Cdc42 Interacts with the Exocyst and Regulates Polarized Secretion

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Abbreviations: GTP, Guanosine 5'-triphosphate; GTPγS, Guanosine 5'-γ-thio-triphosphate; GDP, Guanosine 5'-diphosphate; GST, Glutathione-S-transferase; MBP, Maltose-binding protein; aa, amino acids; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; Con A, Concavanine A.
ABSTRACT

Polarized delivery and incorporation of proteins and lipids to specific domains of the plasma membrane is fundamental to a wide range of biological processes such as neuronal synaptogenesis and epithelial cell polarization. The exocyst complex is specifically localized to sites of active exocytosis and plays essential roles in secretory vesicle targeting and docking at the plasma membrane. Sec3p, a component of the exocyst, is thought to be a spatial landmark for polarized exocytosis. In a search for proteins that regulate the localization of the exocyst in the budding yeast Saccharomyces cerevisiae, we found that certain cdc42 mutants affect the polarized localization of the exocyst proteins. In addition, we found that these mutant cells have a randomized protein secretion pattern on the cell surface. Biochemical experiments indicated that Sec3p directly interacts with Cdc42 in its GTP-bound form. Genetic studies demonstrated synthetic-lethal interactions between cdc42 and several exocyst mutants. These results have revealed a role for Cdc42 in exocytosis. We propose that Cdc42 coordinates the vesicle docking machinery and the actin cytoskeleton for polarized secretion.
Introduction

Spatial regulation of exocytosis is fundamental to many biological processes such as epithelial cell polarization and neuronal growth cone formation\(^1\). The budding yeast *Saccharomyces cerevisiae* undergoes polarized growth, which requires directional transport and incorporation of newly synthesized materials to specific domains of the plasma membrane for cell surface expansion. Using the budding yeast as a model system, a large number of proteins involved in many essential cellular processes such as cell cycle progression, assembly of the actin cytoskeleton, cell polarization, and membrane traffic have been identified\(^2\text{-}^5\). Most of the proteins involved in these processes are evolutionarily conserved among eukaryotic organisms.

Recent studies have indicated that a multiprotein complex, termed the exocyst, is involved in vesicle targeting and docking at the plasma membrane\(^6\text{-}^{11}\). The exocyst consists of eight components, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p. They are specifically localized to sites of active exocytosis. In developing neurons, exocyst components are localized to growth cones and the tips of growing neurites\(^13\), while in epithelial cells, they are concentrated near the tight junction, a region of active basolateral membrane addition\(^14\). In the budding yeast, the exocyst proteins are localized to regions of active cell surface expansion: the bud tip at the early phases of the cell cycle and the mother/daughter connection during cytokinesis\(^6,^8,^{11}\). The exocyst subunit Sec3p is localized to sites of exocytosis independent of the actin cytoskeleton and ongoing membrane traffic\(^8\). In a variety of mutants blocking secretion at or before the Golgi apparatus, GFP-Sec3p is still localized at bud tips and mother/daughter cell connections. Thus Sec3p, rather than "riding" the vesicles to these sites, appears to be at the exocytic sites in "anticipation" of the arrival of the secretory vesicles\(^5\). Furthermore, the localization of Sec3p appears to be independent of the other subunits of the
exocyst. Based on these observations, it was proposed that Sec3p represents a spatial landmark for polarized secretion. In addition to Sec3p, several other components of the yeast exocytic machinery are also localized to sites of polarized growth. For example, Sec4p, the Rab protein essential for post-Golgi secretion, is localized to exocytic sites through its association with secretory vesicles, which are transported via the actin cytoskeleton. Another exocyst protein, Sec15p, associates with secretory vesicles and interacts with the GTP-bound form of Sec4p. Sec4p promotes protein-protein interactions among the exocyst components, which then link Sec15p to Sec3p. The assembly of the exocyst complex thereby tethers secretory vesicles to specific sites on the plasma membrane for subsequent membrane fusion.

Proper localization of the exocyst complex is important for spatial regulation of secretion. To identify proteins that regulate the polarized localization of the exocyst, we examined the localization of GFP-tagged exocyst proteins in various classes of mutants. Here we report that the polarized localization of the exocyst is controlled by Cdc42. Cdc42 is a member of the Rho family of small GTP-binding proteins, which are master regulators of many cellular activities including polarization, morphogenesis, membrane traffic, cell growth and development. We found in certain cdc42 mutants that the exocyst proteins were depolarized and the exocytosis pattern was randomized. In vitro assays indicated that Sec3p directly interacted with Cdc42 in its GTP-bound form. These results revealed a role for Cdc42 in polarized exocytosis, and help us understand the molecular mechanisms underlying the polarized localization of exocytic machinery and the spatial regulation of secretion.
Methods

Yeast Strains and Media for Growth

Yeast cells were grown in YP medium (1% Bacto-yeast extract, 2% Bacto-peptone) supplemented with 2% dextrose (YPD), or grown in SCD medium (synthetic complete plus 2% dextrose) lacking certain supplements. Strains used in this study are listed in Table 1.

Isolation of cdc42-ts mutants

To isolate temperature-sensitive (ts) mutations in CDC42 that may cause distinct morphological changes at either permissive or non-permissive temperature, we randomly mutagenized the entire CDC42 gene by a PCR-based method. The mutagenic PCR conditions (100 μl reaction volume) were as follows: 1 μl template DNA (0.1mg/ml) of plasmid pKS-CDC42, 2 μl the forward (454 bp upstream of the start codon) and reverse (540 bp downstream of the stop codon) primers (50 mM each), 10 μl Taq polymerase buffer (10 X stock, Mg2+ free), 10 μl Mg2+ (10 X stock, 25 mM), 10 μl mutagenic dNTPs mixture (10 X stock: 2 mM dATP, 2 mM dGTP, 10 mM dCTP, and 10 mM dTTP), 1ml MnCl2 (50 mM), 1 μl Taq (5units/μl) (Promega), and 63 μl of ddH2O. PCR was carried out with the following program: 94°C, 4 min → 94°C, 1 min; 45°C, 1 min; 72°C, 1 min 45 sec for 30 cycles → 72°C, 10 min → 4°C. Multiple PCRs were combined and the ~1.6-kb PCR-amplified fragment was purified by ethanol precipitation and resuspended in ddH2O. The mutagenized PCR products were mixed with an equal amount of linearized pRS314-CDC42 (CEN TRP1), in which the entire CDC42 gene was removed by a restriction digestion with HpaI (67 bp upstream of the start codon) and NsiI (346 bp downstream of the stop codon). This mixture of DNA was transformed into strain YEF1194 (a cdc42Δ::HIS3, pRS316-GAL1-CDC42) (pRS316-GAL1-CDC42 is a centromere-based, URA3-marked plasmid carrying CDC42) grown in SC-Ura medium.
containing 2% glucose and 2% galactose to allow "gap repair" between the PCR products and the linearized pRS314-CDC42, and transformants were selected on SC-Trp plates at 24°C. The transformants were replicated onto SC-Trp plates containing 5-FOA to select for the loss of plasmid pRS316-GAL1-CDC42 at 24°C. These Trp⁺ Ura⁻ transformants were replicated onto two sets of SC-Trp plates that were incubated at 24°C and 37°C, respectively, to allow the identification of ts mutants. Plasmids were rescued from mutants that displayed interesting morphologies at either permissive or non-permissive temperature, and were sequenced to confirm the existence of mutation(s) in CDC42. These plasmids were transformed into strain YEF1194 and processed as described above to confirm that the ts and morphological phenotypes were indeed caused by mutations on the plasmids.

YEF1961 (cdc42-201) and YEF1962 (cdc42-13) were constructed through homologous recombination at the cdc42Δ::HIS3 locus by transforming a ~1.7-kb BamHI-SalI fragment from pRS314-CDC42-201 or pRS314-CDC42-13 into YEF1194, selecting for colony formation on YPD plates (to repress the expression of CDC42 from the plasmid) at 24°C. These colonies were then screened for His⁻ and temperature-sensitivity for growth. Finally, the colonies were "cured" of plasmid pRS316-GAL1-CDC42 by streaking out cells on synthetic complete medium containing 1 mg/ml 5-FOA at 24°C, thus, forming the strains YEF1961 and YEF1962. The mutations in cdc42-201 are C6S, D31V, D65G, and F90C; The mutation in cdc42-13 is R120G.

Localization of GFP-tagged Exocyst Components and Cdc42 in Yeast Cells

The exocyst-GFP plasmids were transformed into wild-type and various mutant yeast strains by integration into the chromosomal loci of the exocyst genes. To localize exocyst-GFP, cells from an early log phase culture grown in SC media lacking uracil were transferred to 37°C for 2 hours. Cells were then pelleted in ice-cold PBS at 4°C. Cells were fixed for 10 minutes in
methanol at -20°C, pelleted and washed with acetone at -20°C. Cells were subsequently rehydrated by washing 3 times with PBS at 4°C. Cells were then immediately examined by microscopy. For Sec3-GFP localization in cells recovering from G₀ arrest, the cells were first grown overnight in liquid culture. 0.2 ml of the culture was then spread onto YPD agar plates. After the yeast cells form a lawn (2-3 days at 25°C), the cells were scraped from the plates and transferred into YPD liquid medium. The cells were resuspended in 20 ml of 1M sorbitol / 50% YPD and then spun for 1 min at 500 g to pellet budded cells. The supernatant containing unbudded cells was collected. These cells were examined under the microscope. If necessary, the cells were further spun to ensure the cells were uniformly unbudded. Aliquots of the unbudded cells were transferred to YPD liquid media and allowed to grow for 3 hours at 37°C. The cells were then harvested and Sec3-GFP localization was examined as described above.

Immunofluorescence localization of Cdc42 in yeast cells was performed using affinity-purified rabbit anti-Cdc42 antibody as described previously. Fluorescence microscopy was performed with a Leica microscope fitted with a 100 x oil immersion objective and standard filter sets. The images were acquired with a Quantix HCCD camera and OpenLab Imaging Software.

*In vitro Binding between Sec3p and Cdc42*

Sec3p (aa. 1-501) was fused with maltose-binding protein (MBP-Sec3p). Cdc42 with its C-terminal four amino acids lipid modification sites deleted was fused to GST. The recombinant fusion proteins were purified from bacterial lysates and used in the in vitro binding assay. The GST fusion proteins were first incubated with 0.05 mM GTPγS or GDP for 15 min at 37°C in a buffer containing 20 mM Tris-HCl (pH7.4), 100 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol. Then, 10 mM MgCl₂ was added and the mixture was further incubated for 30 min at room
temperature. After nucleotide loading, the GST-Cdc42 fusion proteins (10 µg) or GST alone (60 µg) were incubated with the resin containing MBP-Sec3p (10 µg) or MBP (100 µg) for 2 hours at room temperature in the binding buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl₂, 1% Triton X-100, and 1 mg/ml bovine serum albumin (BSA). The GST-Cdc42 fusion proteins bound to the resin were detected by Western blot with anti-GST monoclonal antibody. The binding of various cdc42 mutant proteins to MBP-Sec3p was performed the same as that described above. The cDNAs encoding these mutant proteins was obtained by PCR from mutant yeast cells. All the constructs were confirmed by DNA sequencing.

Monitoring polarized growth by FITC-Con A

For monitoring polarized growth, yeast strains WT (YEF473A), cdc24-4 (YEF313), cdc42-201 (YEF1961), and cdc42-13 (YEF1962) cells were grown in YPD medium exponentially at 24°C, and cells were collected by centrifugation and resuspended in buffer P (10 mM sodium phosphate, 150 mM NaCl, pH 7.2) at ~5.0 X 10⁷ cells/ml. For each time point, 0.9 ml of the cell suspension was mixed with 0.1 ml of fluorescein isothiocyanate (FITC)-conjugated Concavanine A (Con A) (stock concentration: 1 mg/ml in buffer P) (Molecular Probes, Eugene, OR) and incubated at dark for 10 min. Cells were washed with buffer P three times and resuspended in prewarmed, fresh YPD medium to allow new growth (the dark zones on the cell surface correspond to new cell wall growth) at 37°C. Cells were collected at 0, 30, 90, and 215 min, respectively, and fixed with formaldehyde for 30 min at 37°C and additional 30 min at 24°C. Cells were washed with buffer P twice and resuspended in mounting medium for observation of the growth pattern.
Results

The exocyst proteins are mislocalized in a cdc24 mutant

To identify proteins that regulate the polarized localization of the exocyst, we examined the localization of GFP-tagged exocyst components in mutants that are defective in different aspects of the budding process such as bud-site selection, bud-site establishment, cell polarization, actin organization, cell cycle regulation, and septin assembly \(^{18}\). The GFP-tagged exocyst proteins were expressed under the control of their own promoters and were fully functional because yeast cells expressing the tagged version as the sole source of the exocyst protein grew as well as wild-type cells in both rich and synthetic media, and at temperatures ranging from 25 \(^{\circ}\)C to 37 \(^{\circ}\)C. We previously found that the exocyst proteins were mislocalized in several rho1 mutants \(^{18}\). Rho1 is a member of the yeast Rho family of small GTPases. It is involved in such cellular processes as actin organization, yeast cell wall remodeling, and maintenance of polarized cell growth \(^{17}\). Another member of the Rho family of small GTPases is Cdc42. Cdc42 is important for the initial establishment of polarity and functions very early in the signaling cascade that orchestrates polarized morphogenesis \(^{19}\). We therefore speculated that Cdc42 may also control exocyst localization. cdc42-1 is the first identified cdc42 mutant strain \(^{36}\), and it has been widely used in studying the function of Cdc42 and cell polarity. Surprisingly, a previous study showed that there is no mislocalization of Sec3-GFP in cdc42-1 cells \(^{8}\).

To further explore the role of Cdc42 on the exocyst, we decided to examine whether exocyst localization is dependent upon the activity of Cdc24, which is the guanine nucleotide exchange factor (GEF) for Cdc42. Wild-type and cdc24-4 mutant cells synchronized to G\(_0\) phase were incubated for 3 hours in YPD liquid medium at the restrictive temperature (37 \(^{\circ}\)C). The localization of Sec3-GFP in the cells was then examined. As shown in Figure 1, Sec3-GFP
randomly distributed in *cdc24-4* mutant cells but not in the wild-type cells, where it polarized to the bud tips. This result demonstrated that the initial targeting of Sec3 to correct sites in the cells is regulated by Cdc24, the GEF for Cdc42.

**The exocyst proteins are mislocalized in a selective set of *cdc42* mutants**

The effect of Cdc24 on the exocyst may be a result of its role in activating Cdc42. Although *cdc42-1* did not show exocyst mislocalization, this mutant strain may not be the appropriate allele for revealing the specific defect. Very recently, a number of new *cdc42* mutants have been generated which revealed some previously unidentified functions of Cdc42. We examined the localization of GFP-tagged exocyst proteins in some of the mutants. Several of the *cdc42* mutants used in this study were generated as described in the *Methods*; other mutants were recently characterized.

Figure 2 shows the distribution of two GFP-tagged exocyst proteins, Sec3p and Sec5p, after shift to 37°C for 2 hours. In addition, F-actin was also examined using rhodamine-phalloidin. In wild-type cells (Figure 2, upper panel), the exocyst proteins were localized to emerging bud sites, the tips of small daughter cells ("bud tip"), or mother/daughter connections ("necks"). However, in *cdc42-201* and *cdc42-13* mutant cells, Sec3-GFP and Sec5-GFP were either dispersed throughout the cell or formed multiple punctuate spots. In some mutant cells, the GFP-tagged exocyst was concentrated in one or several areas, but not in the bud tip or mother/daughter connection. In the parent wild-type strain, most of the cells scored from a single focal plane had polarized Sec3-GFP (66%) and Sec5-GFP (61%) localization (n=100) (Table 2). However, in *cdc42-201*, only 3% and 2% of the cells, respectively, had polarized localization of Sec3-GFP and Sec5-GFP. In *cdc42-13*, only 9% and 8% of the cells,
respectively, demonstrated polarized localization of Sec3-GFP and Sec5-GFP. All the mutant strains expressed similar levels of Sec3-GFP and Sec5-GFP as the wild-type strain at 37°C (data not shown). Sec3-GFP was partially localized in the other cdc42 mutant strains, cdc42-123 and cdc42-129. Table 2 summarizes the results we obtained using various mutant strains. F-actin was generally depolarized in both mutant alleles (Figure 2).

Since Cdc42 is involved in the establishment of cell polarity, by starting with a population of cells from G0 phase, one should be able to study the effect of Cdc42 on the initial targeting of Sec3p-GFP to the emerging bud tip. We therefore examined the localization of Sec3-GFP in cdc42-201 and cdc42-13 mutant cells after released from G0 arrest for 3 hours. As shown in Figure 3A, Sec3-GFP was depolarized in the cdc42-201 cells at the non-permissive temperature. For cdc42-13, most of the cells lost polarized Sec3-GFP localization. In fact, less than 6% of the cells still had polarized localization. As a control, Sec3-GFP was well localized in the wild-type cells released from G0 for 3 hours. These results suggest that initial targeting of Sec3p is affected in cdc42 mutant alleles. In contrast to these observations, it was found that Sec3p can be correctly targeted in actin mutant cells or in cells treated with Latrunculin-A starting from the G0 phase, indicating that the polarized localization of Sec3p can be established in an actin-independent fashion.

We have also compared the localization of Sec3-GFP and Cdc42 in the yeast cells. Due to technical difficulties with the yeast cells, we were not able to observe the Cdc42 immunofluorescence and Sec3p signals (by GFP signals or immunofluorescence using anti-GFP antibody) in the same cell by double labeling. However, we were able to perform the localization experiments on Cdc42 and Sec3-GFP separately and then compare their localization in the cells at similar cell cycle stages. As shown in Figure 3B, Cdc42 co-localizes with Sec3-
GFP at the bud emerging sites or at the tips of the small buds. This observation is consistent with the hypothesis that Sec3p needs Cdc42 for its initial targeting to the bud tip.

**Sec3p interacts with Cdc42 in vitro**

The effect of *cdc42* mutants on Sec3-GFP localization could reflect either a direct interaction of Sec3p with Cdc42 or an indirect effect mediated by one of the Cdc42 effectors. In order to understand the molecular basis for this effect, we tested for the direct binding of Sec3p with Cdc42 with a biochemical assay using MBP-Sec3p and GST-Cdc42 fusion proteins purified from bacteria lysates. Previous domain analysis indicates that the post-coiled-coil region of Sec3p (aa 500-1336) is responsible for binding to another component of the exocyst complex, Sec5p. The N-terminal portion of Sec3p binds Rho1; and Sec3p lacking its N-terminus diffuses inside cells. We tested whether the N-terminal region of Sec3p also binds to Cdc42. As shown in Figure 4A, GST-Cdc42 preferentially bound to the N-terminal region of Sec3p conjugated to the resin in the presence of GTPγS. The amounts of Cdc42 bound to MBP-Sec3p resin are in average about 20% of the input levels in six independent experiments (not shown). As a negative control, we found that GST alone did not bind MBP-Sec3p under the same reaction conditions. As another negative control, MBP itself, even at levels in great excess of MBP-Sec3p, did not bind GST-Cdc42. Since Sec3p is mislocalized in *cdc42-201* and, to a less extent, in *cdc42-13*, we have compared their binding to MBP-Sec3p with wild-type Cdc42. As shown in Figure 4B, comparing with GST-Cdc42 (wild-type), GST-cdc42-201 had a much weaker interaction with MBP-Sec3p in the presence of either GTP or GDP. This may explain why Sec3-GFP is mislocalized in the *cdc42-201* mutant allele. We have also detected a decrease in GST-cdc42-13 binding to MBP-Sec3p. However the decrease in binding was not as
dramatic as that seen with the GST-\textit{cdc42-201} mutant. Sec3-GFP was mislocalized in \textit{cdc42-13}, though the localization defect was less severe than that of \textit{cdc42-201} (see Table 2). Finally, we did not detect any change in \textit{cdc42-1} mutant binding (Fig. 4B). Overall, the binding properties seem to correlate with the exocyst localization in these \textit{cdc42} mutants. It is possible that the defects in binding account for the Sec3 mislocalization defects in some \textit{cdc42} mutants. However, we do not exclude the possibility that additional mechanisms are involved in mislocalization of Sec3-GFP in these mutant cells.

Since the Rho1 binding region is also localized to the N-terminus of Sec3p\textsuperscript{18}, we tested whether Cdc42 competes with Rho1 binding to MBP-Sec3p. The Rho1 and Cdc42 fusion proteins were first loaded with GTP\textsubscript{\gamma}S. Then various amounts of Cdc42 were added to the Rho1-Sec3p binding reaction as described above. As shown in Figure 4C, the Cdc42 recombinant protein competed with Rho1 in binding Sec3p.

**Depolarized pattern of surface protein incorporation in \textit{cdc42} mutant cells**

Since Cdc42 interacts with Sec3p and controls the localization of the exocyst, the essential secretory vesicle targeting and docking machine, we speculated that Cdc42 might spatially regulate exocytosis in cells. We therefore examined the exocytosis pattern in the wild type and \textit{cdc42} mutant cells by monitoring cell wall glycoproteins using the lectin Concanavalin A (Con A). The cell wall was pre-labeled with FITC-conjugated Con A and then thoroughly washed. As cells grow, newly synthesized cell wall glycoproteins are incorporated into the cell surface through exocytosis, displacing previous FITC-Con A labeled glycoproteins on the cell surface. The spatial distribution of exocytosis thus can be identified by monitoring the development of non-fluorescent regions ("dark zones"). As shown in Figure 5, at 0 min after
washing, 100% of the cells were brightly labeled. At 30 min, most of the wild-type cells were still brightly labeled, with very few cells having a dark growth zone at the bud tip (not shown). At 90 min, the majority of the small- and medium-budded cells had dark buds (the entire buds were dark) or dark zones surrounding the bud tips, indicating that exocytosis was localized in the daughter cells. In contrast, the displacement of fluorescence was randomized on the surface of cdc42 and cdc24 mutant cells. For cdc24-4 cells, almost 100% of the mother and daughter cells were uniformly labeled at 90 min. For cdc42-201, at 0 min after FITC-Con A labeling, 100% of the cells were brightly labeled. At 90 min, ~100% of the cells still had bright FITC-Con A staining in both mother and daughter cells. In some cases, the mother cells were even dimmer than the buds, suggesting that new growth had occurred preferentially in the mother cells. It was obvious that cells at this point were larger than those before the shift, indicating that the cells were growing, but in a depolarized fashion. No polarized growth was found in these mutant cells even by 215 min after washing. For cdc42-13, many small-budded cells or cells with tiny buds had polarized growth. However, some of these cells lost polarized growth. Overall, these results indicate that exocytosis occurs in a depolarized manner when Cdc42 is defective.

**cdc42-201 is synthetic-lethal with the exocyst mutants**

In a previous study, it was reported that cdc42-1 had synthetic interactions with members of the exocyst complex including sec3-2, sec5-24, sec8-9, sec10-2, and sec15-1. Here, we have examined the genetic interactions between cdc42-201 and a set of late sec mutants including: sec1-1, sec2-41, sec3-2, sec3-4, sec4-8, sec5-24, sec6-4, sec8-9, sec9-4, sec12-4, sec10-2, sec15-1. Among these mutants, sec3-4 and sec15-1 were synthetic-lethal with cdc42-201 at 25 °C. Sec3p and Sec15p are members of the exocyst complex. The synthetic-lethal
effects further demonstrate the functional connections between Cdc42 and these late SEC proteins involved in post-Golgi secretion.

**Discussion**

Cdc42 is an important regulator of many biological processes including cell polarization, morphogenesis, membrane traffic, cell growth and development in the eukaryotic cells ⁴, ¹⁵-¹⁷. The budding yeast *S. cerevisiae* undergoes polarized growth, which requires a sophisticated system to coordinate multiple cellular processes, including cell cycle progression, assembly of the actin cytoskeleton, morphogenesis, and polarized membrane traffic. Cdc42 is localized to bud tips, and plays important roles in all of these processes. Like many other members of the Ras super family of small GTP-binding proteins, Cdc42 has multiple downstream effectors ²⁰, ²¹. Through these different effectors, Cdc42 coordinates various cellular activities to carry out specific biological functions. Recently, many new *cdc42* conditional mutants have been generated ²²-²⁶. These mutants have been remarkably useful in revealing the functions of Cdc42 in various cellular processes. Since Cdc42 has multiple effectors, experiments using these mutant alleles have great advantage comparing with those using dominant-negative (*cdc42N17*) or constitutively active (*cdc42V12*) alleles in revealing the individual processes that Cdc42 controls. The phenotypes observed by expressing dominant-negative or constitutively active *cdc42* alleles might result from multiple downstream defects rather than a specific defect. It is also possible that the mutant phenotypes are caused by the loss of one effector system prior to the loss of another effector system. In this study, several newly identified *cdc42* mutants specifically revealed the role of Cdc42 in polarized localization of the exocyst.
A major function of Cdc42 is to control the polarized organization of the actin cytoskeleton, which is involved in polarized exocytosis. In actin mutant cells or in cells treated with Latrunculin-A, secretory vesicles are randomly distributed rather than properly localized to exocytic sites \(^{12, 27, 28, \text{and} 31}\). The Rab protein, Sec4p, resides on secretory vesicles, and its localization to the bud tip requires polarized actin \(^{31, 28}\). Some components of the secretory machinery, however, do not require actin for their localization to exocytic sites. Since Sec3p is able to establish and maintain its localization in both actin mutant cells and cells treated with Latrunculin-A\(^8\), there must be an actin-independent mechanism that controls Sec3p localization. Here we report that Sec3p is a direct downstream target of Cdc42. In this study, we found that polarized localization of Sec3p is regulated by Cdc42. GFP-tagged Sec3p is mislocalized in certain \(c\text{dc}\text{42}\) mutants at the restrictive temperature. Consistent with this result, Sec3-GFP was also found to be mislocalized in a \(c\text{dc}\text{24}\) mutant, \(c\text{dc24-4}\). Since Cdc24 is the guanine nucleotide exchange factor (GEF) for Cdc42, the observed mislocalization pattern is probably due to reduced Cdc42 activity. Among the \(c\text{dc}\text{42}\) mutant alleles we have tested, \(c\text{dc42-201}\) has the most dramatic effect on the localization of the exocyst proteins. Furthermore, exocytosis at the cell surface is depolarized as revealed by the FITC-Con A displacement experiment. Recently, Daniel Lew, Patrick Brennwald, and colleagues have also identified a \(c\text{dc}\text{42}\) allele that accumulates secretory vesicles at restrictive temperatures (personal communication), further supporting the role of Cdc42 in exocytosis.

Using a biochemical approach, we found that Sec3p directly interacts with Cdc42 in the presence of GTP\(\gamma\)S. The N-terminal region of Sec3p mediates this direct interaction with Cdc42. The interactions were defective with \(c\text{dc42-201}\) and \(c\text{dc42-13}\) mutants. We have previously shown that the N-terminus of Sec3p also binds Rho1, and an N-terminally truncated version of
Sec3p (Sec3ΔN) diffused in cells. Thus this region may be responsible for receiving the targeting information by interacting with the Rho family of small GTP-binding proteins. Our results demonstrated that Cdc42 competes with Rho1 in the binding assay, suggesting that these two small G-proteins bind to the similar or overlapping regions of Sec3p. Different Rho family proteins carry out related yet different functions. Many work coordinately in a cascade of related cellular processes. Both Cdc42 and Rho1 are involved in cell polarization and actin cytoskeletal organization. Their interactions with formin family proteins may be important in controlling actin organization (for review, see references 15-17). It is possible that the exocyst needs the signaling from both these proteins for the polarized localization. It is also possible that Cdc42 and Rho1 work at different time points during polarized growth. While Cdc42 is primarily involved in the early establishment of cell polarity, Rho1 may be mainly involved in the maintenance of polarized growth. For example, rho1-104 mutant cells can initialize budding but stop further growth at tiny or small budded stages, suggesting that polarized growth cannot be sustained in this mutant. In addition, GFP-exocyst proteins lost their polarity in many rho1-104 cells at the restrictive temperature even though the cells have polarized budding pattern. Further biochemical and cytological experiments are needed to explore the relationship between Rho1 and Cdc42 during polarized cell growth. It has also been shown that Rho3, another member of the yeast Rho family proteins interacts with the exocyst component, Exo70p; and accumulation of secretory vesicles was observed in a rho3 mutant allele. While mislocalization of the exocyst has been observed in rho1 and cdc42 mutant cells, there is no mislocalization of the exocyst proteins in rho3 mutant cells. We speculate that different Rho family proteins may act on different aspects of exocytosis.
The results presented here reveal that Cdc42 spatially controls the vesicle tethering/docking machine, the exocyst. It is also known that Cdc42 controls the polarized organization of the actin cytoskeleton, which provides the track for directional transport of secretory vesicles \(^{28-30}\). Taken together, we propose that Cdc42 regulates polarized exocytosis through its dual control over the vesicle transport system (actin) and the vesicle docking system (the exocyst). In addition to exocytosis, it is known that Cdc42 also controls cell polarity and cell cycle progression. Cdc42 may therefore serve as a coordinator of different cellular processes, all of which must be intimately coupled.

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Figure Legends:

**Figure 1.** Sec3p is mislocalized in *cdc24-4* cells recovering from G0 arrest at 37°C. The wild-type and *cdc24-4* cells arrested at G0 phase were generated as described in the *Method*. Aliquots of the cells were then transferred to YPD liquid medium and allowed to grow for 3 hours at 37°C. The cells were then harvested and Sec3-GFP localization was examined.

**Figure 2.** Sec3p and Sec5p are mislocalized in *cdc42* mutants. GFP-Sec3p, GFP-Sec5p, and actin were monitored in wild-type and *cdc42* mutants after the cells were shifted to the restrictive temperature (37°C) for 2 hours.

**Figure 3.**

**A.** Sec3-GFP is mislocalized in *cdc42* mutant cells recovering from G0 arrest at 37°C. Cells arrested at G0 phase were generated as described in the *Method*. The cells were then transferred to YPD liquid media and allowed to grow for 3 hours at 37°C. The cells were then harvested and Sec3-GFP localization in the cells was examined.

**B.** Cdc42 and Sec3-GFP are localized to the bud emerging sites or the tips of the small buds. Cdc42 was detected by immunofluorescence using anti-Cdc42 antibody. Sec3p was monitored by GFP fluorescence.

**Figure 4.**

**A.** GTP-dependent in vitro binding between Sec3p and Cdc42. The amino terminus of Sec3p (aa. 1-501) was fused with maltose-binding protein (MBP-Sec3p), purified and conjugated to the resin. Cdc42 was fused with GST and purified from bacteria lysates. These
proteins were used in the in vitro binding assay in the absence of nucleotides (-nt) or in the presence of 0.05 mM GTP\textsubscript{\gamma}S or GDP as described in Methods. The GST-Cdc42 fusion proteins bound to the MBP-Sec3p resins were detected by Western blot with anti-GST antibody (a). As a control, GST alone did not bind to the MBP-Sec3p resin (panel b). Also, GST-Cdc42 did not bind to MBP resin (panel c).

**B.** Defective binding of Cdc42 mutant proteins with MBP-Sec3p. Same amounts of wild type Cdc42, \textit{cdc42-201} and \textit{cdc42-13} GST fusion proteins were used in the binding assay.

**C.** Cdc42 competes with Rho1 in binding to Sec3p. Various amounts of GST-Cdc42 fusion protein were loaded with GTP\textsubscript{\gamma}S and applied to the Rho1-Sec3p binding reaction. The amounts of Rho1 bound to Sec3p resin were measured (n=6). In all the experiments, the amounts of Rho1 bound to Sec3p resin in the absence of Cdc42 competition were calculated as 100%. The bottom panel shows one representative result of the competition experiments.

**Figure 5.** \textit{cdc42} mutant cells lost polarized growth upon shift to 37 °C. Wild-type, \textit{cdc24-4}, \textit{cdc42-201}, and \textit{cdc42-13} cells were grown exponentially in YPD medium at 24 °C, and pulse-labeled with FITC-Con A for 10 min, and then released into fresh YPD medium at 37 °C to monitor the deposition of new cell wall materials at indicated times. The newly incorporated cell wall materials displaced FITC-Con A labeling. Exocytosis occurred at the regions that lost the fluorescence.
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### Table 1. Summary of cdc42 mutant strains used in this study

| Strain   | Mat   | Description                                                                                     |
|----------|-------|-----------------------------------------------------------------------------------------------|
| YEF473A  | Mat a | CDC42, ura3, leu2, his3, lys2, trp1 (from E. Bi)                                                   |
| YEF1961  | Mat a | cdc42-201, ura3, leu2, his3, lys2, trp1 (from E. Bi)                                               |
| YEF1962  | Mat a | cdc42-13, ura3, leu2, his3, lys2, trp1 (from E. Bi)                                               |
| YEF313   | Mat a | cdc24-4, ura3, leu2, his3, lys2, trp1 (from E. Bi)                                               |
| DDY1300  | Mat a | CDC42::LEU2, ura3-52, leu2-3, 112, his3Δ200, lys2-801am (from D. Drubin)                        |
| DDY1336  | Mat a | cdc42-123::LEU2, (R163A, K166A), ura3-52, leu2-3, 112, his3Δ200, lys2-801am (from D. Drubin) |
| DDY1344  | Mat a | cdc42-129::LEU2, (V36T), ura3-52, leu2-3, 112, his3Δ200, lys2-801am (from D. Drubin)           |
Table 2. Sec3-GFP localization in wild-type and cdc42 mutant strains:

| Strain      | Original strain | Polarized Sec3-GFP (%) | Cell Morphology      | Descriptions of Sec3-GFP Localization                              |
|-------------|-----------------|------------------------|----------------------|---------------------------------------------------------------------|
| YEF473A     | CDC42           | 66                     | normal               | Normal polarized localization.                                      |
| YEF1961     | cdc42-201       | 3                      | misshaped cells      | Random peripheral and cytoplasmic localization.                      |
| YEF1962     | cdc42-13        | 9                      | mostly un budded     | Mostly random localization.                                         |
| DDY1300     | CDC42           | 56                     | normal               | Normal polarized localization.                                      |
| DDY1336     | cdc42-123       | 22                     | un budded or small budded | Polarized in small budded cells but slightly diffuse in that region. |
| DDY1344     | cdc42-129       | 30                     | elongated or misshaped buds | Polarized in elongated bud tips.                                   |
Fig. 1

 CDC24  cdc24-4
Fig. 2

|            | Sec3-GFP | Sec5-GFP | Actin |
|------------|----------|----------|-------|
| CDC42      | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| cdc42-201  | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| cdc42-13   | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
Fig. 3

A. 

Sec3-GFP

CDC42

cdc42-201

cdc42-13

B. 

Sec3-GFP

Cdc42
Fig. 4C
Fig. 5

|          | WT          |          |
|----------|-------------|----------|
| 0 min    |             | 90 min   |
|          |             |          |

|          | cdc24-4     |          |
|----------|-------------|----------|
| 0 min    |             | 90 min   |
|          |             |          |

|          | cdc42-201   |          |
|----------|-------------|----------|
| 0 min    |             | 90 min   |
|          |             |          |

|          | cdc42-13    |          |
|----------|-------------|----------|
| 0 min    |             | 90 min   |
|          |             |          |
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