Rho GTPase regulation of exocytosis in yeast is independent of GTP hydrolysis and polarization of the exocyst complex

Olivier Roumanie,[1] Hao Wu,[1] Jeffrey N. Molk,[2] Guendalina Rossi,[1] Kerry Bloom,[2] and Patrick Brennwald[1]

[1]Department of Cell and Developmental Biology and [2]Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Rho GTPases are important regulators of polarity in eukaryotic cells. In yeast they are involved in regulating the docking and fusion of secretory vesicles with the cell surface. Our analysis of a Rho3 mutant that is unable to interact with the Exo70 subunit of the exocyst reveals a normal polarization of the exocyst complex as well as other polarity markers. We also find that there is no redundancy between the Rho3–Exo70 and Rho1–Sec3 pathways in the localization of the exocyst. This suggests that Rho3 and Cdc42 act to polarize exocytosis by activating the exocytic machinery at the membrane without the need to first recruit it to sites of polarized growth. Consistent with this model, we find that the ability of Rho3 and Cdc42 to hydrolyze GTP is not required for their role in secretion. Moreover, our analysis of the Sec3 subunit of the exocyst suggests that polarization of the exocyst may be a consequence rather than a cause of polarized exocytosis.

Introduction

In eukaryotic cells, exocytosis is the major route by which newly synthesized proteins and lipids are delivered to the cell surface. This process involves the directed transport, docking, and fusion of Golgi-derived secretory vesicles with the plasma membrane. In *Saccharomyces cerevisiae*, polarization of exocytosis is tightly coordinated with the overall polarity of the cell. Much of the protein machinery responsible for cell polarity and exocytosis is itself concentrated at sites of polarized growth (Pruyne and Bretscher, 2000).

In yeast, the polarized delivery of vesicles along actin cables is thought to be performed by a type V myosin Myo2 and the Rab GTPase Sec4 (Goud et al., 1988; Pruyne et al., 1998). Elegant genetic and biochemical analyses have shown that a multisubunit complex known as the exocyst complex or Sec6–Sec8 complex is required for the docking and fusion of secretory vesicles at the plasma membrane (Terbush et al., 1996; Guo et al., 1999). The secretory machinery, including Myo2, Sec4, and the exocyst complex, is concentrated at sites of active growth to enable the polarized fusion of secretory vesicles with the plasma membrane. The final step of the exocytic process requires the formation of complexes between the v-SNAREs Snc1/2, located on the secretory vesicles, and the plasma membrane t-SNAREs Sec9 and Sso1/2, distributed around the periphery of the cell (Brennwald et al., 1994).

Recent studies from our laboratory have implicated Rho GTPases in the regulation of exocytosis (Adamo et al., 1999, 2001). In addition, physical interactions between specific Rho proteins and two subunits of the exocyst complex have been reported. Rho1 and Cdc42 have been shown to bind to the NH_2-terminal domain of Sec3 to promote its polarization, and Rho3 interacts with Exo70, another subunit of the exocyst (Robinson et al., 1999; Guo et al., 2001; Zhang et al., 2001). We have shown that Rho3 and Cdc42 have specific and direct roles in post-Golgi secretion, which are independent of their roles in other aspects of polarity and morphogenesis (Adamo et al., 1999, 2001). However, the precise mechanism by which Rho3 and Cdc42 regulate exocytosis is presently unclear.

Here, we report that, like *cdc42-6*, the cold-sensitive *rho3-V51* allele has a secretion defect that does not involve detectable effects on the polarized localization of the exocytic machinery. Moreover, GTP hydrolysis by Rho3 and Cdc42 was found to be dispensable for their function in secretion. This suggests that these GTPases act in vesicle docking and fusion at the plasma membrane through allosteric regulation of the exocytic machinery. We propose that the observed polarization of the exocytic apparatus, including the exocyst complex, may be a result of, rather than a cause of, polarized exocytosis. This

Correspondence to Patrick Brennwald: pjbrennw@med.unc.edu

O. Roumanie's present address is Laboratoire de Biologie Moléculaire et Séquençage, Institut de Biochimie et Genetique Cellulaires, 33077 Bordeaux, France.

Abbreviations used in this paper: DIC, differential interference contrast; RBD, Rho-binding domain.

The online version of this article includes supplemental material.

The Journal of Cell Biology, Vol. 170, No. 4, August 15, 2005 583–594
http://www.jcb.org/cgi/doi/10.1083/jcb.200504108

suggests a model in which the unpolarized exocytic machinery present on the plasma membrane can respond directly to a polarization signal initiated by the presence of a patch of activated Rho GTPase. This mechanism does not require the direct recruitment of factors by the Rho GTPase to the site of polarized growth. Recent evidence has shown that much of the exocytic machinery is associated with transport carriers destined for the plasma membrane (Boyd et al., 2004). Thus, the direct asymmetric regulation of exocytosis by activated Rho proteins would reinforce the delivery of secretory vesicles at the site of active growth and lead indirectly to the appearance of a polarized secretion machinery.

Results

The *rho3-V51* secretory defective mutant does not affect the cellular localization of essential secretion and polarity markers

To determine the function of Rho3 in the cell, we previously identified four mutants in the effector domain of Rho3 that result in conditional growth defects. Of these, *rho3-V51* was the only allele that exhibited a well polarized actin cytoskeleton, but showed a severe defect in secretion after a shift to the restrictive temperature (Adamo et al., 1999). This was the first demonstration that Rho GTPases could function in exocytic transport independent of their effects on actin.

To determine how Rho3 regulates exocytosis, we first examined the effect of the *rho3-V51* mutation on the localization of markers of polarized exocytosis. Our initial analysis included Cdc42, Myo2, and Sec4. Cdc42 is an essential Rho GTPase involved in polarized assembly of the cytoskeleton and in regulation of the late secretory pathway and is known to polarize independently of polymerized actin to sites of active growth (Ayscough et al., 1997). Sec4 is a member of the Rab GTPase family that acts between the Golgi apparatus and the plasma membrane (Goud et al., 1988). Myo2 is an unconventional type V myosin implicated in targeting post-Golgi vesicles to the bud tip (Pruyne et al., 1998). Sec4 and Myo2 localize to the presumptive bud site and the tip of small budded cells through a mechanism that is sensitive to both the integrity of actin cables and on-going secretion. The localization of these three markers was analyzed in the *rho3-V51* mutant and wild-type cells using affinity-purified antibodies against endogenous proteins. Temperature shifts of 5 h at 14°C were used, as these were previously shown to result in a severe post-Golgi secretory defect (Adamo et al., 1999). We observed that Cdc42 gave a similar polarized staining pattern in the *rho3-V51* mutant and wild-type cells before and after a shift to 14°C (Fig. 1 A). Quantitation of the number (i.e., penetrance) of cells exhibiting a polarized Cdc42 staining pattern was found to be virtually identical between the *rho3-V51* mutant and wild-type cells at both 25 and 14°C (Fig. 1 B). Furthermore, quantitation of the average intensity (i.e., magnitude) of Cdc42 patch staining in each condition varied by <5% between the wild-type and the *rho3-V51* mutant. We observed that, as in wild-type cells, Sec4 and Myo2 were polarized in *rho3-V51* cells after a 5-h shift to the restrictive temperature (Fig. 1 A). Both wild-type and mutant cells showed a similar efficiency of polarization for either Sec4 or Myo2 at 25 and 14°C (Fig. 1 B), and there was no significant effect on the staining intensity. Thus, Myo2 and Sec4 are properly localized in the *rho3-V51* mutant, consistent with the fact that the actin cytoskeleton organization in this mutant is largely unaffected at the restrictive or permissive temperature (Adamo et al., 1999; unpublished data). These results demonstrate that *rho3-V51* cells are fully competent in localizing critical factors involved in post-Golgi transport.

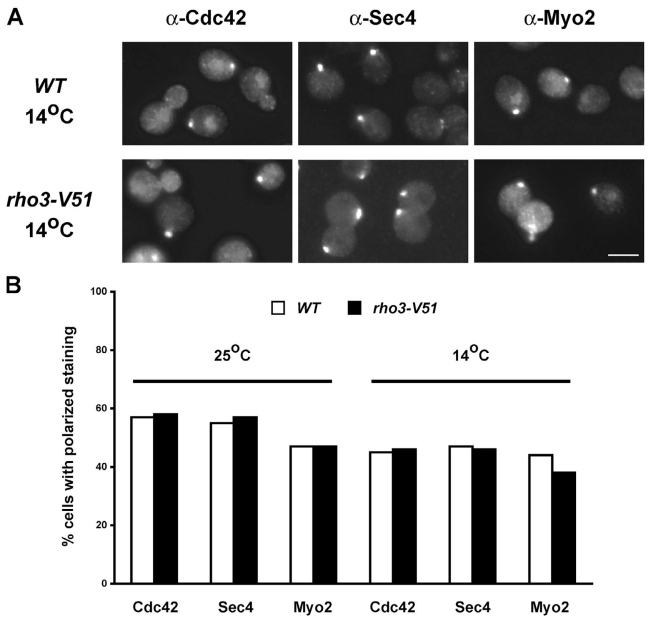

Figure 1. **Distribution of the polarity and secretion markers Cdc42, Myo2, and Sec4 is normal in the *rho3-V51* secretory mutant at both permissive and nonpermissive temperatures.** (A) Wild-type (WT) and mutant (*rho3-V51*) cells were grown at 25°C or shifted to 14°C for 5 h before fixation and then processed for fluorescence microscopy. Visualization of the polarity establishment protein Cdc42, the Rab GTPase Sec4, and the vesicle-transport motor Myo2 was performed using specific purified antibodies. Bar, 2 μm. (B) Quantitation of polarized markers in wild-type and *rho3-V51* cells. Cells were processed as described in A and scored for the polarized localization of Cdc42, Sec4, and Myo2 at the emerging bud sites, bud tips, or mother-daughter neck regions. A minimum of 200 cells were counted for each experiment.

Post-Golgi vesicle tethering and docking to the plasma membrane is dependent on a multisubunit protein complex known as the exocyst complex. Although the aforementioned results suggest that vesicles are properly delivered to sites of polarized growth in *rho3-V51* cells, it is possible that the accumulation of post-Golgi vesicles observed in this mutant could result from a failure in vesicle docking due to the absence of the exocyst complex at sites of polarized growth. To determine if *rho3-V51* cells are defective in polarization of the exocyst complex, we followed the fluorescence associated with three GFP-tagged exocyst subunits Sec3, Sec8, and Exo70. Sec8 and Exo70 are two of the seven essential subunits of the exocyst, and Exo70 has been shown to interact with Rho3 in a GTP-dependent manner (Robinson et al., 1999). Sec3 is the only nonessential subunit of the complex, and it has been proposed to act as a spatial landmark for secretion (Finger et al., 1998).

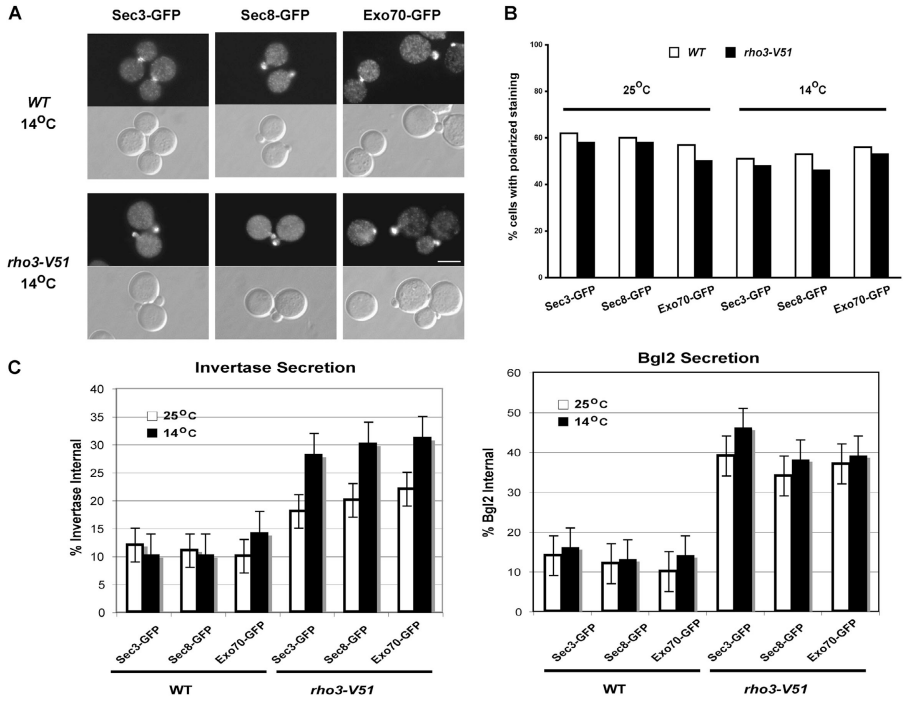

Figure 2. **Exocyst subunits Sec3, Sec8, and Exo70 are polarized in the *rho3-V51* secretory mutant at permissive and nonpermissive temperatures.** (A) Plasmids containing functional, COOH-terminal–tagged GFP versions of Sec3, Sec8, or Exo70 were introduced into wild-type and *rho3-V51* strains. Transformed cells were grown at 25°C or shifted to 14°C for 5 h and immediately fixed and processed for fluorescence microscopy. DIC images of the same cells are shown. Bar, 2 μm. (B) Quantitation of Sec3-GFP, Sec8-GFP, and Exo70-GFP polarized localization in wild-type and *rho3-V51* cells. Cells processed as described in A were scored for the presence of the exocyst components at the emerging bud sites, bud tips, or bud necks. Approximately 50 cells were analyzed under each condition. (C) Invertase and Bgl2 secretion assays on GFP-tagged wild-type and *rho3-V51* strains processed as described in A were performed as described previously (Adamo et al., 1999). The secretion defect associated with each strain is expressed as the percentage of total (internal + external) invertase or Bgl2 found internally. Results shown represent the average of three experiments. Error bars represent SD.

We found that these exocyst components were polarized in *rho3-V51* cells at the restrictive temperature (Fig. 2 A). Furthermore, the percentage of mutant cells displaying polarized subunits was comparable to that observed in wild-type cells (Fig. 2 B). To confirm that the *rho3-V51* strains containing the GFP-tagged exocyst subunits showed secretory defects similar to those described for the parental *rho3-V51* strains, we examined their capacity for secretion of the periplasmic enzymes invertase and Bgl2 after a shift to the restrictive temperature. As can be seen in Fig. 2 C we find that, like the parental *rho3-V51* strain, the GFP-tagged derivatives show a pronounced secretory defect for both enzymes. As previously observed for *rho3-V51*, these strains show a partial secretory defect for both enzymes at the permissive temperature, which correlates with post-Golgi vesicle accumulation by electron microscopy (Fig. 2 C; Adamo et al., 1999). The invertase secretion defect is significantly exacerbated after the shift to 14°C, which also correlates with an increase in vesicle accumulation by EM (Fig. 2 C; Adamo et al., 1999). Also, as previously observed, Bgl2 secretion was similarly defective at both temperatures (Fig. 2 C; Adamo et al., 1999). Thus, we conclude that the secretory defects present in the *rho3-V51* mutant is not due to an inability to properly localize the exocyst complex to sites of polarized growth.

Testing the functional redundancy between the Rho-Sec3 and Rho-Exo70 pathways

Studies on the yeast exocyst complex have revealed interactions of two subunits, Sec3 and Exo70, with Rho GTPases (Fig. 3 A; Robinson et al., 1999; Guo et al., 2001; Zhang et al., 2001). Exo70 interacts specifically with Rho3 (Robinson et al., 1999), and introduction of the V51 mutation in the effector domain of Rho3 leads to a loss of this interaction (Adamo et al., 1999) without affecting the localization of Exo70 or other exocyst components as described in the preceding section. The Sec3 protein has been shown to interact with both Rho1 and Cdc42 through an NH_2-terminal Rho-binding domain (RBD; Guo et al., 2001; Zhang et al., 2001). Surprisingly, although the RBD regulates the polarized localization of Sec3 to sites of polarized growth, its deletion has no effect on the polarized localization of the rest of the exocyst complex. Guo et al. (2001) and Boyd et al. (2004) have suggested that a parallel pathway involving Exo70 may localize the exocyst complex to sites of polarized growth in the absence of the Rho–Sec3 pathway. This suggested that the lack of an effect of the *rho3-V51* mutant on exocyst localization might be due to redundancy with the Rho–Sec3 pathway in localizing the exocyst complex to sites of polarized growth (Fig. 3 A). If such redundancy exists between these pathways, then the combination of mutations impairing both the Rho3–Exo70 and Rho–Sec3 pathways would be expected to be detrimental to the cell. Therefore, we used a mutant *sec3-ΔN* allele lacking the NH_2-terminal RBD of *SEC3* to replace the genomic wild-type *SEC3* locus. The *sec3-ΔN* was expressed behind the *GPD* promoter as in previous studies involving *sec3-ΔRBD* mutants (Guo et al., 2001; Wiederkehr et al., 2003). To detect both wild-type and Sec3-ΔN proteins, we raised and affinity purified antibodies to a hydrophilic region in the central portion of the Sec3 protein (Fig. 3 B). We observed that cells expressing *sec3-ΔN* as the sole copy grew as well as wild-type cells at all temperatures examined. Cellular localization of the endogenous Sec3 by immunofluorescence revealed a characteristic polarized staining at sites of active exocytosis, whereas the *sec3-ΔN* mutant appeared as a diffuse bright staining all over the cell (Fig. 3 C). Under our experimental conditions, no detectable portion of Sec3-ΔN was found to be targeted correctly to sites of cellular growth. Nevertheless,

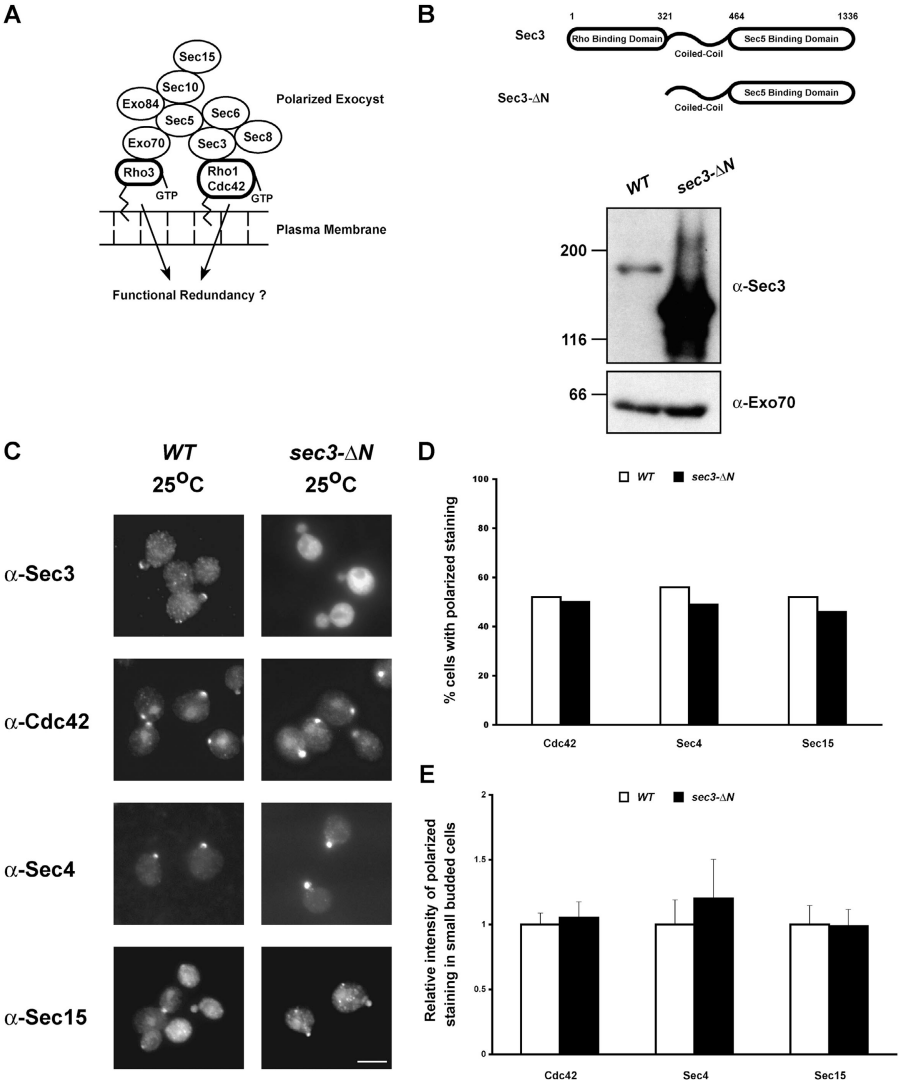

Figure 3. **Testing the redundancy model for Rho function in the polarization of secretory and polarity markers.** (A) Redundancy model for Rho3 and Rho1/Cdc42 pathways in polarizing the exocyst. The Exo70 and Sec3 exocyst subunits interact with distinct Rho GTPases, and these interactions have been proposed to localize the exocyst complex to help promote secretion and polarity. (B) A *sec3* mutant strain lacking the NH_2-terminal RBD (aa 3–320) of Sec3 was placed as the only copy of Sec3 in the cell on high copy expression (see Materials and methods). Correct integration of the construct was checked by Western blot analysis of whole cell lysates from wild-type and *sec3-ΔN* strains using anti-Sec3 antibodies directed against the middle region of the protein. Blotting with anti-Exo70 antibodies was used as loading control. (C) Polarization of distinct secretion markers in the *sec3-ΔN* mutant. Wild-type and *sec3-ΔN* cells were grown at 25°C, fixed, and processed for immunofluorescence. Specific purified antibodies directed against the Sec3 and Sec15 exocyst subunits, the polarity protein Cdc42, or the secretion regulator Sec4 were used. Bar, 2 μm. (D) Quantitation of Cdc42, Sec4, and Sec15 polarization in the *sec3-ΔN* mutant at 25°C. The polarized distribution of the three proteins was scored as described in Fig. 1. (E) Measurement of the relative fluorescence associated with the Cdc42, Sec4, and Sec15 markers in small budded cells at 25°C. Proteins were detected by immunofluorescence microscopy in wild-type and *sec3-ΔN* cells, and the fluorescence intensity of the polarized spots was measured in small budded cells using Metamorph software. Results for each marker are reported as an intensity of fluorescence relative to the fluorescence observed in the wild-type strain for the same marker. Error bars represent SD.

staining of polarity and secretion markers confirmed that, as previously reported (Guo et al., 2001), the exocytic machinery was properly localized in the *sec3-ΔN* strain (Fig. 3, C and D). In addition, the average signal intensity of these markers did not display any decrease in the mutant compared with wild type (Fig. 3 E), demonstrating that the proteins were fully competent for targeting to sites of polarized secretion.

Analysis of *rho3-V51,sec3-ΔN* double mutants reveals no synthetic effects on growth or polarity

To assay for redundancy between the Rho3–Exo70 and the Rho–Sec3 pathways, we crossed strains containing the *sec3-ΔN* and *rho3-V51* alleles to evaluate the phenotype of cells containing both mutations as the only source of Sec3 and Rho3 in the cell. No synthetic effects on growth were observed, as growth of the *rho3-V51,sec3-ΔN* double mutant is identical to that of the *rho3-V51* mutant at both permissive (25°C) and nonpermissive (14°C) conditions (Fig. 4 A). None of the double mutants showed sensitivity to growth at high temperature (37°C), and both *rho3-V51,sec3-ΔN* and *rho3-V51* grew slightly slower than wild-type or *sec3-ΔN* strains at 25°C. To determine the extent to which secretion was blocked in the *rho3-V51,sec3-ΔN* mutant cells, the export of the Bgl2 exoglucanase was monitored at both 25 and 14°C (Fig. 4 B). Wild-type and *sec3-ΔN* cells displayed similar small amounts of internal pools of the enzyme, indicating that deletion of the Sec3 RBD motif did not impair secretion. The *rho3-V51,sec3-ΔN* strain was found to have a defect in secretion comparable to *rho3-V51*. As expected, a large amount of Bgl2 accumulated in the *rho3-V51* cells and a comparable fraction of enzyme was detected within the *rho3-V51,sec3-ΔN* double mutant at both temperatures. Therefore, the growth and secretion defects displayed by *rho3-V51* cells were not amplified in combination with the *sec3-ΔN* mutation.

Next, we determined whether the loss of Sec3's ability to interact with Rho proteins had any effect on the localization of components of the exocytic machinery in *rho3-V51* cells. Immunofluorescence studies on the *rho3-V51,sec3-ΔN* double mutants demonstrated that Cdc42, Sec4, and Myo2 were polarized normally after a shift to the nonpermissive temperature of 14°C (Fig. 5 A). In addition, the ability of mutant cells to main-

tain the localization of these markers was normal (Fig. 5, B and C). The exocyst complex polarization was then investigated using GFP-tagged Sec8 and Exo70 subunits and antibodies directed against Sec15. No defect in the localization of these exocyst components was observed at the restrictive temperature (Fig. 5, A and D), showing that the exocyst was polarized in *rho3-V51,sec3-ΔN* cells at 14°C. The amount of cells showing polarized exocyst subunits was determined to be similar in wild-type and mutant strains (Fig. 5 E). Also, examination of the *rho3-V51,sec3-ΔN* mutant actin cytoskeleton revealed that both patches and cables were polarized at 25 and 14°C (unpublished data).

To determine if there was an effect of *RHO3* mutants on the dynamics of exocyst polarization, photobleaching was performed on Sec8-GFP–expressing cells and FRAP was analyzed. Wild-type strains expressing Sec8-GFP from a *CEN* plasmid were photobleached at the bud tip of small or medium budded cells (Fig. 6 A). Sec8-GFP had a half-recovery time of ~17 s that was fit by a single exponential curve (Fig. 6 B and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200504108/DC1), similar to previous FRAP measurements (Boyd et al., 2004). The corrected fluorescence recovery was measured as ~82% of the prebleach fluorescence intensity (Table S1). Photobleaching and FRAP measurements of Sec8-GFP in *rho3-V51* mutants demonstrated that both the $t_{1/2}$ and fluorescence recovery values were not significantly different from wild-type cells (Fig. 6, C and D). Likewise, expression of the GTP-locked *CEN-RHO3*^{Q74L} as the only source of Rho3 in the cell did not significantly alter Sec8-GFP recovery rates. Nor did we detect any significant differences in the Sec8-GFP recovery rates in the *sec3-ΔN* single mutant or *rho3-V51,sec3-ΔN* double mutants from that seen in wild-type cells (Fig. 6, C and D). Therefore, the analysis of exocyst dynamics using FRAP strongly supports our observations that polarization of this complex is unperturbed in the *rho3-V51* mutants or in the *rho3-V51,sec3-ΔN* double mutants. Thus, the Rho1–Sec3 and Rho3–Exo70 pathways do not appear to act redundantly to determine the localization or dynamics of the exocyst complex at sites of polarized growth. Interestingly, we also find kinetics similar to wild-type cells with the *RHO3*^{Q74L} GTP-locked mutant, suggesting that activation of Rho3 function has no dramatic effect on the overall dynamics of exocyst polarization. Rho3, therefore, does not appear to directly regulate exocyst polarization. Moreover, we also find that Rho1–Sec3 and Rho3–Exo70 pathways do not act redundantly to determine the localization or dynamics of the exocyst complex at sites of polarized growth.

To determine if the loss of the RBD has any effect on Sec3 function in combination with other late secretory mutants, we introduced a *sec3-ΔN* or a control *SEC3* construct into *cdc42-6* and several late *sec* mutants (Table S1). As with the *rho3-V51* mutant, we found no obvious synthetic effect of the *sec3-ΔN* allele on growth of *sec9-4* cells. However, synthetic effects at semipermissive temperatures were apparent in *sec1-1*, *sec8-9*, and, most severely, *sec15-1* mutant cells. Strikingly, we were unable to obtain transformants of the *sec3-ΔN* construct in *cdc42-6* cells, which we confirmed by meiotic analysis was

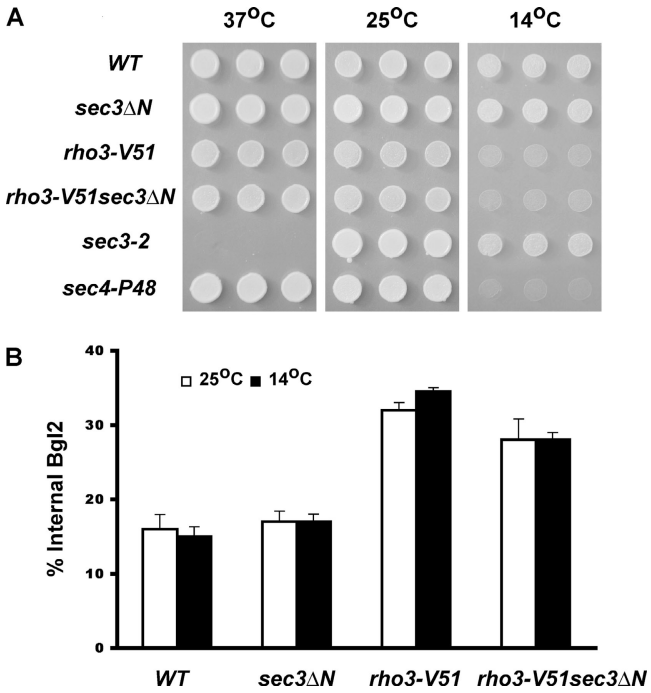

Figure 4. ***rho3-V51* and *sec3-ΔN* mutations do not demonstrate any synthetic effects on growth and secretion.** (A) Analysis of the growth phenotypes of the *rho3-V51,sec3-ΔN* double mutant. The *sec3-2* and *sec4-P48* strains were used as growth controls. Plates at 37 and 25°C were incubated for 2–3 d or at 14°C for 6 d. (B) Analysis of Bgl2 protein secretion in wild-type, *sec3-ΔN*, *rho3-V51*, and *rho3-V51,sec3-ΔN* strains. Cells were grown at 25°C or shifted to 14°C for 5 h before analyzing the distribution of the secreted protein Bgl2 as described previously (Adamo et al.,1999). Quantitation of the bands was performed using ImageQuant software. The percentage of the total Bgl2 that is found internally in different strains is depicted. The data represent the average of three independent experiments. Error bars represent SD.

due to the synthetic lethality of this allele with *cdc42-6*. Therefore, when secretory function is compromised in certain ways, a positive role for the function of the Rho binding domain of Sec3 is apparent. The synthetic lethality with *cdc42-6* may indicate that this function is especially important in the highly polarized membrane growth during bud emergence, when the secretory defect of *cdc42-6* is manifested (Adamo et al., 2001).

Sec3 polarization is sensitive to the same perturbations as other exocyst subunits and other polarity markers

A previous study using GFP-tagged Sec3 suggested that the polarization of this subunit of the exocyst was uniquely and remarkably insensitive to perturbations in vesicle transport and actomyosin polarity. These observations led to the suggestion that this subunit may act as a spatial landmark for localization of the exocyst complex and consequently polarization of membrane transport (Finger et al., 1998). In light of our previous results, we reexamined the polarization of the native Sec3 protein by using purified antibodies raised against recombinant Sec3 (see Materials and methods). To determine if the localization of Sec3, like other exocyst components, is sensitive to ongoing secretion, we examined Sec3 immunostaining in mutants defective for ER to Golgi transport (*sec21-1* and

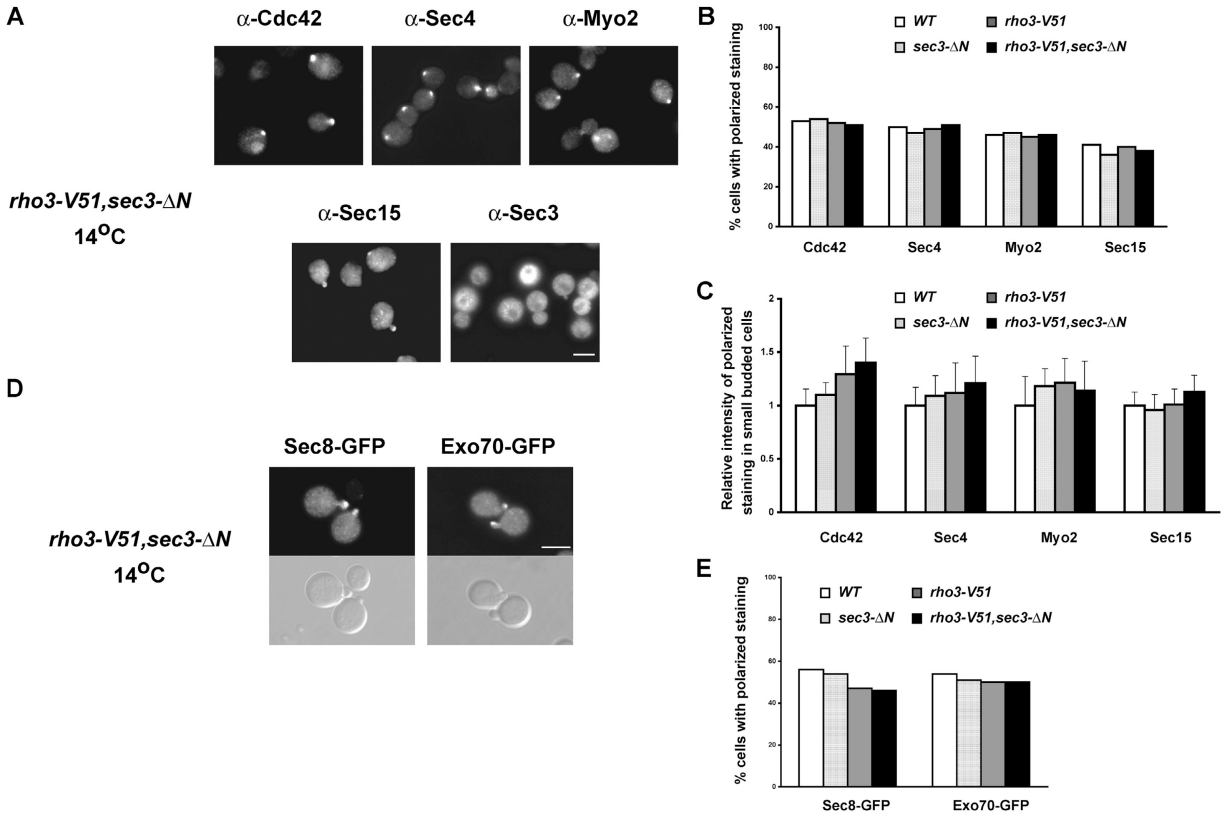

Figure 5. ***rho3-V51,sec3-ΔN* cells show no defect in the polarization of polarity or secretory markers.** (A) The *rho3-V51,sec3-ΔN* cells were grown at 25°C and shifted to 14°C for 5 h before being analyzed by fluorescence microscopy. Purified antibodies were used to visualize polarity/secretion markers (Cdc42, Sec4, and Myo2) and exocyst subunits (Sec3 and Sec15). Bar, 2 μm. (B) Quantitation of polarized markers at 14°C in the indicated wild-type or mutant strains. Cells were scored for the polarized localization of Cdc42, Sec4, Myo2, and Sec15 as described in Fig. 1. (C) Measurement of relative fluorescence associated with the polarity/secretion markers in small budded cells at 14°C. Polarized staining was analyzed as described in Fig. 3. Error bars represent SD. (D) The plasmids carrying the GFP-tagged exocyst subunits Sec8 or Exo70 were transformed into the *rho3-V51,sec3-ΔN* double mutant strain. The cells were grown at 25°C, shifted to 14°C for 5 h, fixed, and observed by fluorescence and DIC microscopy. Bar, 2 μm. (E) Quantitation of Sec8-GFP and Exo70-GFP polarized localization in wild-type and mutant strains at 14°C. Under each condition, ~50 cells were analyzed and scored for the presence of the exocyst components at the emerging bud sites, bud tips or bud neck.

sec22-3) or for post-Golgi trafficking (*sec1-1*, *sec6-4*, *sec9-4*, and *myo2-66*). We also examined the localization of Sec4, Myo2, and Sec15 markers in these strains. As previously reported (Walch-Solimena et al., 1997), Sec4 was found to be redistributed in *myo2-66* and ER-to-Golgi mutants and to be polarized in post-Golgi *sec* mutants after a shift to the restrictive temperature (Fig. 7 and not depicted). Myo2 and Sec15 concentration in the bud was lost in early or late secretion mutants after a 1-h shift to the restrictive temperature (Fig. 6), suggesting that Myo2 and Sec15 polarized localization is dependent on an active secretory pathway. These results are consistent with observations that suggest that both proteins are associated with post-Golgi vesicles (Pruyne et al., 1998; Guo et al., 1999), and therefore their polarized localization on the plasma membrane would be expected to depend on ongoing secretion. In contrast to previous studies using GFP-Sec3, we find that whereas immunostained, native Sec3 was well polarized in wild-type cells, a complete loss of polarized staining was observed in both early and late secretion mutants at the restrictive temperature (Fig. 7). These data strongly suggest that, like the other subunits of the exocyst, ongoing secretion is required for the polarization of Sec3.

To determine the dependence of native Sec3 polarization on actomyosin function, we made use of a temperature-sensitive tropomyosin mutant strain, *tpm1-2* (in a *tpm2Δ* background), which rapidly loses polarized actin cables after a temperature shift to 34°C. Previous studies have demonstrated that both Sec4 and the exocyst subunit Sec8 rapidly depolarize upon loss of actin cables (Pruyne et al., 1998). In contrast, GFP-tagged Sec3 has recently been shown to be resistant to the *tpm1-2*–induced depolarization (Zajac et al., 2005). To determine if native Sec3 behaves similarly to the Sec3-GFP, we followed its polarization in this strain by immunofluorescence staining at different times after the temperature shift. We also examined the effects of this mutant on Sec4 and Sec15 polarization. As expected, Sec4 and Sec15 were found to rapidly depolarize in the *tpm1-2* strain with kinetics similar to that seen previously by Pruyne et al. (1998) for Sec4 and Sec8 in this mutant. However, in contrast to the results of Zajac et al. (2005), we find that untagged native Sec3 rapidly depolarizes in this strain with kinetics that closely match that found for Sec15. As expected, all three markers remained polarized in the control *tpm2Δ* strain (Fig. 8). Together with the aforementioned results, we conclude that the behavior of native Sec3 in these experiments is distinct

from that of Sec3-GFP, as its polarization appears to require ongoing polarized exocytosis and polarized actin cables as found for the other exocyst subunits examined.

The functions of Rho3 and Cdc42 in secretion do not require GTP hydrolysis

The ability of Cdc42 to hydrolyze GTP has been shown to be essential for its ability to promote septin ring assembly and polarity establishment (Gladfelter et al., 2002; Irazoqui et al., 2003). Local cycles of GTP loading and hydrolysis by Cdc42 have been proposed to be critical for the proper assembly of multisubunit complexes and in producing an initial asymmetric patch of Cdc42. Our analysis of the *cdc42-6* allele revealed a role for Cdc42 in docking and fusion of secretory vesicles that was independent of its other major functions in cell polarity, including polarization of actin cytoskeleton, septin ring assembly, and polarization of Cdc42 itself (Adamo et al., 2001). Similar to what we observed previously for the *rho3-V51* mutant, *cdc42-6* cells have a severe exocytic defect but show normal localization of all the markers for polarized exocytosis. This suggests that the mode of action for both Rho GTPases is not in the recruitment of the exocytic machinery but rather by direct allosteric activation of this machinery at sites of growth. One prediction of this allosteric mechanism is that it would not be expected to require GTP hydrolysis to regulate exocytosis.

We investigated whether the specific roles of Rho3 and Cdc42 in exocytosis required cycling between GTP and GDP bound forms using mutations analogous to the RAS^{Q61L} allele, which block the ability of Rho GTPases to hydrolyze GTP, as well as the mutants analogous to RAS^{S17N}, resulting in a GDP-locked form of the GTPase. We first examined whether a single-copy (*CEN*) plasmid containing $RHO3^{Q74L}$ could suppress several secretion-deficient strains. Although the cold-sensitive Rab GTPase mutant *sec4-P48* was weakly suppressed by *CEN-RHO3*, $CEN\text{-}RHO3^{Q74L}$ strongly rescued growth at the restrictive temperature, whereas the $RHO3^{T30N}$ GDP-locked mutant failed to suppress (Fig. 9 A, i). These results are similar to those obtained previously using a different activating mutation in *RHO3* (Adamo et al., 1999). In complementation tests, wild-type and GTP-locked *RHO3* alleles were able to equally restore growth of *rho3-V51* cells at 14°C (Fig. 9 A, ii). $CEN\text{-}RHO3^{Q74L}$ was found to be a strong suppressor of the growth defect associated with the *cdc42-6* mutation at 32°C, and the level of suppression was significantly better than *CEN-RHO3* (Fig. 9 A, iii). At 37°C, *cdc42-6* cells have been shown to be altered for both the actin cytoskeleton and secretion (Adamo et al., 2001). We observed that constitutively activated *RHO3* was not able to restore growth of *cdc42-6* at 37°C (Fig. 9 A, iii), suggesting a secretion-based specificity in suppression. These results show that GTP hydrolysis by Rho3 is not required for its secretory function. *RHO3* is known to have multiple genetic interactions with the exocytic apparatus. Indeed, wild-type *RHO3* has the ability to suppress different late-acting *sec* mutants, and, importantly, the *rho3* null allele is suppressed by components of the secretion pathway such as *SEC4*, *SRO7,* and *SEC9* (Adamo et al., 1999). We examined the ability of various nucleotide forms of Rho3 to function as the only source of Rho3 in the cell using a plasmid shuffle assay. Complementation of a *rho3* deletion was observed by following growth on 5-FOA–containing media, which selects against a plasmid containing the wild-type *RHO3* gene, leaving the indicated *RHO3* allele as the sole source of Rho3 in the cell. Although empty vector shows no growth at 30°C, introduction of *CEN-RHO3* (Fig. 9 A, iv, middle) or activated $CEN\text{-}RHO3^{Q74L}$ (Fig. 9 A, iv, bottom) fully complemented the growth defect associated with loss of chromosomal *rho3*. Moreover, complementation by $RHO3^{Q74L}$ was

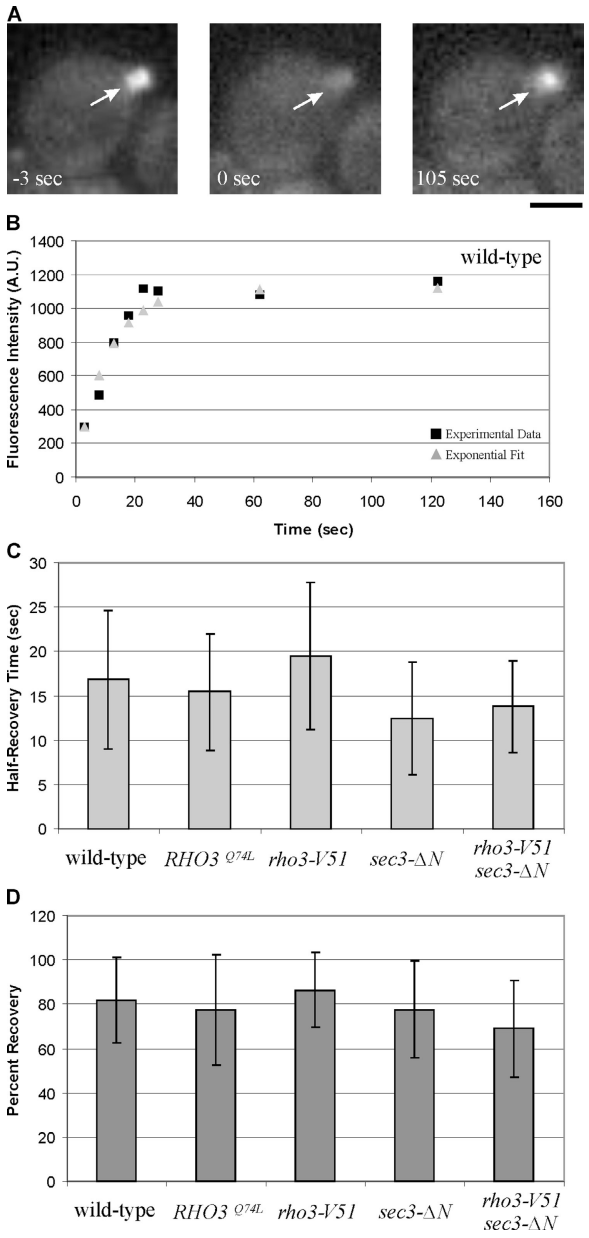

Figure 6. **Sec8-GFP photobleaching and FRAP measurements in *rho3-V51,sec3-ΔN* cells show wild-type recovery times.** (A) Representative example of Sec8-GFP photobleaching experiment. Arrow denotes photobleached area. Prebleach, t = −3 s; photobleaching, t = 0 s; maximum recovery, t = 105 s. Bar, 2 μm. (B) Measured fluorescence recovery of Sec8-GFP (black squares) compared with single exponential fit of recovery (gray triangles). Fluorescence intensity is recorded in arbitrary units (A.U.). See Materials and methods for details of analysis. (C) Average half-recovery times measured for Sec8-GFP. (D) Average percent recovery values for Sec8-GFP. Error bars represent SD.

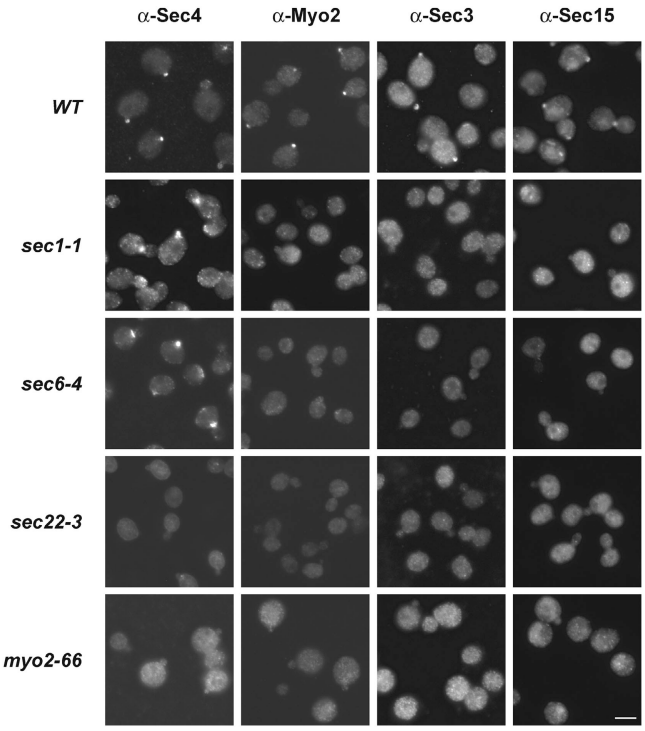

Figure 7. **A functional secretory pathway is required to polarize the exocyst subunits Sec3 and Sec15 and the myosin Myo2.** *Sec1-1*, *sec6-4*, and *myo2-66* mutants, which are blocked at a late stage in transport, and the early secretion mutant *sec22-3* and wild-type cells were grown at 25°C and shifted to 37°C for 1 h, fixed, and processed for fluorescence microscopy. Purified antibodies directed against the Rab protein Sec4, the type V myosin Myo2, and the Sec3 and Sec15 exocyst subunits were used for immunostaining. Bar, 2 μm.

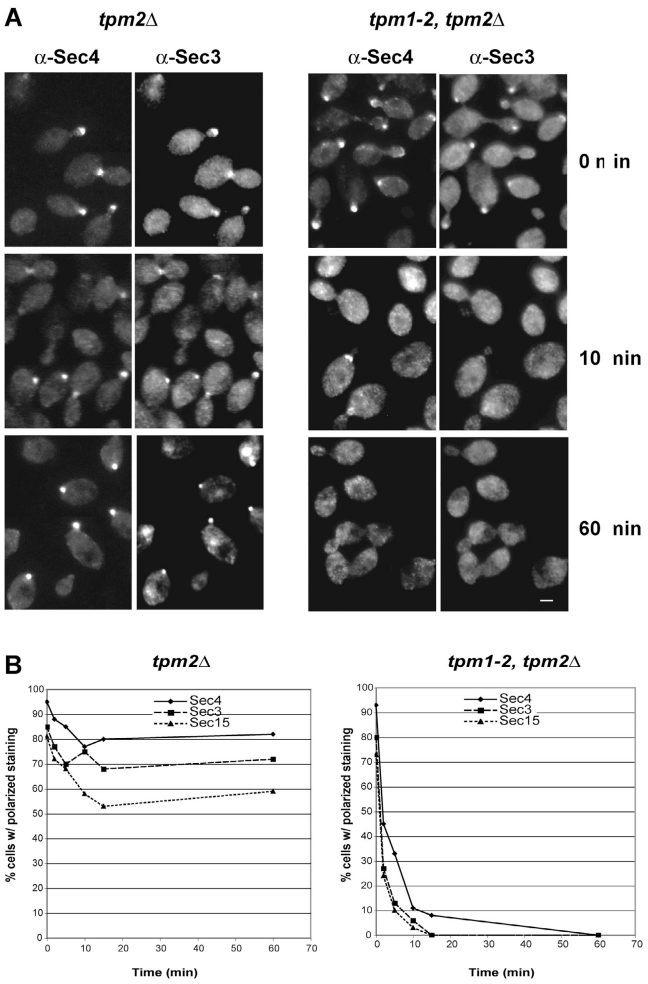

Figure 8. **The polarized localization of Sec3, Sec15, and Sec4 is dependent on actin cables.** (A) Polarization of Sec3 and Sec4 in *tpm1-2, tpm2Δ* homozygous diploids and control *tpm2Δ* diploid cells (with functional *TPM1*; both gifts of A. Bretscher, Cornell University, Ithaca, NY) was examined by double-label immunofluorescence after temperature shifts to 34°C for the indicated times. Bar, 1 μm. (B) Shift to the restrictive temperature rapidly disrupts the polarization of Sec3, Sec15, and Sec4 in tropomyosin mutant strains. Cells were shifted for the indicated times and immediately fixed. Small budded cells were scored for the polarization of Sec3, Sec15, and Sec4 after immunofluorescent staining. Small budded cells were defined as cells whose buds were less than half the size of the mother cell. 200–300 cells were scored per data point.

indistinguishable from *RHO3* at high (37°C) or low (14°C) temperatures (unpublished data). In contrast, the GDP-locked mutant $RHO3^{T30N}$ failed to show any complementation activity. These results demonstrate that all the essential functions of Rho3 in yeast, including regulation of polarized exocytosis, are fulfilled by a GTPase inactive allele. These data suggest that GTP hydrolysis and cycling are not critical to the regulatory functions of Rho3, which further supports the notion that Rho3 functions primarily through allosteric regulation of its effectors.

In contrast to Rho3, many of the functions of Cdc42 are known to require GTP hydrolysis (Irazoqui et al., 2003). To determine if the specific role of Cdc42 in exocytosis requires GTP hydrolysis, we examined the ability of a GTPase-deficient allele to complement growth defects associated with various temperature-sensitive mutations in Cdc42. Because high levels of $Cdc42^{Q61L}$ protein are known to be lethal to yeast, we made use of a construct that allows production of sublethal amounts of activated $CDC42^{Q61L}$ from a crippled version of the *GAL1* promoter (Gladfelter et al., 2002). The thermosensitive mutants of *cdc42* were transformed with this construct and then scored for growth under a variety of conditions. We have shown previously that the *cdc42-6* mutant shows a highly allele-specific defect in exocytosis after shifts to 32°C, but demonstrates pleiotropic polarity defects when shifted to 37°C (Adamo et al., 2001). We observed that *cdc42-6* growth and secretion defects at 32°C were rescued when expression of wild-type *CDC42* or $CDC42^{Q61L}$ was induced on galactose medium (Fig. 9 B; unpublished data). As expected, only wild-type *CDC42* was able to complement *cdc42-6* growth defects at 37°C because the phenotype of this mutant at high temperature resembles that of the pleiotropically defective *cdc42-1* allele. Consistent with a previous paper (Irazoqui et al., 2003), we found that at the restrictive temperatures GTP-locked Cdc42 was unable to rescue defects in *cdc42-17* and *cdc42-27* (Fig. 9 B, bottom) or *cdc42-1* (not depicted), all of which show general defects in Cdc42-mediated polarity. Together, these results show that GTP-locked Cdc42 specifically rescues the highly allele-specific secretory defect associated with *cdc42-6*. Hence, both Rho3 and Cdc42 do not require GDP/GTP cycles to regulate exocytosis, which is consistent with the allosteric mechanism suggested by our phenotypic analysis of *rho3-V51* and *cdc42-6*.

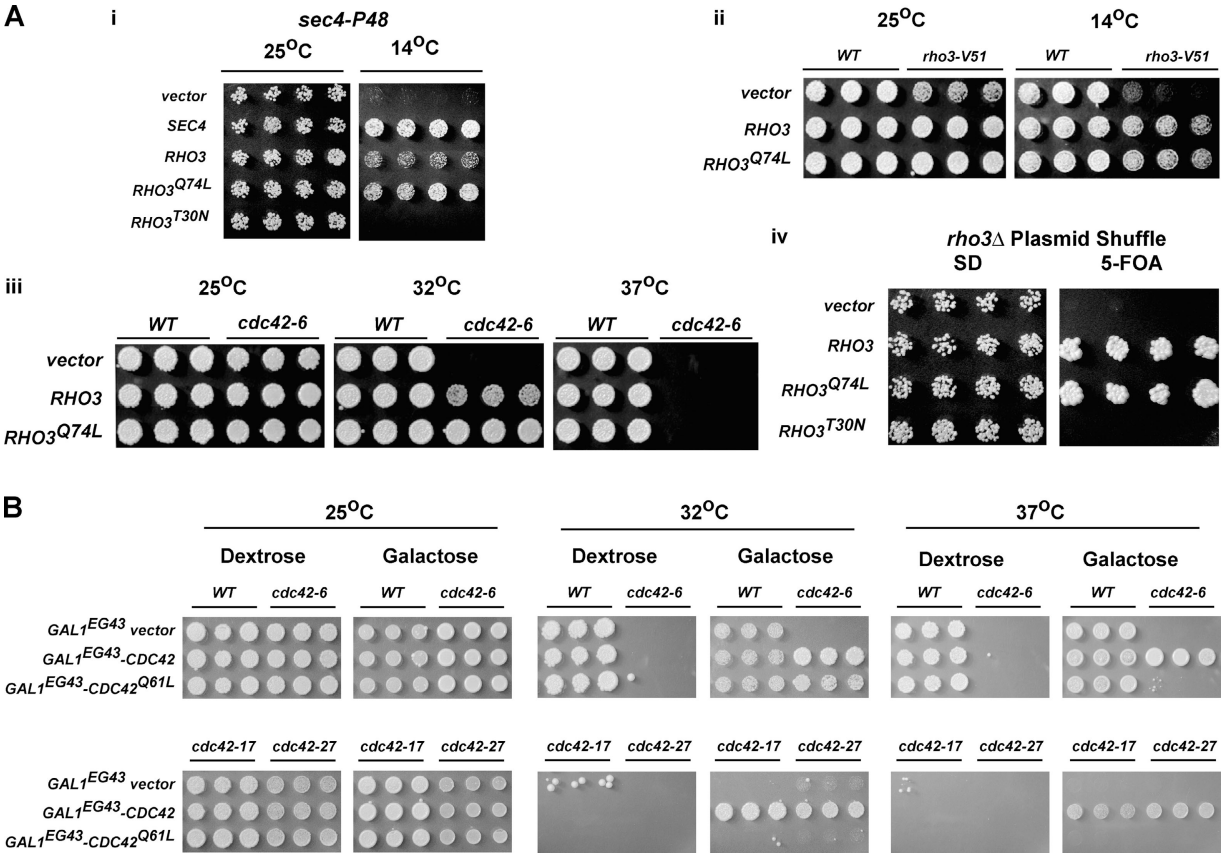

Figure 9. **GTP hydrolysis by Rho3 and Cdc42 is not essential for their secretory function.** (A, i) *sec4-P48* cs phenotype is efficiently suppressed by the activated *RHO3*^{Q74L} allele. Wild-type and mutant *sec4-P48* strains were transformed with single-copy (*CEN*) plasmids with the indicated gene, picked into microtiter wells, and transferred onto SC-ura plates at 25 and 14°C. Similar results were obtained on YPD medium. (ii) GTP hydrolysis by Rho3 is not necessary for its function in secretion. Centromeric plasmids containing the wild-type or activated alleles of *RHO3* were introduced into the secretion-defective mutant *rho3-V51* and wild-type strain. Suppression of cs phenotype was assayed as described for A (i). (iii) Constitutively activated Rho3 is a strong suppressor of the *cdc42-6* secretion mutant. *RHO3* and *RHO3*^{Q74L} centromeric plasmids were transformed into wild-type and *cdc42-6* cells. Suppression of the growth defect was tested on YPD media at the restrictive temperatures of 32 and 37°C. (iv) Complementation of the *rho3* deletion by activated *RHO3*^{Q74L}. A *rho3Δ* strain containing *CEN/URA3/RHO3* plasmid was used in a plasmid shuffle complementation assay. This strain was transformed with an empty *CEN/HIS3* plasmid or an identical plasmid containing wild-type *RHO3*, activated *RHO3*^{Q74L}, or the GDP-blocked mutant *RHO3*^{T30N}. Transformants were tested for growth on control (SD) plates or 5-FOA plates, which select against the *CEN/URA3/RHO3* plasmid, to monitor growth of each strain with the *CEN/HIS3* plasmid as the sole source of *RHO3* in the cell. Plates were grown for 3 d at 30°C. (B) GTP hydrolysis by Cdc42 is not necessary for suppression of the *cdc42-6* secretion defect. *GAL1*^{EG43} plasmid carrying the wild-type or activated (Q61L) allele of *CDC42* was integrated into the *URA3* locus of the wild-type, *cdc42-6, cdc42-17*, and *cdc42-27* strains. Empty *GAL1*^{EG43} vector was used as a growth control. Colonies from each transformation were picked into microtiter wells and transferred onto YP plates containing either 2% glucose or 2% galactose to repress or induce the *EG43* crippled *GAL1* promoter, respectively. The cells were then incubated at the permissive temperature (25°C) or at the nonpermissive temperatures (32°C and 37°C) for 2–3 d.

Discussion

We have previously characterized a mutant in the Cdc42 GTPase, which is specifically defective in post-Golgi vesicle docking and fusion with the plasma membrane but exhibits no detectable defect in the polarized localization of the exocytic machinery. Based on these results, we suggested that Cdc42 acts as a positive allosteric regulator of the late secretory apparatus at sites of polarized growth. Here, we show that another Rho GTPase in yeast, Rho3, acts in a similar manner to positively regulate exocytosis independent of any effect in the polarization of the exocytic machinery. Together, these findings suggest a novel mechanism for the action of Rho GTPases in polarized secretion. This new model, depicted in Fig. 10, is distinct from previous models in which Rho proteins were thought to act in polarization of exocytosis by sequestering components of the secretion machinery at a specific site on the plasma membrane (Fig. 10, A and B, top; Guo et al., 2001; Symons and Rusk, 2003). In this model, a polarized patch of activated Rho GTPase would act directly on the initially unpolarized late secretory machinery (Fig. 10, A and B, bottom). This localized signal would increase the activity of the machinery and hence the likelihood of a productive secretory vesicle fusion event at a precise site on the membrane. Because many components of the docking and fusion machinery—including the exocyst complex (Guo et al., 1999; Folsch et al., 2003; Vik-Mo et al., 2003), Cdc42, and Rho1 (Abe et al., 2003; Wedlich-Soldner et al., 2003)—have been found to associate with secretory vesicles and other transport intermediates, ongoing allosteric regulation would be expected to lead to the polarization of the secretion apparatus and to a further increase in allosteric activation. According to this view, the polarization of the secretory machinery would then be a consequence, rather than a cause, of ongoing polarized delivery of vesicles to the plasma membrane. Based on this model,

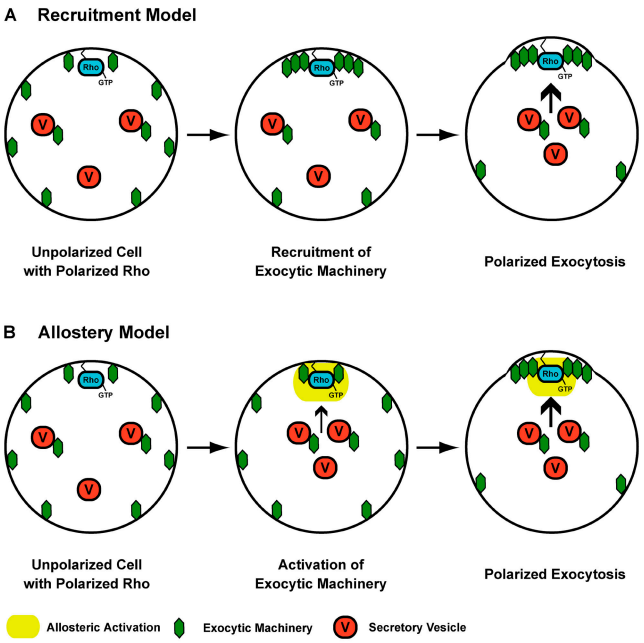

Figure 10. **Models for the spatial regulation of exocytosis by Rho GTPases.** (A) Recruitment model for regulation of exocytosis. A polarized patch of GTP-bound Rho protein would recruit specific components of the exocytic machinery, including the exocyst complex, to a specific site on the plasma membrane. Once polarized, the exocyst complex would act as a spatial landmark for exocytosis, restricting the docking and fusion of post-Golgi vesicles at this site. (B) Allostery model for regulation of exocytosis. A polarized patch of activated Rho GTPase would act directly as a spatial landmark for exocytosis by locally activating the late secretory machinery, including the exocyst complex. This locally activated machinery would increase the likelihood of productive docking and fusion at this site. Many components of the exocytic machinery, including Rho GTPases and exocyst subunits, have been shown to be associated with secretory vesicles. Therefore, increased exocytosis at sites of allosteric activation would lead to both reinforcement of the activation signal as well as polarization of the exocytic machinery.

the activated Rho GTPases, rather than their effectors, would serve as the major spatial landmarks for polarized secretion.

Previous work on the Sec3 component of the exocyst complex has led to the hypothesis that the localization of this protein might serve as a spatial landmark for polarization of vesicle docking and fusion events on the plasma membrane (Finger et al., 1998). However, in the current study, we found that polarization of Sec3, through its NH_2-terminal RBD, is dispensable for its overall function in the cell, although a positive role for this domain was detected in combination with certain other secretory defective mutants. Indeed, although the NH_2-terminal RBD domain is essential for the polarization of Sec3, localization and function of the exocytic machinery is not dependent on Sec3 polarization. Our analyses of Sec3 localization using antibodies to examine the localization of native Sec3 suggest that its polarization is determined in the same way as the rest of the exocyst complex. This is in strong agreement with our model for Rho-dependent polarization of exocytosis. It is not presently clear why we see such distinct differences in the polarization of Sec3 to those seen in the previous studies done primarily with GFP-tagged forms of the protein. It is possible that either the presence of the GFP tag itself influences the localization of Sec3 or that our Sec3 antibodies recognize an epitope whose accessibility is sensitive to a conformational change in the protein.

Guo et al. (2001) has suggested that, in the absence of polarized Sec3, the rest of the exocyst complex may be polarized through a parallel pathway involving Exo70. Because Exo70 binds to Rho3, this interaction could represent a redundant pathway for exocyst localization. We tested this hypothesis directly using an allele of Rho3 that has been shown to be specifically defective in interacting with Exo70. We find no evidence for redundancy between the Rho3–Exo70 and Rho1–Sec3 pathways. To exclude an indirect effect due to *sec3-ΔN* overexpression, we performed similar experiments using a strain producing a wild-type level of Sec3-ΔN protein as the only source of Sec3. Again, no redundancy between the two pathways was observed (unpublished data). Thus, we find no evidence to suggest that either Sec3 alone or the exocyst as a whole act as spatial landmarks for exocytosis. Rather, we propose that the exocyst polarization is a consequence of ongoing polarized exocytosis (Fig. 10, A and B, bottom). Sec3 is the only component of the exocyst whose localization has been reported to be independent of the secretory pathway and the actin cytoskeleton (Finger et al., 1998). In yeast, the Sec8 and Sec15 subunits are delivered to the plasma membrane through the secretory pathway, and the other exocyst subunits, with the exception of Sec3, act similarly (Finger et al., 1998; Guo et al., 1999, Boyd et al., 2004). However, we report here that the mechanism by which Sec3 becomes polarized appears to be similar to that of the other exocyst subunits and involves polarized delivery of vesicles to the plasma membrane. Thus, it is unlikely that additional interactions between Rho GTPases and the exocyst would be involved in the initial polarization of this complex, but these signals would serve to regulate the assembled complex at sites of growth.

A prediction of the allostery model is that the function of Rho GTPases in exocytosis would not require GTP hydrolysis, as allosteric regulation is expected to be maximal in the GTP-bound state (Buck et al., 2004; Peterson et al., 2004). In support of this model, we found that Rho3 efficiently fulfills its function in exocytosis when locked in a GTP-bound form, as measured by suppression of specific late secretory mutants. In fact, the GTPase-deficient mutant can fulfill all the functions of Rho3 in the cell because it completely rescues a deletion in the gene in a manner that is genetically and morphologically indistinguishable from the wild-type *RHO3* gene. In contrast, Cdc42 is known to require GTP hydrolysis for many of its functions in the cell, including septin ring assembly and early polarization (Gladfelter et al., 2002; Irazoqui et al., 2003). Consistent with this, we and others have found that most *cdc42* mutant alleles fail to be rescued by a mutant form of Cdc42 unable to hydrolyze GTP. In contrast, we found that the *cdc42-6* allele, which has a very specific defect in exocytosis, is well complemented by a GTPase-deficient form of Cdc42. These results provide further support for the fact that Rho3 and Cdc42 function in a similar allosteric fashion in the spatial regulation of exocytosis.

Overall, our analysis of the *rho3-V51* and *cdc42-6* mutants show that these Rho GTPases regulate secretion independent of their ability to polarize the exocytic machinery and to

hydrolyze GTP. These observations lead us to propose a model in which Rho proteins work as allosteric regulators of the unpolarized secretion machinery by activating the fusion of vesicles with the plasma membrane. As a consequence of this activation, components of the secretion machinery, carried on post-Golgi vesicles, would themselves become polarized (Boyd et al., 2004). This would be expected to result in the reinforcement of the polarization of this process by a positive feedback mechanism. Importantly, this model is applicable to polarization events observed in mammalian cells. In particular, the basolateral membrane recruitment of the Sec6/8 complex in epithelial cells is known to be a consequence of cell–cell adhesion. The polarized localization of the Sec6/8 complex correlates with, but does not precede, the development of polarized transport to the basolateral membrane (Grindstaff et al., 1998). In the developing neuron, the polarized patches of Sec6/8 observed in the synaptic region disappear upon maturation of the synapse (Hazuka et al., 1999). This phenomenon may simply reflect a decrease in the delivery of the exocyst components to the membrane, as trafficking strongly decreases after maturation of the synapse.

Determining the precise nature of allosteric regulation by Rho3 and Cdc42 on the exocytic machinery and the exocyst will be critical to understanding the molecular mechanism by which Rho proteins regulate polarized secretion. A likely mechanism for allosteric regulation would involve relief of an autoinhibitory interaction similar to that found for the effect of Rho GTPases in regulating the activity of members of the formin family (Zigmond, 2004). By analogy, binding of the Rho3 GTPase to the RBD of Exo70 (for example) would relieve an inhibitory interaction within Exo70 or perhaps between Exo70 and another exocyst subunit. The conformational change created by the binding of Rho3 would then promote the ability of this complex to drive downstream events through direct effects on SNAREs (Sivaram et al., 2005) or through an intermediary (Lehman et al., 1999). In either case, the result would be a direct increase in the rate of vesicle docking and fusion at sites populated by the activated Rho GTPase.

Materials and methods

Genetic techniques and yeast strains construction

Standard yeast genetic manipulations were performed as described by Guthrie and Fink (1991). Cells were grown in complete YP media (1% Bacto yeast extract and 2% Bacto peptone containing 2% glucose or galactose) or in minimal media (0.67% yeast nitrogen base without amino acids and 2% glucose) supplemented with appropriate metabolites. For all assays performed, 25°C was the permissive temperature, whereas the restrictive temperature of 14 or 32°C was used to visualize *rho3-V51* and *cdc42-6* growth defects, respectively.

To construct the *sec3-ΔN* strain, a region encoding amino acids 321–653 of Sec3 was PCR amplified from yeast genomic DNA and cloned into the BamHI and SalI sites of the integrative, *HIS3*, pRS303 plasmid (Sikorski and Hieter, 1989). A 0.7-kb PCR fragment containing the glyceraldehyde-3-phosphate hehydrogenase 3 promoter (*GPD*p) sequence fused to the first two Sec3 codons was ligated into the BamHI and NotI sites. Digestion of the resulting plasmid at the unique PmlI site was used to target integration into the *SEC3* gene, and thus create a modified locus expressing truncated *sec3-Δ3-320* under the dependence of *GPD*p. The PmlI-linearized plasmid was transformed into a diploid wild-type yeast strain (**a**/α, *ura3-52/ura3-52, leu2-3, 112/leu2-3, 112, his3Δ200/his3Δ200*), and his^+ transformants were sporulated. The tetrads were dissected on complete media, and the haploid progeny were analyzed by replica plating for the presence of wild-type (scored as his^-) or modified (scored as his^+) *SEC3* locus. Correct replacement of wild-type *SEC3* locus by *GPD*p-*sec3-Δ3-320* was further confirmed by immunoblot analysis using anti-Sec3 antibodies directed against the middle region of the protein (aa 279–474).

To assay for the genetic interaction between *rho3-V51* and *sec3-ΔN*, an integrative plasmid containing the *rho3-V51* allele (Adamo et al., 1999) was first linearized with PstI and integrated into the *URA3* locus of a haploid *sec3-ΔN* strain (**a**, *sec3::HIS3::GPD*p-*sec3-ΔN, ura3-52, leu2-3, 112, his3Δ200*). The resulting strain was then crossed to *rho3-V51* (α, *ura3::rho3-V51, rho3Δ::LEU2, leu2-3, 112, his3Δ200*), and the diploid cells were sporulated and dissected on complete media. Progeny were analyzed for the presence of *GPD*p-*sec3-ΔN* (scored as his^+) and *RHO3* deletion (scored as leu^+) by replica plating and were analyzed for a growth defect at 14°C by dilution assays. Sec3 and Sec3-ΔN proteins production was monitored by immunoblot analysis using anti-Sec3 antibodies.

Wild-type and activated forms of *CDC42* expressed from a crippled version of the *GAL1* (*EG43*) promoter (a gift of J. Irazoqui and D. Lew, Duke University, Durham, NC) were integrated into the *URA3* locus of temperature-sensitive *cdc42* mutants as previously described (Gladfelter et al., 2002). Expression was induced in the presence of galactose and repressed in the presence of glucose in the medium.

Immunoblot analysis

Western blot analyses were performed on either yeast whole-cell lysates, to detect Sec3 and Exo70 proteins, or on internal/external cellular fractions, to analyze Bgl2 secretion. Blots were probed with polyclonal α-Sec3 or α-Exo70 antibodies at 1:1,000 dilution or affinity-purified α-Bgl2 antibody diluted 1:100, depending on the experiment. Primary antibodies were detected with radiolabeled ^{125}I-protein A at 1:660 dilution. Quantitation of bands was done on a STORM phosphoimager using ImageQuant software (Molecular Dynamics).

Invertase and Bgl2 secretion assays

Invertase assays were performed as described previously (Adamo et al., 1999). Exoglucanase Bgl2 secretion was tested essentially as described previously (Adamo et al., 1999). In brief, yeast strains were grown overnight to midlog phase at 25°C in YPD and then either shifted to 14°C for 5 h or kept at 25°C. NaF and NaN_3 were added to a final concentration of 20 mM. 25 ODs of cells were washed in Tris/NaF/NaN3 and spheroplasted, and the internal and external fractions were separated and boiled in 2× or 6× sample buffers, respectively. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with affinity-purified α-Bgl2 antibody.

Immunofluorescence microscopy

Cells were grown overnight to mid-log phase and then either fixed immediately with 3.7% formaldehyde or shifted to the restrictive temperature. Fixed cells were spheroplasted, permeabilized with 0.5% SDS, and affixed to the slides as described previously (Brennwald and Novick, 1993). The following primary antibodies were incubated for 1 h at RT: Sec4 mAb (1:100 dilution), affinity-purified polyclonal Cdc42 (1:75 dilution), Myo2 (1:100 dilution), Sec3 (1:50 dilution), or Sec15 (1:100 dilution). The secondary antibodies used were FITC-conjugated goat anti–mouse at 1:50 dilution for detection of Sec4, and rhodamine-conjugated goat anti–rabbit at 1:50 dilution for detection of Cdc42, Myo2, and Sec3 or at 1:100 dilution for detection of Sec15 (secondary antibodies were obtained from Jackson ImmunoResearch Laboratories). After a 1-h incubation at RT, the slides were washed extensively and mounting media (90% glycerol with 4′, 6-diamidino-2-phenylindole to visualize DNA and *o*-phenylenediamine to retard photobleaching) was added. Stained cells were viewed on a microscope (model E600; Nikon) equipped with a 512 × 512 back illuminated frame-transfer charge coupled device camera (Princeton Instruments) and Metamorph software (Universal Imaging Corp.) to capture images. Pictures were processed for presentation using Adobe Photoshop.

Intensity of fluorescence associated with polarized immunostained proteins was measured in small budded cells using Metamorph software. A minimum of 25 distinct spots from wild-type and mutant cells were measured for each experimental condition, and the average and SD values were determined. Result for each marker was then expressed as a fluorescence intensity in mutant cells relative to the fluorescence observed in wild type.

Visualization of GFP-tagged exocyst components

GFP experiments were performed on wild-type or mutant cells transformed with a plasmid containing Sec3, Sec8, or Exo70 fused to a single GFP molecule at the COOH-terminal extremity (Adamo et al., 2001). Yeast

strains were grown overnight in selective media to an early log phase and then either shifted to 14°C for 5 h or kept at 25°C. Cells were fixed by a 10-min treatment in −20°C methanol, washed with acetone at −20°C, and rehydrated by three washes with ice-cold PBS before being observed by a fluorescence microscope.

Photobleaching experiments and FRAP analysis

Mid-logarithmic Sec8-GFP cells grown at 32°C in selective media were placed on a slab of selective media supplemented with 25% gelatin and imaged at RT (~22°C). Photobleaching of Sec8-GFP was performed on an inverted microscope (Eclipse TE2000-U; Nikon) equipped with a 1.4 NA 100× differential interference contrast (DIC) objective. Single focal plane images were captured by a digital camera (Orca ER; Hamamatsu) using a 400-ms (ms) epifluorescence exposure time (binned 2 × 2) and a 200-ms DIC exposure time. Cells were photobleached using the 488-nm line from a 100-mW argon laser (Spectra-Physics) controlled by a sliding filter cube set (Conix Research). The bud tip signal was photobleached to ~25% of the prebleach fluorescence intensity. The photobleaching exposure time was 50–175 ms. During image acquisition, one prebleach data point was recorded, followed by eight data points acquired every 5 s, and then five data points every 20 s. The total imaging time was ~140 s. Aperiodic DIC images were also acquired. FRAP measurements were performed as previously described (Molk et al., 2004).

Online supplemental material

Table S1 details the data obtained from the FRAP analysis with Sec8-GFP. Table S2 shows the synthetic genetic effects of combining the *sec3-ΔN* mutant with various *rho3*, *cdc42*, and late *sec* mutants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200504108/DC1.

The authors are grateful to Anthony Bretscher, Javier Irazoqui, and Daniel Lew for the gift of plasmids and yeast strains, Chad Pearson for construction of the photobleaching microscope, E.D. Salmon for support, Akanksha Gangar and Anne Andreeva for discussions and critical reading of the manuscript, and Robert Hales and Will Smith for technical assistance. We also thank members of the Pringle and Lew laboratories for stimulating discussions.

This work was supported by National Institutes of Health grants GM 54712 (P. Brennwald) and GM 32238 (K. Bloom).

Submitted:19 April 2005
Accepted: 12 July 2005

References

Abe, M., H. Qadota, A. Hirata, and Y. Ohya. 2003. Lack of GTP-bound Rho1p in secretory vesicles of *Saccharomyces cerevisiae*. *J. Cell Biol.* 162:85–97.

Adamo, J.E., G. Rossi, and P. Brennwald. 1999. The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. *Mol. Biol. Cell.* 10:4121–4133.

Adamo, J.E., J.J. Moskow, A.S. Gladfelter, D. Viterbo, D.J. Lew, and P.J. Brennwald. 2001. Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. *J. Cell Biol.* 155:581–592.

Ayscough, K.R., J. Stryker, N. Pokala, M. Sanders, P. Crews, and D.G. Drubin. 1997. High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. *J. Cell Biol.* 137:399–416.

Boyd, C., T. Hughes, M. Pypaert, and P. Novick. 2004. Vesicles carry most exocyst subunits to exocytic sites marked by the remaining two subunits, Sec3p and Exo70p. *J. Cell Biol.* 167:889–901.

Brennwald, P., and P. Novick. 1993. Interactions of three domains distinguishing the Ras-related GTP-binding proteins Ypt1 and Sec4. *Nature.* 362:560–563.

Brennwald, P., B. Kearns, K. Champion, S. Keranen, V. Bankaitis, and P. Novick. 1994. Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. *Cell.* 79:245–258.

Buck, M., W. Xu, and M.K. Rosen. 2004. A two-state allosteric model for autoinhibition rationalizes WASP signal integration and targeting. *J. Mol. Biol.* 338:271–285.

Finger, F.P., T.E. Hughes, and P. Novick. 1998. Sec3p is a spatial landmark for polarized secretion in budding yeast. *Cell.* 92:559–571.

Folsch, H., M. Pypaert, S. Maday, L. Pelletier, and I. Mellman. 2003. The AP-1A and AP-1B clathrin adaptor complexes define biochemically and functionally distinct membrane domains. *J. Cell Biol.* 163:351–362.

Gladfelter, A.S., I. Bose, T.R. Zyla, E.S. Bardes, and D.J. Lew. 2002. Septin ring assembly involves cycles of GTP loading and hydrolysis by Cdc42p. *J. Cell Biol.* 156:315–326.

Goud, B., A. Salminen, N.C. Walworth, and P.J. Novick. 1988. A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. *Cell.* 53:753–768.

Grindstaff, K.K., C. Yeaman, N. Anandasabapathy, S.C. Hsu, E. Rodriguez-Boulan, R.H. Scheller, and W.J. Nelson. 1998. Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. *Cell.* 93:731–740.

Guo, W., D. Roth, C. Walch-Solimena, and P. Novick. 1999. The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. *EMBO J.* 18:1071–1080.

Guo, W., F. Tamanoi, and P. Novick. 2001. Spatial regulation of the exocyst complex by Rho1 GTPase. *Nat. Cell Biol.* 3:353–360.

Guthrie, C., and G. Fink, eds. 1991. Guide to yeast genetics and molecular biology. Methods in Enzymology. Vol. 194. Academic Press, San Diego. 863 pp.

Hazuka, C.D., D.L. Foletti, S.C. Hsu, Y. Kee, F.W. Hopf, and R.H. Scheller. 1999. The sec6/8 complex is located at neurite outgrowth and axonal synapse-assembly domains. *J. Neurosci.* 19:1324–1334.

Irazoqui, J.E., A.S. Gladfelter, and D.J. Lew. 2003. Scaffold-mediated symmetry breaking by Cdc42p. *Nat. Cell Biol.* 5:1062–1070.

Lehman, K., G. Rossi, J.E. Adamo, and P. Brennwald. 1999. Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. *J. Cell Biol.* 146:125–140.

Molk, J.N., S.C. Schuyler, J.Y. Liu, J.G. Evans, E.D. Salmon, D. Pellman, and K. Bloom. 2004. The differential roles of budding yeast Tem1p, Cdc15p, and Bub2p protein dynamics in mitotic exit. *Mol. Biol. Cell.* 15:1519–1532.

Sivaram, M.V.S., J.A. Saporita, M.L.M. Furgason, A.J. Boettcher, and M. Munson. 2005. Dimerization of the exocyst protein Sec6p and its interaction with the t-SNARE Sec9p. *Biochemistry.* 44:6302–6311.

Peterson, F.C., R.R. Penkert, B.F. Volkman, and K.E. Prehoda. 2004. Cdc42 regulates the Par-6 PDZ domain through an allosteric CRIB-PDZ transition. *Mol. Cell.* 13:665–676.

Pruyne, D., and A. Bretscher. 2000. Polarization of cell growth in yeast. *J. Cell Sci.* 113:571–585.

Pruyne, D.W., D.H. Schott, and A. Bretscher. 1998. Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. *J. Cell Biol.* 143:1931–1945.

Robinson, N.G., L. Guo, J. Imai, E.A. Toh, Y. Matsui, and F. Tamanoi. 1999. Rho3 of *Saccharomyces cerevisiae*, which regulates the actin cytoskeleton and exocytosis, is a GTPase which interacts with Myo2 and Exo70. *Mol. Cell. Biol.* 19:3580–3587.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 122:19–27.

Symons, M., and N. Rusk. 2003. Control of vesicular trafficking by Rho GTPases. *Curr. Biol.* 13:R409–R418.

Terbush, D.R., T. Maurice, D. Roth, and P. Novick. 1996. The Exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *EMBO J.* 15:6483–6494.

Vik-Mo, E.O., L. Oltedal, E.A. Hoivik, H. Kleivdal, J. Eidet, and S. Davanger. 2003. Sec6 is localized to the plasma membrane of mature synaptic terminals and is transported with secretogranin II-containing vesicles. *Neuroscience.* 119:73–85.

Walch-Solimena, C., R.N. Collins, and P.J. Novick. 1997. Sec2p mediates nucleotide exchange on Sec4p and is involved in polarized delivery of post-Golgi vesicles. *J. Cell Biol.* 137:1495–1509.

Wedlich-Soldner, R., S. Altschuler, L. Wu, and R. Li. 2003. Spontaneous cell polarization through actomyosin-based delivery of the Cdc42 GTPase. *Science.* 299:1231–1235.

Wiederkehr, A., Y. Du, M. Pypaert, S. Ferro-Novick, and P. Novick. 2003. Sec3p is needed for the spatial regulation of secretion and for the inheritance of the cortical endoplasmic reticulum. *Mol. Biol. Cell.* 14:4770–4782.

Zajac, A., X. Sun, J. Zhang, and W. Guo. 2005. Cyclical regulation of the exocyst and cell polarity determinants for polarized cell growth. *Mol. Biol. Cell.* 16:1500–1512.

Zhang, X., E. Bi, P. Novick, L. Du, K.G. Kozminski, J.H. Lipschutz, and W. Guo. 2001. Cdc42 interacts with the exocyst and regulates polarized secretion. *J. Biol. Chem.* 276:46745–46750.

Zigmond, S.H. 2004. Formin-induced nucleation of actin filaments. *Curr. Opin. Cell Biol.* 16:99–105.