdc42p by these extractions (Figure 6B). Therefore, there is presumbably a unique GTPase or other GTPγS-senstive target that functions within the fusion step and remains to be identified.

Discussion

Previous studies indicated that, in addition to the Rab GTPase Ypt7p, vacuole fusion requires a late-acting,

Table I. Yeast strains

Strain	Genotype	Source
BJ3505	*Mat α, ura3, trp1, his3, lys2, gal2, can, prb1-Δ1.6R, pep4::HIS3*	Elizabeth Jones
DKY6281	*Mat α, ura3, leu2, trp1, his3, lys2, suc2, pho8::TRP1*	Dan Klionsky
ONHY1	*Mat a, ura3, leu2, trp1, his3, ade2*	Enrico Cabib
NHY21	*Mat a, ura3, leu2, trp1, his3, ade2, rho1-104*	Enrico Cabib
TD4	*Mat a, ura3, leu2, trp1, his4, can*	Gerald Fink
DJTD216A	*Mat a, ura3, leu2, trp1, his4, gal2, cdc42-1*	ATCC
BY4742	*Mat α, his3Δ1, leu2Δ0, ura3Δ0, lys2Δ0*	Research Genetics
GEY6048	ONHY1, *pho8::URA3*	this study
GEY1658	NHY21, *pho8::URA3*	this study
GEY6038	TD4, *pho8::URA3*	this study
GEY2298	DJTD2-16A, *pho8::URA3*	this study
GEY6028	BY4742, *pho8::URA3*	this study
GEY0908	BY4742, *rho2::kanMX, pho8::HIS3*	this study
GEY1188	BY4742, *rho3::kanMX, pho8::URA3*	this study
GEY0558	BY4742, *rho4::kanMX, pep4::LEU2*	this study
GEY1808	BY4742, *rho5::kanMX, pho8::URA3*	this study

GTPγS-sensitive factor (Eitzen *et al*., 2000). We have now identified two additional GTPases, Rho1p and Cdc42p, which are required for homotypic vacuole fusion, although each acts before the GTPγS-sensitive step. Four lines of evidence prove that both Rho1p and Cdc42p are required for the fusion reaction: (i) Rho1p and Cdc42p antibodies inhibit vacuole fusion (Figure 1A; Müller *et al*., 2001); (ii) Rdi1p treatment, which extracts Rho1p and Cdc42p from vacuole membranes, blocks vacuole fusion (Figures 2A and 3A); (iii) vacuoles isolated from *RHO1* and *CDC42* temperature-sensitive strains are thermolabile for fusion (Figure 1B and C); and (iv) these strains show vacuole fragmentation after a brief incubation at the non-permissive temperature (Figure 1E and F).

Our results and those of Müller *et al*. (2001) show that Cdc42p is required for vacuole docking, functioning between Ypt7p-mediated tethering and *trans*-SNARE pairing. Müller *et al*. (2001) have identified a specific site-directed cdc42-ts allele, *cdc42-123*, which affects vacuole fusion, whereas another, *cdc42-124*, does not. This also demonstrates that Rho proteins are capable of mediating multiple downstream effects. Additionally, Cdc42p interacts genetically with Nrf1p, which is involved in vacuole trafficking (Murray and Johnson, 2000, 2001). Nrf1p is a subunit of the VTC complex that interacts with the V-ATPase (Cohen *et al*., 1999), which has an important role in membrane fusion (Peters *et al*., 2001). Rho1p also functions to regulate the targeting of Sec3p, a component of the exocytic machinery, to the plasma membrane (Guo *et al*., 2001).

The Rho family of GTPases are thought to have a central role in vesicular trafficking pathways by controlling the organization of the actin cytoskeleton to spatially direct the transport of vesicles and to regulate endocytosis (Murphy *et al*., 1996; Kroschewski *et al*., 1999; Merrifield *et al*., 1999; Guo *et al*., 2001). However, our current study shows that Rho GTPases can also function to promote the fusion of isolated vacuoles in the absence of cytoskeleton or cytosol.

There are several possible roles for Rho proteins within our reaction. As was shown for Rho3p (Adamo *et al*., 1999; Robinson *et al*., 1999), Rho1p interacts with tethering complexes (Guo *et al*., 2001). Vacuole tethering complexes form specialized membrane docking domains (L.Wang, A.Merz, S.Seeley and W.Wickner, manuscript submitted), and Rho1p might be required for the maintenance of these complexes. Rho GTPases also regulate phosphatidylinositol 4,5-bisphosphate (PIP_2) synthesis (Chong *et al*., 1994; Weernink *et al*., 2000). Vacuole docking needs PIP_2 and this lipid is synthesized from PI by isolated vacuoles (Mayer *et al*., 2000). Rho proteins can also be stimulated by PIP_2 to control actin dynamics (Higgs and Pollard, 2000; Rohatgi *et al*., 2000). Localized actin remodeling may be necessary as F-actin can either block or promote membrane fusion (Muallem *et al*., 1995; Ayscough, 2000; Jahraus *et al*., 2001).

Rho GTPases control actin structure through effector cascades (Hall, 1998). Cdc42p-regulated kinases (Cla4p and Ste20p in *Saccharomyces cerevisiae*) act on type I myosins (Myo3p and Myo5p in yeast) and regulate the complex of Wiskott–Aldrich syndrome proteins (Bee1p and Vrp1p) and Arp2/3p that controls actin polymerization and branching (Naqvi *et al*., 1998; Madania *et al*., 1999; Winter *et al*., 1999; Higgs and Pollard, 2000). Studies that exploit vacuole fragmentation as a phenotype of defective vacuole fusion have shown vacuole fragmentation in cells with deletions in the genes for Cla4p and Arc18p, as well as for Sac2p and Sac6p, which also modulate actin structure. Actin is found on the surface of our isolated vacuoles (P.Slusarewicz, A.Merz and W.Wickner, unpublished) and undergoes G to F conversion in conditions of our fusion reaction. Further studies will be needed to establish whether actin participates in vacuole docking and whether Rho GTPases regulate PIP_2 biosynthesis and actin polymerization on vacuoles.

Materials and methods

Yeast strains and genetic modifications

Yeast strains used in this study are listed in Table I. To create *PHO8* or *PEP4* gene deletions, strains were grown in YPD to an OD_{600} ~ 1.5 and transformed by the lithium acetate method (Gietz and Schiestl, 1995) using a linear DNA fragment containing the *URA3* or *HIS3* gene for *PHO8* and the *LEU2* gene for *PEP4* flanked by 40 nucleotides of homology to the upstream and downstream region of the *PHO8* or *PEP4*

genes. The oligonucleotides CCAGCATTACGGGACATTATTTGAACGCGCATTAGCAGCAAGATTGTACTGAGAGTGCAC and ATTAAATAATATGTGAAAAAAGAGGGAGAGTTAGATAGGACTGTGCGGTATTTCACACCG were used to PCR amplify the *URA3* gene from plasmid pRS406 and the *HIS3* gene from plasmid pRS403, and the oligonucleotides ACCTAGTATTTAATCCAAATAAAATTCAAACAAAAACCAAAGATTGTACTGAGAGTGCAC and TAGATGGCAGAAAAGGATAGGGCGGAGAAGTAAGAAAAGTCTGTGCGGTATTTCACACCG were used to PCR amplify the *LEU2* gene from plasmid pRS405 (Brachmann *et al*., 1998). Transformants were selected on SD –ura, –his or –leu (Bio101) agar plates and screened for proper integration by PCR, using primers TAGCGATAAGCTTCGCGC and CGCGGATCCACGTGTCATGCGGTTAG for PHO8 and TGAGAAGCCTACCACGTA and AGCAGCATAGAACAATGGA for PEP4, which anneal to the upstream and downstream regions, respectively. Vacuoles were visualized *in vivo* by FM4-64 staining (Vida and Emr, 1995) with a Zeiss fluorescence microscope and Tmax 400 film (Kodak) used at an exposure index of 1600.

Biochemicals

All biochemical reagents were dissolved or dialyzed in PS buffer (20 mM PIPES–KOH pH 6.8, 200 mM sorbitol). Anti-Rho1p (Drgonova *et al*., 1999), anti-Vam3p, anti-Sec17p, anti-Sec18p and anti-SecF antibody inhibition experiments used IgG, purified as previously described (Mayer *et al*., 1996). Anti-Ypt7p antibody was affinity purified. Anti-Cdc42p antibodies were purchased from Santa Cruz Biochemicals. Inhibitors were used at the following final concentrations: affinity-purified anti-Ypt7p antibodies (50 μg/ml), anti-Vam3p IgG (150 μg/ml), anti-Sec18p IgG (250 μg/ml), anti-Sec17p IgG (250 μg/ml), anti-Rho1p IgG (280 μg/ml), Gdi1p (100 μg/ml), Gyp7p (30 μg/ml), Rdi1p (500 μg/ml), Mg-GTPγS (3 mM) and BAPTA (3 mM).

Synthesis and purification of recombinant proteins

His_6-Sec18p, Gyp7p and Gdi1p were purified as previously described (Garrett *et al*., 1994; Haas and Wickner, 1996; Eitzen *et al*., 2000). The *RDI1* gene was PCR amplified from yeast genomic DNA using primers CGCGGATCCGCCGAAGAAAGTACCGACTT and CCGGAATTCATTAGTGATCTACTAGCTAAATC. The amplification product was digested with *Bam*HI and *Eco*RI and cloned into pGEX-4T1 (Pharmacia) to create plasmid pGST-RDI. pGST-RDI was used to transform *Escherichia coli* BL21 and glutathione *S*-transferase (GST)–Rdi1p was purified (Ausubel *et al*., 1997). Proteins were buffer exchanged with P6 columns (Bio-Rad) into PS buffer.

Vacuole fusion reactions and reversible inhibition

Vacuoles were isolated from strains grown in YPD (plus 20 μg/ml adenine for ade2 strains) to an OD_{600} ~ 0.9 (Haas, 1995). Standard vacuole fusion reactions contain 3 μg of vacuoles without proteinase A, 3 μg of vacuoles without alkaline phosphatase, 1 mM ATP, 40 mM creatine phosphate, 0.5 mg/ml creatine kinase, 125 mM KCl, 6 mM $MgCl_2$, 10 μM CoA, 200 mM sorbitol, 20 mM PIPES–KOH pH 6.8 in 30 μl. Reactions were incubated for 90 min at 27°C prior to assaying for alkaline phosphatase activity (Haas, 1995). For reversible blocks of the priming, docking and fusion stages, 200 μg/ml anti-Sec18p IgG, 64 μg/ml anti-Ypt7p antibodies (raised against the peptide TEAFEDDYNDAINIRC) or 3 mM BAPTA was added, respectively. To reverse these blocks, 0.1 μg/ml His_6-Sec18p, 67 μg/ml Ypt7 peptide or 3.5 mM $CaCl_2$ was added after 30 min from 30× concentrated stock solutions.

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