The Exo70 Subunit of the Exocyst Is an Effector for Both Cdc42 and Rho3 Function in Polarized Exocytosis

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The Rhö3 and Cdc42 members of the Rho GTPase family are important regulators of exocytosis in yeast. However, the precise mechanism by which they regulate this process is controversial. Here, we present evidence that the Exo70 component of the exocyst complex is a direct effector of both Rhö3 and Cdc42. We identify gain-of-function mutants in EXO70 that potently suppress mutants in RHÖ3 and CDC42 defective for exocytotic function. We show that Exo70 has the biochemical properties expected of a direct effector for both Rhö3 and Cdc42. Surprisingly, we find that C-terminal prenylation of these GTPases both promotes the interaction and influences the sites of binding within Exo70. Finally, we demonstrate that the phenotypes associated with novel loss-of-function mutants in EXO70, are entirely consistent with Exo70 as an effector for both Rhö3 and Cdc42 function in secretion. These data suggest that interaction with the Exo70 component of the exocyst is a key event in spatial regulation of exocytosis by Rho GTPases.

INTRODUCTION

The ability of eukaryotic cells to grow asymmetrically relies on the delivery of lipids and proteins to specific sites on the cell surface. This delivery is largely mediated by the docking and fusion of secretory vesicles with a precisely demarcated region of the plasma membrane. The budding yeast Saccharomyces cerevisiae undergoes polarized growth throughout its cell cycle, which makes it an attractive model for studying the regulation of polarized exocytosis and its role in overall cell polarity. An important factor in targeting of secretory vesicles to the plasma membrane is an evolutionary conserved eight-subunit protein complex, known as the exocyst complex, which is composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (for review, see Nelson and Yeaman, 2001; Hsu et al., 2004; Munson and Novick, 2006; Wu et al., 2008).

Both Rab and Rho family small GTPases have been shown to be the upstream regulators of exocyst function in vesicle docking and fusion. The Rab family small GTPase, Sec4, directly interacts with the Sec15 subunit of the exocyst, and it is this interaction that is thought to help link the exocyst complex to the vesicle during docking (Guo et al., 1999). The Rho family small GTPases are also important regulators of polarized exocytosis in yeast. Previous studies have shown that specific conditional mutations in Rhö3 or Cdc42 result in actin-independent defects in exocytosis (Adamo et al., 1999, 2001). Although Rhö3 and Cdc42 seem to have partially overlapping functions, they are also individually required for polarized exocytosis; loss of function in either GTPase shows a specific set of phenotypes when their secretory function is impaired. The secretory-defective mutant form of Cdc42, cdc42-6, accumulates post-Golgi vesicles only during bud emergence (i.e., small-budded cells) and displays a pronounced defect in secretion of Bgl2 but not in secretion of invertase. In contrast, the secretory defective form of Rhö3, rho3-V51, accumulates post-Golgi vesicles throughout the cell cycle and has pronounced defects in secretion of both Bgl2 and invertase pathways.

Exo70 has been implicated as a possible effector for Rhö3 based on its ability to interact with Rhö3 in a GTP-dependent manner (Robinson et al., 1999), and the fact that the interaction of Rhö3 with Exo70 is blocked by the presence of the rhö3-V51 effector domain mutation (Adamo et al., 1999). Interestingly, recent studies have identified exo70 mutant alleles, exo70-35 and exo70-38, that demonstrate a small-bud-specific and Bgl2-specific secretion defect, phenotypes that are remarkably similar to that of cdc42-6. These results could suggest a role for Exo70 as a target for Cdc42 during exocytosis, however, a physical interaction between activated recombinant Cdc42 and Exo70 was not detected (He et al., 2007a).

The recent determination of the crystal structure of Exo70 proteins from yeast and mice revealed a conserved rod structure with four domains arranged sequentially from the N to the C terminus (Dong et al., 2005; Hamburger et al., 2006; Moore et al., 2007). Structural similarity also seems to exist between the C-terminal domain of Exo70 and other members of the exocyst complex (Dong et al., 2005; Munson and Novick, 2006; Sivaram et al., 2006). However, despite the conservation in overall structure of yeast and mammalian Exo70, the site of interaction with Rhö family GTPases seemed to be quite distinct. Although recombinant Rhö3 was found to bind primarily through interactions within the C domain of yeast Exo70, the site of interaction between the mouse Cdc42 homologue TC10 and mouse Exo70 was mapped to the N-terminal AB domains (Chiang et al., 2001).

Recent work has called into question the role of Exo70 as an effector of Rhö3 function in exocytosis. Examination of two alleles in EXO70 suggested that Exo70 function was...
required only in small-budded cells (He et al., 2007a), in contrast to studies on Rho3 demonstrating exocytic defects in both small and large budded yeast (Adamo et al., 1999, 2001). More importantly, mutants or deletions in Exo70 predicted to block the interaction with Rho3 in vitro, showed little to no effect on growth or secretory function of EXO70 (He et al., 2007b; Hutagalung et al., 2009). Together, these results suggested that Exo70 was unlikely to be the effector for Rho3 function in exocytosis.

In the current study, we report a combination of genetic and biochemical examinations to determine the possible role of Exo70 as a target of Rho3 and Cdc42 function in exocytosis. First, we describe the isolation of dominant gain-of-function alleles of EXO70 that strongly suppress the loss of either Rho3 or Cdc42 function in the exocytic pathway. Second, we describe a biochemical assay using posttranslationally modified forms of Rho3 and Cdc42 to demonstrate that Exo70 has the biochemical properties of a direct effector for both of these GTPases. Interestingly, we found that C-terminally prenylated forms of Rho3 and Cdc42 are able to interact with Exo70 in a manner that is structurally distinct from that of unmodified recombinant Rho3. Finally, we describe the isolation of novel recessive alleles of EXO70 that demonstrate that the spectrum of phenotypes associated with Exo70 loss of function is in strong agreement with its role as an effector for both Cdc42 and Rho3 function in polarized exocytosis.

MATERIALS AND METHODS

Yeasts, Reagents, and Genetic Techniques

Cells were grown in YPD media containing 1% bacto-yeast extract, 2% bacto-peptone, and 2% glucose. The components of the media were from Thermo Fisher Scientific (Waltham, MA). For all assays performed, 25°C was the permissive temperature, whereas 14°C and 37°C were used as the restrictive temperatures. Sorbitol, sodium azide (Na3), N-ethylmaleimide (NEM), β-mercaptoethanol, d-ribose, glucose oxidase, peroxidase, Triton X-100, NP-40, and His-Select Nickel Affinity Gel were obtained from Sigma-Aldrich (St. Louis, MO). Zymolyase (100T) was from Seikagaku (Tokyo, Japan). Biovine serum albumin, yeast nitrogen base, raffinose galactose, and 5-fluoroorotic acid (5-FOA) were from United States Biologicals (Swampscott, MA). Glutathione-Sepharose beads and protein A beads were from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). Guanosine 5′-O-(3-hydroxymethyl) ribonucleotide and YPD plates at 32°C for 2 d. One percent of the cultures were shifted to restrictive temperature for 3 d. The exo70-113 and sec6-4 mutant cells were then shifted to prechilled YP media containing 0.1% glucose for 9 h. The cells were then pelleted, resuspended in cold water, and cold-sensitive alleles as the only copy of EXO70, the constructs were integrated into the chromosome of the diploid strain BY861 (exl: exo70::LEU2/RHO3; his3::HIS3/EXO70; his3::HIS3/EXO70; his3::HIS3/EXO70). Transformants were selected by replica plating onto synthetic minimal media containing 5-FOA while simultaneously selecting for the temperature sensitivity by incubation of replica plates at 37, 35, 17, and 14°C. From 30 colonies, 4 colonies exhibited cold sensitivity. Plasmids from these colonies were isolated and transformed back into the original EXO70 plasmid shuttle strain (BY61) to confirm that the plasmids were responsible for the temperature sensitivity and cold sensitivity. One plasmid was found to give rise to a cold-sensitive phenotype (exo70-188) and one plasmid was found to have a temperature sensitive phenotype (exo70-113). Sequence analysis demonstrated that the cold-sensitive mutant, exo70-188, contained the three mutations S305G, T523P, and D575N. Site-directed mutagenesis of the cold-sensitive mutation in exo70-113 contained two mutations: S79K and incorporation of a stop codon at Y327 leading to truncation of the C-terminal half of the predicted protein.

Genetic Analysis of Mutants

To study the phenotypes of exo70-188 and exo70-113 alleles as the only copy of Exo70, the constructs were integrated into the chromosome of the diploid strain BY861 (exl: exo70::LEU2/RHO3; his3::HIS3/EXO70; his3::HIS3/EXO70; his3::HIS3/EXO70). Transformants were selected by replica plating onto synthetic minimal media containing 5-FOA, viability, and conditional growth defects.

Electron Microscopy

The exo70-113 and exo70-113 mutants were grown overnight to mid-log phase in YPD media. Half of the cultures were shifted to restrictive temperature for various time periods. The exo70-113 mutant was shifted to 37°C water bath shaker for 9 h, where the exo70-113 mutant was shifted to 37°C water bath shaker for 1.5 h. Shifted and unshifted cells were processed as described previously (Adamo et al., 1999).

Invertase Assay and Bgl2 Assay

The exo70-113, exo70-113, rho3-51 and sec6-4 cells were grown overnight to mid-log phase at 25°C in YPD liquid media. For the cold-sensitive strains exo70-188 and rho3-51, the cells were preshifted to the restrictive temperature of 14°C for 1 h. After the preshift, one sample was taken as T0 and the rest of the cells were shifted to prechilled YP media containing 0.1% glucose for 9 h. Samples after the temperature shift were collected as T1t, T2t, T3t, and T4t, respectively. For temperature-sensitive alleles exo70-113 and sec6-4, the cells were directly shifted to warm YP media containing 0.1% glucose for 1.5 h at 37°C. Cells sampled before and after the temperature shift were collected as T0eg and T4eg, respectively. After the temperature shift, the cells were spheroplasted and the internal and external fractions were separated. Samples were processed as described previously (Adamo et al., 1999; Lehman et al., 1999). The percentage of internal invertase accumulation is calculated by: [Δinternal/Δ internal + Δ external].

To analyze the accumulation of Bgl2 enzyme, cells were grown in YPD media overnight to mid-log phase. The exo70-113 mutant cells were then shifted to a 14°C water bath shaker for 10 h. The exo70-113 mutant cells were shifted to 37°C water bath shaker for 2 h. The cells were then pelleted, resuspended in cold water, and cold-sensitive alleles as the only copy of EXO70, the constructs were integrated into the chromosome of the diploid strain BY861 (exl: exo70::LEU2/RHO3; his3::HIS3/EXO70; his3::HIS3/EXO70). Transformants were selected by replica plating onto synthetic minimal media containing 5-FOA, viability, and conditional growth defects.

Construction of pGEX-6His Vector and Protein Expression, Purification, and Quantification

The pGEX6p1 vector was digested with Ncol and SalI. An additional PreScission enzyme site and a 6-histidine tag were generated by fusion PCR reaction and introduced into the Ncol and SalI sites of pGEX6p1 to create pGEX-6pHis (pB1579). The resulting plasmid contained an N-terminal glutathione transferase (GST) tag and a C-terminal 6xHis tag flanking the polylinker and was used to

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create the following GST-fusion constructs: GST-Seq9 (aa 402-451), GST-Ste20 (aa 314-432), GST-Exo70 (aa 1-263), GST-Exo70-1521, GST-Exo70-DC, GST-Seq9 (aa 1-263), and GST-Seq9 (aa 1-367) were cloned into pGEX-3p-1 vector. All constructs were confirmed by sequencing and protein expression was performed in Escherichia coli BL21 cells. Cells were grown at 37°C in Terrific broth medium to an OD600 2.0–2.5. Cells were shifted to 25°C, and protein expression was induced with 0.1 mM isopropyl β-D-thiogalactoside for 3 h at 25°C. To maximize protein solubility, GST-Exo70-ABD was induced at 22°C for 4 h. Cells were pelleted and frozen at ~80°C until lysis.

6xHis-tag purification was performed by binding the bacterial lysate to HIS-Select Nickel Affinity Gel (Sigma-Aldrich) and then eluting with 500 mM imidazole. The 6xHistidine eluates were then incubated with glutathione-HIS-Select Nickel Affinity Gel (Sigma-Aldrich) and then eluting with 500 mM glutathione. The purified protein was analyzed by SDS-PAGE gel and Western blotting.

**RESULTS**

**Isolation of Dominant Gain-of-Function Alleles of EXO70**

We have suggested previously a model for localized stimulation of exocytosis by the guanosine triphosphate (GTP)-bound forms of Rho3 and Cdc42. For Rho3, we proposed this activation would involve interaction with the Exo70 subunit of the exocyst complex, whereas the effector for Cdc42 was unknown (Roumanie et al., 2005; Wu et al., 2008). Using other Rho-effectors such as formins, Wiskott–Aldrich Syndrome protein, or p21-activated kinase kinases, as a model, one might expect that downstream activation would involve “relief of autoinhibition,” i.e., that binding of Rho3-GTP to the Exo70 subunit of the exocyst would activate this complex by both inhibiting interactions between Exo70 and other components of the complex, thus reorganizing the complex into a more active form (Wu et al., 2008). A prediction of this model is that mutations in Exo70 that block or perturb inhibitory interactions of Exo70 within the exocyst should act as “activated” or gain-of-function alleles and would behave genetically as dominant-suppressors of loss of Rho3 function. Furthermore, if Rho3 and Cdc42 share a common pathway in regulating exocyst function then mutants that suppress loss of Rho3 function in exocytosis should also suppress loss of Cdc42 function in exocytosis.

To test these predictions, we performed two independent screens to look for dominant suppressing forms of EXO70. One screen made use of the rho3Δ mutant, which is sensitive to increased gene dosage of several components of the exocytic apparatus (Matsui and Toh, 1992; Imai et al., 1996; Lehman et al., 1999). The other screen made use of the cdc42Δ mutant that has a highly allele-specific defect in exocytosis that genetically overlaps with that of rho3Δ (Adamo et al., 2001). EXO70 was randomly mutagenized by PCR amplification and introduced into the mutant yeast strains by cotransformation with a gap-repair plasmid containing the flanking sequences of the EXO70 gene (see Materials and Methods). Approximately 6000 transformants were assayed from each strain and ~45 plasmids containing dominant suppressing forms of EXO70 were isolated from both screens. Our initial work focused on the 41 strongest suppressors. When we examined the strongest 16 EXO70 dominant suppressors isolated from the cdc42Δ-6 screen in the rho3Δ strain, we found that they also strongly suppressed (Supplemental Figure S1). When we examined the ability of the 3 strongest EXO70 dominant suppressors isolated from the rho3Δ screen to suppress cdc42Δ-6 we found that all but...
Figure 1. Dominant mutations of EXO70 are able to suppress rho3Δ and cdc42-6 mutants. (A) The structure of S. cerevisiae Exo70 (residues 62-623) is in a transparent surface representation with mutant residues in a space-filing model. The domains are labeled A–D from the N terminus to the C terminus. The arrows point to the residues involved in Exo70 gain of function mutations. Residues colored in blue represent highly conserved residues, residues colored in yellow represent residues conserved only within fungi, and residues colored in pink represent variable residues. Three residues, I114, G388, and N479, are buried within the structure, whereas the remaining seven mutations are surface-exposed residues. The image was generated using the PDB file 2B1E. (B) The gain-of-function mutations of EXO70 are capable of suppressing a rho3Δ and the cdc42-6 mutant at the restrictive temperature. Dominant mutants of EXO70 were transformed into a rho3Δ/H9004 and cdc42-6/H9004 strain and an cdc42-6 strain on CEN plasmids. The growth of three independent transformants is shown for each EXO70 mutant in both mutant strains under restrictive conditions. (C) The EXO70 dominant mutations can function as the sole copy of Exo70 in the cell. The dominant mutations were introduced into the EXO70 plasmid shuffle strain on a CEN-LEU2 plasmid. Three individual colonies were picked to show the growth of each EXO70 mutant under restrictive conditions when the plasmid containing wild-type EXO70-URA3 was lost by counterselection on 5-FOA plates. (D) EXO70 dominant mutants express similar amounts of Exo70 compared with wild-type cells. Whole cell lysate were made from the strains containing Exo70 gain of function mutants as the only source of EXO70 in the cell. The lysates were subjected to SDS-PAGE analysis and blotted with anti-Myo2 and anti-Exo70 antibodies.

one of these also strongly suppressed cdc42-6 (Supplemental Figure S1A). We also examined all 16 mutants in rho3Δ, rho4Δ and found they all strongly suppressed (Supplemental Figure S1B). Therefore, each of these two genetic screens appeared to be selecting for mutants in EXO70 with similar properties. The ability of these suppressors to potently suppress growth defects associated with both rho3 and cdc42 loss-of-function strongly supports the idea that Exo70 is a functionally important downstream target of regulation by both of these Rho GTPases.

Sequence analysis of 41 of the strongest suppressors identified an average of five to eight coding sequence changes per dominant plasmid shuffle allele. This was not unexpected, as the predicted frequency of mutations for the amplifying conditions used was 1- to 2-base pair changes/kb. To identify specific amino acid substitutions linked to the suppression activity, we scanned the mutant sequences for missense mutations that appeared in two or more of the suppressing plasmids. This identified 10 single residue mutations as candidate residues to confer dominant suppression on Exo70. These mutations were then prepared as single residue changes and tested for dominant suppression in rho3Δ or cdc42-6 (Figure 1).

The placement of the dominant gain-of-function mutations in the crystal structure of yeast Exo70 revealed a number of possible sites of regulation. Three residues, I114, G388, and N479, are buried within the structure. The other seven mutations are all surface exposed residues. Charged residues at the D domain form two patches—D541 and E547 form one patch and E557, K564, and R623 form another patch. N479 and G388 are located at the interface of the B and C domains, which potentially cause rearrangements of the two domains relative to each other within the structure of Exo70. An alignment of Exo70 protein sequences from yeast, mouse, worm, and fly is shown in Supplemental Figure S2. The site of each dominant suppressing mutation is indicated on the alignment along with the overall conservation of each residue. As can be seen in Supplemental Figure S2, six of the dominant suppressing mutations occur in highly conserved residues (I114, N479, D541, E547, E557, K564)—four of which reside in a single helix (H17) within the Exo70 crystal structure—suggesting this may be a “hot spot” for activation of the exocyst by Exo70. Together, however, the results of this screen overall suggest that there are likely to be multiple, structurally distinct mechanisms by which EXO70 gain of function can be achieved.

One possible mechanism for activation of the exocyst complex by the gain-of-function mutants is by effecting the assembly or disassembly of individual subunits of the complex. To determine whether this was the case, we examined the assembly state of the exocyst complex in the presence of EXO70 gain-of-function alleles as the sole source of Exo70 in the cell. We immunoprecipitated wild-type and mutant exocyst complexes using strains containing a C-terminally myc-tagged form of SEC8 (Sec8-myc) and anti-myc antibody-coated beads.
(TerBush and Novick, 1995), and we assayed for the presence of the other exocyst subunits by quantitative Western blot analysis. As can be seen in Figure 2, all six of the gain-of-function alleles of \textit{EXO70} examined resulted in nearly identical amounts of exocyst subunits being coprecipitated with Sec8-myc. An exception was the \textit{EXO70-E557K} allele which had a small (\approx 25\%) reduction in the mutant Exo70 protein coprecipitated with the complex. However, this seems to mirror a similar small reduction in the overall steady-state amounts of this protein in the \textit{EXO70-E557K} cells rather than an effect on assembly or disassembly of the complex. Because the activation of the exocyst complex with these gain-of-function mutants seems to mimic the effect of Rho3/Cdc42 activation, these data suggest that assembly and/or disassembly of the complex is unlikely to be the central means of regulation by Rho3 and Cdc42 GTPases.

\textbf{The GTP-bound Forms of Rho3 and Cdc42, Produced in Yeast, Interact with Exo70}

The dominant suppressor analysis described above suggests that Exo70 is probably a critical downstream target of both Rho3 and Cdc42 regulation of exocytosis. However, work using recombinant forms of these GTPases produced in \textit{E. coli} is inconsistent with this notion. First, recombinant Cdc42-GTP fails to show any detectable interaction with Exo70 (Figure 3A; He \textit{et al.}, 2007a). Recombinant Rho3 does interact with Exo70 in a GTP-dependent and effector domain-dependent manner (Figure 3, A and B) but with relatively low affinity (Hamburger \textit{et al.}, 2006). Second, mutations or deletions in Exo70 that severely diminish this interaction in vitro fail to recapitulate growth or secretory defects associated with either the \textit{rho3-V51} or \textit{cdc42-6} cells (He \textit{et al.}, 2007b; Hutagalung \textit{et al.}, 2009). Because nearly all small GTPases are subjected to posttranslational modifications that do not occur after expression in \textit{E. coli}, we sought to determine whether we could detect an interaction after their overexpression in yeast. To determine the nucleotide dependence of this interaction, we made use of mutant forms of Rho3 and Cdc42 which are predicted to “lock” the GTPases in the either the GTP-bound conformation (Rho3-L74 or Cdc42-L61) or GDP-bound conformation (Rho3-N30 or Cdc42-N17). The nucleotide-locked forms of each GTPase were overexpressed (\approx 20-fold) using an inducible GAL1/10 promoter, and the induced cells were used to make detergent lysates (see Materials and Methods). These lysates were then used in binding experiments with purified full-length Exo70 fused to GST or various control GST-fusion proteins. A GST-fusion containing

\begin{figure}[h]
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\caption{Dominant mutations in Exo70 do not affect the assembly of the exocyst complex. (A) Analysis of coimmunoprecipitation of exocyst subunits in \textit{EXO70} dominant mutant strains. A single copy of C-terminal myc-tagged Sec8 (\textit{SEC8-MYC}) was introduced by integrative transformation into strains containing dominant mutants of \textit{EXO70} (on CEN plasmid) as the only source of Exo70 in the cell. Cells were grown, lysed, and subjected to native immunoprecipitations with myc antibody to analyze the integrity of the exocyst complex (see Materials and Methods for details). We loaded 12\% of total lysate and 10\% of myc immunoprecipitate on SDS-PAGE gel for Western blot analysis with antibodies against each exocyst components. (B) Quantitation of Western blots described in A were performed by Odyssey infrared imaging system. Top, quantitation of each individual subunit in the Sec8 Myc immunoprecipitation organized by strain. Bottom, comparison of wild type and each gain of function strain organized by individual subunit. Error bars represent SD of four independent experiments.}
\end{figure}
the C-terminal CRIB domain of the Ste20 protein was included as a positive control for Cdc42. As can be seen in Figure 3C, both the GTP-locked forms of Rho3 and Cdc42, Rho3-L74 and Cdc42-L61, are specifically pulled down by GST-Exo70 and this binding is nearly completely lost in lysates overexpressing the GDP-mutant forms of Rho3 and Cdc42, Rho3-N30, and Cdc42-N17. In contrast to Rho3 and Cdc42, no binding to Exo70 was detected with lysates overexpressing GTP-locked Rho1, indicating that the interaction between Exo70 and Rho3/Cdc42 obtained from yeast is sensitive to mutations in the effector domain of the two GTPases. The rho3-V51, L74 and cdc42-6 effector domain mutations (cdc42-L29, V31, H32, and L61) were expressed behind a galactose inducible promoter in yeast. Lysates generated from the overexpressing yeast strains were subjected to binding experiments as described above. (D) The interaction between Exo70 and Rho3/Cdc42 obtained from yeast is sensitive to mutations in the effector domain of the two GTPases. The rho3-V51, L74 and cdc42-6 effector domain mutations (cdc42-L29, V31, H32, and L61) were expressed behind a galactose inducible promoter in yeast. Lysates generated from the overexpressing yeast strains were subjected to binding experiments as described above. (E) Bar graphs represent quantitation of the blots using the Odyssey infrared imaging system. Data were analyzed by two-tail Student’s t test with error bars representing SD from four independent experiments.
with recombinant Rho3 reflects only a residual level of interaction seen with the modified form of the protein.

To further examine the apparent requirement for CAAX prenylation in the interaction between these Rho GTPases and Exo70, we made use of rabbit reticulocyte lysates that have been shown to be active in prenylating CAAX motifs in vitro (Hancock, 1995). We examined whether we could detect prenylation of either yeast GTPase after in vitro translation. When Rho3-L74 (GTP) or Rho3-N30 (GDP) were translated in reticulocyte lysates and further incubated for 6 h, a faster migrating form of the protein appeared over time. When activated Rho3 containing a mutation of the critical cysteine (C228) in the CAAX motif was used, the faster migrating form failed to appear (Figure 4B). The addition of mevalonic acid—a metabolic precursor of both farnesyl and geranylgeranyl moieties used to modify CAAX motifs—has been shown to stimulate both types of prenylation in reticulocyte lysates. As can be seen from Figure 4B, the addition of mevalonic acid to the reaction after translation results in a dramatic increase in the conversion of Rho3-L74 and Rho3-N30 proteins to the faster migrating form but has no effect on the Rho3-L74,A228 mutant lacking the prenylation acceptor cysteine.

We examined the ability of in vitro prenylated Rho3 from reticulocyte lysates to bind to GST-Exo70 under conditions similar to that used for the yeast lysate binding studies described above. The results show that in vitro prenylated Rho3-L74 (GTP-locked) binds well to the GST-Exo70, whereas prenylated Rho3-N30 (GDP form) fails to bind (Figure 4C). Importantly, only very weak binding is seen when unprenylated but activated Rho3-L74,A228 is present. Unlike Rho3, we were unable to detect a cysteine-dependent mobility shift in a parallel set of translations with Cdc42 translated in the reticulocyte system. However, when we examined the binding of translations of activated Cdc42 (Cdc42L61) in the presence or absence of mevalonate and the presence or absence of the C-terminal cysteine we find that binding to Exo70 is dependent on GTP, the addition of mevalonate, and the CAAX box. This provides strong evidence that, like Rho3, prenylation of Cdc42 CAAX motif is required for interaction with Exo70 in this system. Together with the binding assays using GTPases from yeast lysates, these data clearly demonstrate that prenylation of Rho3 and Cdc42 is an important determinant in promoting the binding between these Rho GTPase and Exo70.

**Figure 4.** Prenylation of Rho3 and Cdc42 promotes the GTP-dependent interaction with Exo70. (A) The interaction between Exo70 and the two Rho GTPases, Rho3 and Cdc42, from yeast lysate is dependent on C-terminal prenylation. Activated forms of Rho3 and Cdc42 (Rho3L74, Cdc42L61) containing a cysteine-to-alanine mutation in the CAAX box (Rho3-L74, A228, and Cdc42-L61, A188) were overexpressed in yeast. Lysates were prepared in parallel for these mutants and for the activated GTPases containing the intact CAAX motif (Rho3-L74 and Cdc42-L61) and used in binding experiments (see Materials and Methods). Ste20 binding assays with Cdc42 (but not Rho3) were diluted 20-fold before loading. As expected, Ste20 binding was unaffected by the prenylation site mutation. Bar graphs represent quantitation of binding experiment with error bars representing SD of three independent experiments. Coomassie Blue staining of the GST-fusion proteins used in the experiment is shown below the quantitation. (B) In vitro prenylation of Rho3 results in a change in mobility and is stimulated by addition of mevalonic acid to reticulocyte lysate. Rho3 was in vitro translated in rabbit reticulocyte lysates (see Materials and Methods) and then subjected to extended incubation (for the time shown) in the presence or absence of mevalonic acid (±MVA). The appearance of a faster migrating species appears during the extended incubation and is strongly stimulated by the addition of mevalonic acid. (C) The interaction between Exo70 and Rho3 and Cdc42 produced in reticulocyte lysates is dependent on C-terminal prenylation. Immobilized GST-fusion proteins were incubated with prenylated Rho3 or Cdc42 obtained from the in vitro translation described in Materials and Methods. Input lanes for Rho3 binding experiments represent signal equivalent to 2.5% binding. Input lane for Cdc42 binding experiments represent signal equivalent to 10% binding. The Cdc42-N17 binding experiment was exposed to film twice as long as the other experiments due to lower efficiency of translation for this mutant.
eight residue flexible linker (Hutagalung et al., 2009). We compared the binding of these two forms of Exo70 fused to GST to equivalent amounts of wild-type full-length Exo70 by using the Rho GTPases produced in yeast lysates. Remarkably, we found that both GST-Exo70-1521 and GST-Exo70-ΔC mutant proteins showed binding activity to yeast Rho3-L74 and Cdc42-L61 that was indistinguishable from that of wild-type Exo70 (Figure 5B). As before, this binding was highly dependent on the activation status of the Rho3 and Cdc42. We repeated these binding assays using the in vitro translated/in vitro prenylated Rho3 and Cdc42 obtained from the in vitro translation as described in Materials and Methods. Input lanes for Rho3 binding experiments represent signal equivalent to 2.5% binding. Input lane for Cdc42 binding experiments represent signal equivalent to 10% binding. The Cdc42-N17 binding experiment was exposed to film twice as long as the other experiments due to lower efficiency of translation for this mutant. Shown below is Coomassie Blue staining of the GST fusion proteins used for binding and bar graphs representing quantitation of the binding with error bars representing SD of three independent experiments.
lated Rho3 protein. Using the in vitro modified Rho3, we see a small loss of binding activity to the GST-Exo70-1521 and GST-Exo70-ΔC mutant proteins, but both mutants demonstrate very clear binding which is highly dependent on both the activation (i.e., GTP) and prenylation status of Rho3. Therefore, both of these assays reveal that Exo70 is able to interact with Rho GTPases in a manner that does not depend on the C domain and that the prenylation state of these GTPases seems to be critical in revealing this mode of interaction. It is worth noting that mammalian TC10 GTPase (produced in NIH 3T3 cells and hence probably prenylated) also has been shown to interact with mammalian Exo70 in a manner that does not depend on the C domain (Chiang et al., 2001).

**Isolation of Novel Conditional-Lethal Alleles of EXO70**

A previous screen for mutants in EXO70 resulted in the identification of two alleles, exo70-38 and exo70-35, with unusual phenotypes nearly identical to those described for cdc42-6 (He et al., 2007a). Like cdc42-6 mutants, exo70-38 and exo70-35, exhibit defects in secretion of Bgl2 but not invertase and show accumulation of post-Golgi vesicles in small-but not large-budded cells. Invertase and Bgl2 are thought to be delivered to the cell surface through two distinct populations of post-Golgi vesicles, and both populations are blocked by all of the original 10 late sec mutants (Harsay and Bretscher, 1995). The remarkable similarity of the unusual phenotypes of the exo70-38 and exo70-35 mutants with the cdc42-6 mutant gives strong in vivo support for the notion that Exo70 is a direct effector of Cdc42 function in exocytosis, as suggested by the dominant suppressor and biochemical interaction studies described above. However, the phenotypes associated with the exo70-38 and exo70-35 mutants are not consistent with the model that Exo70 is a downstream effector of the Rho3 GTPase. The rho3-V51 mutant has significantly more expansive secretory defects than seen in these two mutants. Specifically, the rho3-V51 mutant shows pronounced defects in both invertase and Bgl2 secretion and accumulates post-Golgi vesicles in both small and large budded cells. Because these phenotypes are at odds with the genetic and biochemical studies described above linking Exo70 as a downstream effector of both GTPases, we postulated that the exo70-38 and exo70-35 mutants might represent hypomorphic alleles of EXO70, defective in transmitting Cdc42 function but still permissive for promoting Rho3 function. To test this idea, we set out to identify and characterize novel conditional-lethal alleles in EXO70 and describe the loss-of-function phenotypes associated with them.

To identify additional alleles of EXO70 with recessive conditional growth defects, we screened a library of random PCR-generated exo70 mutants by using a plasmid shuffle strategy (see Materials and Methods). After growth on 5-FOA media at 25°C (to evict the EXO70 plasmid and reveal possible recessive phenotypes), 57 independent colonies were identified that exhibited low-temperature–sensitive growth at 14°C (es−), and 40 colonies were identified that exhibited high-temperature–sensitive (ts−) growth. After recovery of the mutant plasmids from these strains and retransformation into the exo70Δ/CEN-URA3-EXO70 plasmid shuffle strain, we identified one plasmid containing a cold-sensitive allele (exo70-188), and one plasmid containing a temperature-sensitive allele (exo70-113). These alleles (as well as wild-type EXO70 control) were then integrated at the EXO70 locus by replacing a disruption in a heterozygous diploid. After sporulation and tetrad dissection, the pattern of spores showing conditional growth was seen to be tightly linked to the newly introduced allele. Thus the phenotypes of the integrated alleles were similar to that of the original plasmid-borne mutants. These integrated mutant strains were then used for all the subsequent analyses of the growth and secretion phenotypes associated with these new alleles of exo70.

We examined the exo70-188ts− allele for its ability to secrete invertase and Bgl2 at permissive temperature (25°C) and after a 9-h shift to the nonpermissive conditions (14°C). A wild-type EXO70 and cold-sensitive rho3-V51 strain were assayed in parallel as negative and positive controls for cold-sensitive secretory defects. As can be seen in Figure 6, B and C, the exo70-188 mutant shows pronounced invertase and Bgl2 secretion defects at 14°C (relative to EXO70), and both the magnitude and temperature dependence of these secretory defects are remarkably similar to that seen in rho3-V51 cells (Roumanie et al., 2005). When exo70-188 cells were shifted to 14°C and then examined by thin-section electron microscopy, a highly penetrant (>80% of cells) and dramatic (>75 vesicles/cell on average) accumulation of 80- to 100-nm post-Golgi vesicles was apparent (Figure 6, D and E). As we had seen previously with rho3-V51, and consistent with the secretion data, a lower—and less penetrant—level of vesicle accumulation was observed in exo70-188 mutants grown at the permissive temperatures (Figure 6, D and E).

To determine whether there was an effect of the stage of the cell cycle on vesicle accumulation in this mutant, we compared the vesicle accumulation in cells with small (<1-μm-diameter) buds compared with larger (>1-μm-diameter) buds, as we have previously with cdc42-6 and exo70-38. The results, shown in Figure 6D clearly demonstrate that both large- and small budded exo70-188 cells have a similar high degree of penetration and vesicle accumulation at both permissive and nonpermissive temperatures.

The temperature-sensitive exo70-113 mutant was characterized in a similar manner except that cells were shifted to 37°C for 2 h to examine the effect of temperature-shift on exocytic function. An isogenic wild-type EXO70 and sec6-4 mutant were examined in parallel as controls. As can be seen in Figure 7, B and C, the exo70-113ts− mutant shows pronounced invertase and Bgl2 secretion defects after a shift to 37°C and when examined by thin section electron microscopy, a highly penetrant (>80% of cells) and strong (>80 vesicles/cell, on average) accumulation of 80- to 100-nm post-Golgi vesicles was apparent in both large- and small-budded cells (Figure 7, D and E). Together, our analysis of these two new alleles demonstrates that Exo70 function is required for all the secretory functions seen with other late-acting sec genes including secretion of both the Bgl2 and the invertase class of vesicles, and exocytosis in early bud emergence as well as in later stages of bud growth. These observations are important to the present study because they demonstrate that the spectrum of phenotypes associated with loss-of Exo70 function is entirely consistent with Exo70 being a downstream effector of both Cdc42 and Rho3 function in exocytosis.

**DISCUSSION**

We have previously characterized a role for Cdc42 and Rho3 as positive regulators of exocytosis and found that this role was independent of polarization of both the actin cytoskeleton and of the exocyst complex (Roumanie et al., 2005). We suggested a model where these GTPases would work by locally increasing the activity (or throughput) of the exocytic apparatus at sites occupied by GTP-bound Rho/Cdc42 proteins (Roumanie et al., 2005; Wu et al., 2008). However, the precise effector pathway by which such a mechanism would take place was either unknown—as for Cdc42—or highly controversial—as for Rho3. In this report, we provide three independent lines of evidence that strongly supports this model of regulation and demon-

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Figure 6. Characterization of a novel cold-sensitive mutant exo70-188. (A) Growth properties of the cold sensitive mutant, exo70-188 at permissive and nonpermissive temperatures. Wild-type EXO70 and exo70-188 mutant alleles were integrated at the EXO70 locus. Ten-fold serial dilutions of yeast cells were grown on YPD plates at the indicated temperatures. (B) The exo70-188 mutant is defective in secretion of the periplasmic enzyme invertase. Invertase assays were conducted on cells shifted to low glucose media at the permissive temperature (25°C) for 2 h or the restrictive temperature (14°C) for 9 h. The graph shows the percentage of internal invertase at 25 and 14°C in each strain analyzed. The error bars represent SD of five independent experiments. (C) The exo70-188 is defective in Bgl2 secretion. Bgl2 assays were performed on cells kept at permissive temperature or shifted to restrictive temperature for 9 h. The percentage of distribution of Bgl2 was determined by immunoblot analysis using affinity-purified Bgl2 antibody. The graph depicts the percentage of Bgl2 protein that remains internal in EXO70, exo70-188, and rho3-V51 strains. Quantitations were performed by Odyssey infrared imaging system. Error bars represent SD of four independent experiments. (D) The exo70-188 mutant accumulates post-Golgi vesicles in both large-budded cells and small-budded cells. The exo70-188 mutant cells kept at the permissive temperature or shifted to restrictive temperature for 9 h were fixed and processed for electron microscopy. Examples of both small- (<1-μm) and large-budded (≥1-μm) cells are shown. Cells were scored for number of vesicles accumulated throughout the cell and bar graphs show the percentage of small-budded or large-budded cells containing different number of vesicles. Bar, 2 μm. (E) Overall post-Golgi vesicle accumulation was determined for electron microscopy sections by scoring cells >3 μm in diameter (irrespective of bud or bud size) for 80- to 100-nm vesicles. For each condition, micrographs were divided into three groups representing 20–25 cells each to determine the SD in this assay.

strates that the Exo70 component of the exocyst complex represents a critical and direct effector for both Rho3 and Cdc42 function in spatial regulation of exocytosis.

Several studies have been carried out to investigate the interaction of Exo70 and Rho family GTPases in yeast and mammalian cells. However, despite the fact that both yeast and mammalian Exo70 have been reported to bind Rho family GTPases and the overall structural similarity between yeast and mammalian Exo70 proteins is very high throughout their length, the sites of interaction reported for the
GTPases seemed to be quite distinct. Studies using tagged-TC10 in transfected mammalian cells identified a region within domains A and B in mouse Exo70 as the major site of binding, whereas studies using recombinant Rho3 have identified a binding site within C domain as the major binding site for the yeast Exo70 protein (Dong et al., 2005; He et al., 2007b; Hutagalung et al., 2009). To make matters more difficult, mutations or deletions in Exo70 that block recombinant Rho3 binding had little or no effect on growth or secretion of yeast cells containing these mutant exo70 alleles as the sole source of Exo70 in the cell (He et al., 2007b; Hutagalung et al., 2009). Finally, phenotypic analysis of two

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**Figure 7.** Characterization of a novel temperature-sensitive mutant exo70-113. (A) Growth defect of a novel temperature sensitive mutant exo70-113. Wild-type EXO70 and exo70-113 alleles were integrated at the EXO70 locus. Ten-fold serial dilutions of yeast cells were grown on YPD plates at the indicated temperatures. (B) The exo70-113 mutant is defective in secretion of the periplasmic enzyme invertase. Invertase assays were performed on cells after a 2-h shift to low glucose media at permissive (25°C) or a 1.5-h shift to restrictive temperature (37°C). The graph shows the percentage of internal invertase in the strains analyzed. The error bars represent SD of four independent experiments. (C) exo70-113 is defective in Bgl2 secretion. Bgl2 assays were performed on cells kept at permissive temperature or shifted to the restrictive temperature of 37°C for 2 h. The percentage of distribution of Bgl2 was determined by immunoblot analysis using affinity-purified Bgl2 antibody. The graph depicts the percentage of Bgl2 protein that remains internal in EXO70, exo70-113, and sec6-4 strains. Quantitations were performed by Odyssey infrared imaging system. Error bars represent SD of four independent experiments. (D) exo70-113 mutant accumulates post-Golgi vesicles in both large-budded cells and small-budded cells. exo70-113 mutant cells kept at the permissive temperature or shifted to restrictive temperature for 2 h were fixed and processed for electron microscopy. Examples of both small- (<1 μm) and large-budded (>1 μm) cells are shown. Cells were scored for number of vesicles accumulated throughout the cell, and bar graphs show the percentage of small-budded or large-budded cells containing different number of vesicles. Bar, 2 μm. (E) Overall post-Golgi vesicle accumulation was determined for electron microscopy sections by scoring cells >3 μm in diameter (irrespective of bud or bud size) for 80- to 100-nm vesicles.

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et al., 2007b; Hutagalung et al., 2009). To make matters more difficult, mutations or deletions in Exo70 that block recombinant Rho3 binding had little or no effect on growth or secretion of yeast cells containing these mutant exo70 alleles as the sole source of Exo70 in the cell (He et al., 2007b; Hutagalung et al., 2009). Finally, phenotypic analysis of two
conditional alleles of exo70 showed exocytic phenotypes that were remarkably similar to that of cdc42-6 but very distinct from that of rho3-V51 (He et al., 2007a). However, despite the similarity in phenotypes with the Cdc42 mutant, biochemical analyses failed to show any detectable interaction between Exo70 and recombinant GTP-bound forms of Cdc42 (He et al., 2007a, this study). Collectively these results seemed to be entirely incompatible with the notion that Exo70 was a direct effector of Cdc42 or Rho3 in yeast. Moreover, the ability of Exo70 family members to interact with Rho GTPases is highly conserved in yeast and mammals, suggesting that structurally divergent mechanisms had evolved into interactions with completely distinct regions of the yeast and mammalian Exo70 proteins.

We have spent considerable effort to find conditions for examining the interaction of Exo70 and either Rho3 or Cdc42 by coimmunoprecipitation from detergent lysates. Despite numerous attempts we have been unable to detect this interaction by this method. This may reflect the relatively low affinity of these interactions because, in vivo, these interactions occur on the plasma membrane where the local concentrations of both GTPases and the Exo70 effector are much higher than after lysis in detergent-containing buffer.

A key finding to unraveling these apparently contradictory observations was the use of post-translationally modified forms of Rho3 and Cdc42 in our Exo70 binding studies, a strategy similar to that used to map the TC10 interaction with mammalian Exo70. In particular, we found that mutation of the cysteine present in the C-terminal CAAX motif of either Cdc42 or Rho3 resulted in a dramatic loss of interaction with Exo70. Because this modification does not occur in bacterially-produced forms of these GTPases, the absence of this modification is probably responsible for the dramatic differences seen in the binding assays described here from those described previously. Furthermore, because the mapping of the binding of the Cdc42 homologue TC10 to mouse Exo70 made use of a mammalian expression system (hence, prenylated TC10), this may explain the apparent distinction in the site of binding between the yeast and mammalian forms of Exo70. Further mapping of the Rho GTP binding site in both yeast and mammalian Exo70 will be necessary to determine how structurally similar these binding events are.

Because Rho3 and Cdc42 are predicted to be prenylated with different prenyl moieties—Rho3 with farnesyld and Cdc42 with geranylgeranyl—we think it unlikely that the prenyl groups themselves are part of the effector interaction. Rather, we suggest that the presence of the prenyl group is likely to affect presentation of the GTP-bound Rho protein to Exo70 in the context of the detergent micelles present in the binding assays. This may reflect the nature of the interaction that probably occurs in proximity to the membrane in vivo, because both Exo70 and the GTPase have C-terminal membrane-targeting motifs. A role for prenylation in effector binding is unusual but not unprecedented. Ohya and colleagues (Inoue et al., 1999) demonstrated that the physical interaction of Rho1 with its effector Fks1 was highly dependent on the C-terminal geranylgeranylation. It remains to be seen whether other Rho effector relationships depend on prenylation because this is not a feature that is commonly examined.

What does “activation” of the exocyst by Rho GTPases do to regulate exocytosis? In its simplest form, this activation would involve an increase in the rate of vesicle docking and fusion at a specific place on the plasma membrane demarcated by the GTP-bound form of Cdc42 and/or Rho3. Because the loss of function mutants in cdc42 and rho3 fail to show a detectable effect on polarization of Sec4 or exocyst subunits to sites of polarized growth, and the mutants are strongly suppressed by increased levels of the target membrane-soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) Sec9, we favor the model that these GTPases act through the exocyst to increase localized SNARE assembly leading to more rapid vesicle fusion. The details of how this activation mechanism works remain to be elucidated. However, because dominant alleles of Exo70 described here act as though they are “constitutively active” forms of Exo70 they will be valuable tools in helping to understand the nature of this activation. For example, all of the gain-of-function mutants seem to have similarly fully assembled exocyst complex, suggesting that assembly and/or disassembly of the complex is unlikely to represent the major mode of regulation by these GTPases.

In addition to Exo70, Sec3—another component of the exocyst complex—has been proposed to be an effector in regulating polarized growth downstream of the Rho1 and Cdc42 GTPases (Guo et al., 2001; Zhang et al., 2008; Hutaglung et al., 2009). However, it is unlikely that Sec3 is the principal effector for Cdc42 function in exocytosis. First, deletion of the N-terminal Rho-binding domain of Sec3 is nonessential and has no detectable impact on polarized growth or secretion at any temperature, whereas the cdc42-6 mutant results in a severe temperature-sensitive growth and secretion defect. Second, we have shown previously that deletion of the Sec3 N-terminal domain results in a synthetic lethal phenotype when combined with the cdc42-6 mutant, which strongly suggests that this domain of Sec3 has an important role that acts in parallel (perhaps through Rho1 signaling) with Cdc42 signaling through Exo70 (Roumanie et al., 2005). In agreement with the idea that the Cdc42–Exo70 pathway functions in parallel with a Sec3–Rho pathway, several groups have shown synthetic lethality between sec3-DNT and several EXO70 loss-of-function mutants (Zhang et al., 2008; Hutaglung et al., 2009). It also important to note that although the Exo70 effector activity seems to be regulated by both Rho3 and Cdc42 but not Rho1, the N terminus of Sec3 has been reported to interact with both Rho1 and Cdc42 but not Rho3. Therefore, this process seems to be regulated by a complex network of partially overlapping Rho GTPase/effector relationships which may provide increased robustness to the overall spatial regulation of polarized exocytosis.

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