An Internal Domain of Exo70p Is Required for Actin-independent Localization and Mediates Assembly of Specific Exocyst Components

Alex H. Hutagalung,* Jeff Coleman,[†] Marc Pypaert,^{†‡} and Peter J. Novick*

*Department of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA 92093; and [†]Department of Cell Biology, Yale University, New Haven, CT 06510

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The exocyst consists of eight rod-shaped subunits that align in a side-by-side manner to tether secretory vesicles to the plasma membrane in preparation for fusion. Two subunits, Sec3p and Exo70p, localize to exocytic sites by an actin-independent pathway, whereas the other six ride on vesicles along actin cables. Here, we demonstrate that three of the four domains of Exo70p are essential for growth. The remaining domain, domain C, is not essential but when deleted, it leads to synthetic lethality with many secretory mutations, defects in exocyst assembly of exocyst components Sec5p and Sec6p, and loss of actin-independent localization. This is analogous to a deletion of the amino-terminal domain of Sec3p, which prevents an interaction with Cdc42p or Rho1p and blocks its actin-independent localization. The two mutations are synthetically lethal, even in the presence of high copy number suppressors that can bypass complete deletions of either single gene. Although domain C binds Rho3p, loss of the Exo70p-Rho3p interaction does not account for the synthetic lethal interactions or the exocyst assembly defects. The results suggest that either Exo70p or Sec3p must associate with the plasma membrane for the exocyst to function as a vesicle tether.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* grows by budding and therefore serves as an excellent model system for studying cell polarity during cell division and the contribution of polarized membrane traffic to this process. Once the site of bud emergence is determined, secretory vesicles are directed to it to form the plasma membrane and cell wall of the daughter cell. Late in the cell cycle, this polarity is reversed, directing vesicular traffic to the neck separating the mother and daughter cell, thereby facilitating cytokinesis and cell separation (Pruyne *et al.*, 2004).

Secretory vesicles derived from the Golgi or endosome are transported by the type V myosin Myo2p along polarized actin cables to sites of cell surface growth (Pruyne *et al.*, 2004) where they fuse with the plasma membrane via the action of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins and their regulatory factors (Novick *et al.*, 2006). This stage of vesicular traffic also requires a large protein complex called the exocyst. The exact function(s) served by the exocyst is unknown, but the loss of function of any exocyst component blocks the secretory pathway after polarized vesicle delivery, but before SNARE complex assembly, leading to an accumulation of secretory vesicles preferentially concentrated in the daughter cell (Novick *et al.*, 1980; Walch-Solimena *et al.*, 1997; Guo *et al.*, 1999a; Grote *et al.*, 2000). The exocyst is composed of eight

subunits, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p, and although the significance of this subunit structure is unclear, it has been evolutionarily conserved (TerBush *et al.*, 1996; Kee *et al.*, 1997; Guo *et al.*, 1999a).

Insight into the mechanism by which the exocyst performs its tethering function has come from the determination of the structure of several exocyst subunits. The structure of almost the entire *S. cerevisiae* Exo70 protein (amino acids 62–623) is that of an extended rod, composed of α -helical bundles, arranged into four domains (Dong *et al.*, 2005). This structure is remarkably similar to that of *Mus musculus* Exo70 despite the low level of amino acid sequence conservation (Moore *et al.*, 2007). The structures of domains corresponding to the C termini of Sec6p, Exo84p, and *Drosophila* Sec15 show a high degree of architectural similarity to Exo70p in as much as they are each formed of two helical bundles, although they show little sequence similarity with one another (Dong *et al.*, 2005; Sivaram *et al.*, 2005; Wu *et al.*, 2005). Even the amino terminal regions of these three subunits are predicted to be largely helical in structure as are the four other exocyst subunits. Because this structural feature is shared among at least several subunits, and possibly all, and has been evolutionarily conserved, it is likely to perform an essential function. Recent results imply that Exo70p interacts with Sec8p and Sec10p along the length of the protein in an elongated, side-to-side manner (Dong *et al.*, 2005). Quick freeze/deep etch images of the purified mammalian exocyst suggest that the other exocyst components may also adopt extended, rod-shaped structures and further suggest that the side-to-side assembly of these rod-like subunits may be a general feature of the exocyst (Hsu *et al.*, 1999; Munson and Novick, 2006). Yet, how this structural feature mediates the tethering function of the exocyst is unclear.

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‡ Deceased.

Address correspondence to: Peter J. Novick (pnovick@ucsd.edu).

All subunits of the exocyst are concentrated at sites of cell surface expansion, most prominently, the tip of the bud, early in the cell cycle and the neck, near the time of cytokinesis (Finger *et al.*, 1998; Boyd *et al.*, 2004). Nonetheless, several lines of evidence indicate that there are at least two different means by which these subunits can achieve this pattern of localization. Most subunits are delivered to exocytic sites on secretory vesicles; therefore, their localization is strongly dependent upon the actin cables that serve as tracks for vesicle delivery. In contrast, Sec3-green fluorescent protein (GFP) and a portion of Exo70-GFP still localize to these sites in the presence of the actin-depolymerizing drug latrunculin A (LatA) (Ayscough *et al.*, 1997; Finger *et al.*, 1998; Boyd *et al.*, 2004). They can even associate with new sites formed in the presence of latrunculin A, implying that they can be delivered to exocytic sites in an actin-independent manner, and fluorescence recovery after photobleaching analysis directly confirms this conclusion (Finger *et al.*, 1998; Boyd *et al.*, 2004). The localization of Sec3p relies on its interaction with either the Rho1p or Cdc42p GTPases, working in conjunction with the phosphoinositide phosphatidylinositol-4,

5-bisphosphate (PI4,5P₂) (Guo *et al.*, 2001; Zhang *et al.*, 2008). Superficially, Exo70p seems to localize by a similar mechanism. At the extreme carboxy-terminal tip of the Exo70p structure lies a cluster of highly conserved positively charged residues that were proposed to function as a lipid binding site (Dong *et al.*, 2005) and recent studies confirm their role in binding PI4,5P₂ (He *et al.*, 2007; Liu *et al.*, 2007). In addition Exo70p binds Rho3p in its GTP-bound form (Robinson *et al.*, 1999). The Rho3 binding site is associated with the third helical bundle, domain C (amino acids 346-515) (Dong *et al.*, 2005).

In this study, we report that the N- and C-terminal domains of Exo70p (domains A and D, respectively) are essential for viability, deletion of domain B confers a dominant lethal phenotype, whereas the deletion of domain C, containing the Rho3 binding site, confers surprisingly mild phenotypic effects. Nonetheless, this deletion leads to genetic interactions with multiple genes on the secretory pathway, defects in assembly of specific exocyst components and loss of the actin-independent localization pathway. In contrast, disruption of the interaction between Rho3p and

Table 1. Strains used in this study

Strain	Genotype	Reference
NY2704	<i>MATa/α exo70ΔdD::HIS3/EXO70 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2705	<i>MATa/α exo70ΔdD-GFP::HIS3/EXO70 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2706	<i>MATa exo70ΔdC::LEU2 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2707	<i>MATa exo70ΔdC-TAP::LEU2 SEC5-3xHA::HIS3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2708	<i>MATa EXO70-TAP::LEU2 SEC5-3xHA::HIS3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2709	<i>MATa exo70ΔdC-GFP::LEU2 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2710	<i>MATa SEC8-TAP::LEU2 SEC5-3xHA::HIS3 exo70ΔdC::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2711	<i>MATa SEC10-TAP::LEU2 SEC5-3xHA::HIS3 exo70ΔdC::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2712	<i>MATa SEC8-TAP::LEU2 SEC6-13xmyc::HIS3 exo70ΔdC::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2713	<i>MATa EXO84-TAP::LEU2 SEC6-13xmyc::HIS3 exo70ΔdC::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2714	<i>MATa SEC15::LEU2 SEC6-13x myc::HIS3 exo70ΔdC::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2715	<i>MATa SEC10-TAP::LEU2 SEC5-3xHA::HIS3 exo70(K354A, K355A)::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2716	<i>MATa SEC10-TAP::LEU2 SEC5-3xHA::HIS3 exo70(K370A)::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2717	<i>MATa SEC10-TAP::LEU2 SEC5-3xHA::HIS3 exo70(D374A)::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2718	<i>MATa rho3-1::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2719	<i>MATa SEC8-TAP::LEU2 SEC5-3xHA::HIS3 rho3-1::URA3 leu2-3,112 ura3-52 his3-Δ200</i>	This study
NY2720	<i>MATa P_{MET17}::GFP-sec3ΔN::HIS3 leu2-3,112 ura3-52 his3-Δ200</i>	P. Novick collection
NY2721	<i>MATa/α P_{MET17}::GFP-exo70ΔdA::HIS3/EXO70 leu2-3,112 ura3-52 his3-Δ200</i>	P. Novick collection
NY2722	<i>MATa/α P_{GALI}::GFP-exo70ΔdA::HIS3/EXO70 leu2-3,112 ura3-52 his3-Δ200</i>	P. Novick collection
NY2723	<i>MATa P_{TEF}::3xHA- sec3ΔN::kanMX6 exo70ΔdC::LEU2 SEC5-GFP::URA3 [P_{MET17}::EXO70::HIS3, CEN]</i>	P. Novick collection
NY768	<i>MATa sec1-1 ura3-52 leu2-3,112</i>	P. Novick collection
NY770	<i>MATa sec2-41 ura3-52 leu2-3,112</i>	P. Novick collection
NY772	<i>MATa sec3-2 ura3-52 leu2-3,112</i>	P. Novick collection
NY774	<i>MATa sec4-8 ura3-52 leu2-3,112</i>	P. Novick collection
NY776	<i>MATa sec5-24 ura3-52 leu2-3,112</i>	P. Novick collection
NY778	<i>MATa sec6-4 ura3-52 leu2-3,112</i>	P. Novick collection
NY780	<i>MATa sec8-9 ura3-52 leu2-3,112</i>	P. Novick collection
NY782	<i>MATa sec9-4 ura3-52 leu2-3,112</i>	P. Novick collection
NY784	<i>MATa sec10-2 ura3-52 leu2-3,112</i>	P. Novick collection
NY786	<i>MATa sec15-1 ura3-52 leu2-3,112</i>	Wiederkehr <i>et al.</i> (2003)
NY2448	<i>MATa sec3Δ::kanMX leu2-3,112 ura3-52 his3-Δ200</i>	P. Novick collection

Exo70p is not deleterious to the cell and affects neither the localization nor assembly of the exocyst.

MATERIALS AND METHODS

Strain Construction

Strains used in this study are listed in Table 1. Deletions of the C-terminal domain of Exo70p (amino acid residues 542–623) was performed by genomic insertion of a polymerase chain reaction (PCR) product amplified from pFA6-GFP(S65T)-HIS3MX6 that, after recombination, replaced residues 542–623 of Exo70p with GFP (Longtine *et al.*, 1998). N-Terminally GFP-tagged 1) Sec3p deleted of the first 320 amino acids and 2) Exo70p deleted of the first 152 amino acids were made by amplification of an insertion cassette from the plasmid pFA6-[MET17 promoter]-GFP(S65T)-HIS3MX6. To make this vector, the MET17 promoter (Kerjan *et al.*, 1986) was amplified from genomic DNA and used to replace the GAL1 promoter in pFA6-[GAL1 promoter]-GFP(S65T)-HIS3MX6 by using the sites PacI and BglII. The same procedure was performed to make GFP-Sec3ΔNp driven by the TEF promoter, which was amplified from p413TEF (Mumberg *et al.*, 1995). To make a construct with EXO70 under the MET17 promoter, the MET17 promoter was inserted in front of full-length EXO70 in pRS413 to make a CEN HIS3 vector. Deletion of internal domains of Exo70p were performed by cloning of two PCR fragments flanking the region to be deleted (amino acids 346–515 that correspond to domain C and amino acids 153–302 that correspond to domain B). The two fragments were ligated together with a flexible linker containing a SalI site (GTG GGT ACC GGT TCG GGT) and inserted into pRS305 or pRS306 containing the ADHI terminator cloned between the SacI and SacII sites for integration. To create a tandem affinity purification (TAP)-tagged or GFP-tagged construct, the TAP tag was amplified by PCR from pBS1479 (Puig *et al.*, 2001), and GFP was amplified from pFA6-[GAL1 promoter]-GFP(S65T)-HIS3MX6. The resulting fragments inserted into the above-described integration vectors to create GFP- or TAP-tagged C-terminal fusion proteins.

Electron Microscopy

Cells were grown in YPD at 30°C, and 10 OD₆₀₀ units of cells were washed with 10 ml of 0.1 M sodium cacodylate, pH 6.8 (buffer A). The cells were fixed in 10 ml of 3% glutaraldehyde in buffer A for 1 h at room temperature and then overnight at 4°C. The cells were then treated with zymolyase (0.25 mg/ml zymolyase in 50 mM potassium phosphate, pH 7.5; Seigaku, Tokyo, Japan) at 37°C for 25 min while shaking. The cells were then washed twice in buffer A and treated with 2% osmium tetroxide in buffer A and incubated for 1 h in the hood. The cells were then washed three times with water and stained with 2% uranyl acetate for 1 h at room temperature in the dark. After washing twice with water, the cells were dehydrated in increasing amounts of ethanol followed by 100% acetone and embedded in Spurr resin (Electron Microscopy Sciences, Fort Washington, PA) for serial sections. The samples were poststained with 2% uranyl acetate and lead citrate and examined on a Philips Tecnai 12 electron microscope.

Rho3p Binding Assays

Binding of Exo70p or mutant constructs of the protein to Rho3p were performed as described previously (Dong *et al.*, 2005). Briefly, glutathione transferase (GST)-Rho3p, immobilized on glutathione beads, was loaded with either guanosine diphosphate (GDP) or guanosine 5'-O-(3-thio)triphosphate (GTPγS) and then combined with either Exo70p or various point mutants. The negative control contained GST instead of GST-Rho3p. The mixtures were incubated at room temperature for 1 h, washed, and then analyzed by Western blot.

TAP Tag Binding Assays

Units (100 OD₆₀₀) of the respective yeast strains were lysed in 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.8, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM antipain, 20 μM aprotinin, 20 μM chymostatin, 20 μM leupeptin, 20 μM pepstatin A, and 10 mM β-mercaptoethanol by using a bead beater. NP-40 was added to the lysate to a final concentration of 1% and incubated at 4°C for 20 min. The lysate was then spun down at 20,000 × g for 20 min. Immunoglobulin G (IgG)-Sepharose beads were added to the resulting supernatant and incubated at 4°C for 3 h. The beads were washed in the above-mentioned lysis buffer, and the samples were analyzed by Western blot or treated with tobacco etch virus (TEV) protease and further purified using calmodulin beads (Puig *et al.*, 2001). Quantitation of Western blots was performed using ImageJ (Abramoff *et al.*, 2004).

Fluorescence Microscopy

Epifluorescence microscopy and video microscopy of strains harboring EXO70, *exo70ΔdC*, *exo70ΔdA*, or *exo70ΔdD* fused to GFP tag were conducted on cells grown overnight in SC medium at 25°C, and then strains were diluted to OD₆₀₀ = 0.2 in fresh SC medium and grown for 4 h at 25°C. For *exo70ΔdA*, overnight cultures were grown in SC containing 2% raffinose and then diluted into SC media containing 2% galactose to induce expression of the construct. To examine *sec3ΔN;exo70ΔdC;SEC5-GFP* rescued by EXO70 under the MET17

promoter, cells were initially grown in 1 mM methionine to suppress expression of EXO70 and then centrifuged and resuspended in methionine-free media for 4 h. Samples were collected by centrifugation, resuspended in fresh SC medium, and 7 μl of the suspension was placed on a slide with coverslip. The cells were allowed to settle on the bottom surface of the slide for 5–10 min before being examined with an Axioplan2 upright fluorescence microscope (Carl Zeiss, Thornwood, NY) by using a 63× or 100× Plan Neofluor apochromatic oil immersion objective with numerical aperture 1.3. Images were captured with an ORCA ER cooled charge-coupled device camera (Hamamatsu, Bridgewater, NJ) and analyzed with OpenLab software from Improvision (Lexington, MA). In some experiments, latrunculin A (10 mM in DMSO; Invitrogen, Carlsbad, CA) was added to a concentration of 100 μM and visualized 5 min after treatment to determine localization in the absence of actin cables.

RESULTS

The N and C Termini of Exo70p Are Essential

The extended rod-shaped structure of Exo70p is composed of four domains (Figure 1A) that interact with other exocyst proteins in an elongated side-to-side manner (Dong *et al.*,

Figure 1. The N and C termini of Exo70p are essential. (A) A space-filled model of the structure of amino acid residues 62–623 of *S. cerevisiae* Exo70p. The domains are labeled A through D from the N terminus to the C terminus. Residues marked in blue or red indicate a positive or negative charge, respectively. The arrows point to the cluster of positively charged residues in the C terminus of Exo70p. The image was rendered using Cn3D 4.1 (NCBI) and the PDB file 2B1E (Dong *et al.*, 2005). (B) Tetrad dissections of diploid yeast heterozygous for a deletion of the C terminus or domain D (amino acids 537–623, ΔdD), domain C (amino acids 346–515, ΔdC), and the N-terminal domain A (amino acids 1–153, ΔdA). The two viable:two inviable arrangement of spores demonstrate that the N and C termini of Exo70p are essential, whereas domain C is not essential. (C) Fluorescence microscopy of Exo70-GFP (wt) and *exo70ΔdD*-GFP (ΔdD) in diploid cells. Left, bright field images. Right, fluorescence images. (D) Fluorescence microscopy of GFP-*exo70ΔdA* in diploid cells before (UN) and after 4 h of galactose induction (IN). (E) Western blot analysis of internal and external pools of Bgl2p in wild-type versus mutant cells. To control for protein loading, Western blots against alcohol dehydrogenase (ADH) were performed.

Figure 2. Electron microscopy of *wt* (A) and *exo70ΔdC* (B) cells. Bars, 1 μ m.

2005). To understand how the domains of Exo70p contribute to exocyst assembly and function, we systematically deleted each domain. We first deleted the C-terminal domain D of Exo70p (residues 537–623) generating *exo70ΔdD*. Tetrads derived from a diploid strain heterozygous for this domain D deletion were dissected, and the resulting haploid spores showed a two viable:two nonviable dissection pattern (Figure 1B). These results demonstrated that domain D of Exo70p is essential and that *exo70ΔdD* is a recessive loss of function mutation. A GFP-tagged construct of this truncated protein was also created and inserted into a diploid strain, and it localized to sites of secretion at the bud tip and bud neck (Figure 1C). The C terminus contains a patch of positive residues that are evolutionarily conserved across various species and seems to play a role in binding to phospholipids at the plasma membrane (Dong *et al.*, 2005; He *et al.*, 2007; Liu *et al.*, 2007). Therefore, the C terminus of Exo70p is required for its function, but if absent, the truncated Exo70 protein can still localize to exocytic sites.

We used a similar approach to generate *exo70ΔdA*, a deletion of amino acids 1–153 of Exo70p. Tetrads from a diploid strain heterozygous for *exo70ΔdA* under the control of the *MET17* promoter produced a two viable:two nonviable pattern after dissection (Figure 1B). Like domain D, domain A performs an essential function and *exo70ΔdA* is a recessive loss of function mutation. A GFP fusion of this construct did not show any signal under the microscope and was likely due to low levels of expression. To alleviate this problem, the construct was overexpressed as an N-terminal GFP fusion protein under the *GAL1* promoter and showed a diffuse localization pattern in diploid cells (Figure 1D). Diploid cells containing this construct were also sporulated and the resulting tetrads showed a two viable:two nonviable pattern after dissection (data not shown). Therefore, even when expressed under the stronger inducible *GAL1* promoter, *exo70ΔdA* cannot support cell growth.

Deletion of Domain B Produces a Dominant-Negative Protein

To test the function of domain B of Exo70p (amino acids 153–302), an internal deletion was constructed that replaced domain B with an eight amino-acid flexible linker generating *exo70ΔdB*. Attempts to isolate wild-type (*wt*) diploid transformants that had integrated the domain B deletion at the *EXO70* locus were unsuccessful even though this would still leave one wild-type copy of the *EXO70* gene. One explanation

for the lack of transformants is that *exo70ΔdB* acts as a dominant-negative construct. To test this proposal, we attempted to introduce the construct behind the regulated *GAL1* promoter at the *LEU2* locus. However, this yielded very few transformants and these grew very poorly even on 2% glucose, which represses expression from the *GAL1* promoter. The poor growth phenotype of these cells was exacerbated when grown on galactose, which induces expression from the *GAL1* promoter (data not shown). Therefore, these results suggest that *exo70ΔdB* produces a highly toxic protein.

Deletion of Domain C in Exo70p Produces a Localization Defect

The results described above show the requirement for the N- and C-terminal domains of Exo70p and that removal of the internal domain B yields a dominant-negative construct. To test the function of the intervening domain C, we next replaced the region between residues 346 and 515 with an eight amino-acid flexible linker to generate *exo70ΔdC*. This construct was introduced into a wild-type diploid yeast strain, making it heterozygous at the *EXO70* locus. On dissection of tetrads derived from this strain, four viable spores were obtained from each dissected tetrad (Figure 1B), and there were no obvious growth defects associated with the *exo70ΔdC* mutant when tested at various temperatures and growth conditions. Electron microscopy of *exo70ΔdC* cells compared with *wt* cells showed no measurable accumulation of vesicles (Figure 2), although there was a small reduction in secreted invertase ($97 \pm 1\%$ secretion for wild-type cells vs. $89 \pm 2\%$ secretion for *exo70ΔdC* cells). Previously published results of a specific effect of *exo70* mutants on Bgl2p secretion prompted testing of Bgl2p secretion in the *exo70ΔdC* mutant. There was no detectable defect in Bgl2p secretion in *exo70ΔdC* cells compared with wild-type cells unlike that seen in *sec6-4* cells at the restrictive temperature of 37°C (Figure 1E).

These were surprising results considering the size of the deletion and the functional importance of all the other domains. Exo70p is an effector of the small GTPase Rho3p that plays an important role in cell polarity and secretion and domain C of Exo70p is required for this interaction in addition to mediating an interaction with the exocyst component Sec6p (Imai *et al.*, 1996; Robinson *et al.*, 1999; Dong *et al.*, 2005).

A C-terminally GFP-tagged version of *exo70ΔdC* was constructed and examined for defects in localization. A previous report had shown that the interaction of Exo70p with

Figure 3. Localization of Exo70ΔdC-GFP relies on the actin cytoskeleton. (A) Exo70ΔdC-GFP localizes to sites of secretion in cells treated with DMSO but shows a diffuse localization pattern in cells treated with 100 μ M LatA dissolved in DMSO. Exo70-GFP has a polarized localization in cells treated with DMSO or LatA. (B) Quantitation of Exo70ΔdC-GFP and Exo70-GFP localization from at least 100 cells for each condition. The error bars represent $\pm$ SD.

Rho3p is not required for the polarized localization of Exo70p (Roumanie *et al.*, 2005). Consistent with that report, we found that under normal growth conditions, Exo70ΔdC-GFP localized to bud tips in small buds, isotropically in larger buds and at bud necks in cells undergoing cytokinesis, much like full-length Exo70-GFP (Figure 3A). However, unlike full-length Exo70-GFP, localization of the mutant Exo70ΔdC-GFP protein was sensitive to treatment with LatA, a drug that causes depolymerization of actin filaments (Figure 3A). The polarized localization of Exo70ΔdC-GFP was almost completely lost in small- and medium-sized buds and only partially resistant in larger buds in which the exocyst localizes to bud necks (Figure 3B). These results demonstrate that the polarized localization of Exo70ΔdC-GFP, unlike Exo70-GFP, requires an intact actin cytoskeleton. This situation mirrors the requirement of the N terminus of Sec3p for its actin-independent localization (Zhang *et al.*, 2008). This suggests that Exo70ΔdC-GFP may be delivered to exocytic sites by interacting with other components of the exocyst on the surface of secretory vesicles as they are transported along actin cables. Due to the size of the deletion, this

phenotype could be caused by more than just the loss of the Rho3p-Exo70p interaction and may reflect the loss of interactions with other components on the plasma membrane.

Exo70ΔdC Is Synthetically Lethal with Multiple Mutations in Genes of the Secretory Machinery

The results described above demonstrate that domain C of Exo70p is not essential for growth and that the mutant protein localizes in a polarized manner to sites of secretion. However, Exo70ΔdC is not fully functional because its localization is sensitive to LatA, unlike the wild type protein. Therefore, to determine the nature of the loss-of-function introduced by this internal deletion, *exo70ΔdC* was combined with temperature-sensitive (*ts*) alleles in other exocyst genes as well as several other genes whose products are involved in secretion (Table 2). Surprisingly, *exo70ΔdC* displayed synthetic effects with most of the *ts* mutants tested. This included not only most other exocyst genes but also genes such as *SEC4* (encoding a Rab GTPase required for secretion) and *SEC9* (encoding the yeast target membrane-associated [t]-SNARE homologue of soluble *N*-ethylmaleim-

Table 2. Summary of the genetic interactions between mutations in either *EXO70* or *RHO3* and *ts* mutations of genes in the secretory pathway

Mutants	<i>exo70ΔdC</i>	<i>exo70(K354A, K355A)</i>	<i>exo70(E370A)</i>	<i>exo70(D374A)</i>	<i>rho3-1</i>
<i>sec1-1</i>	NE	NE	NE	NE	N/A
<i>sec2-41</i>	SL	NE	NE	NE	NE
<i>sec3-2</i>	SL	SS	SS	NE	SL
<i>sec4-8</i>	SL	NE	N/A	NE	NE
<i>sec5-24</i>	NE	N/A	N/A	N/A	N/A
<i>sec6-4</i>	SS	NE	NE	NE	NE
<i>sec8-9</i>	SL	NE	NE	NE	NE
<i>sec9-4</i>	SL	NE	NE	NE	SS
<i>sec10-2</i>	SL	NE	NE	NE	SL
<i>sec15-1</i>	SL	NE	NE	NE	SL
<i>sec3Δ</i>	SL	SL	SL	NE	N/A
<i>sec3ΔN</i>	SL ^a	NE	NE	NE	NE

NE, no effect; N/A, not applicable; SL, synthetic lethal; and SS, synthetic sickness.

^a The double mutant *exo70Δ346-515;sec3ΔN* is not rescued by 2 μ plasmid overexpression of *SEC1*, *SEC4*, *SEC5*, *SEC6*, *SEC8*, *SEC10*, *SEC15*, or *SRO7*.

ide-sensitive factor attachment protein [SNAP]-25). However, *exo70ΔdC* showed no enhancement of the *sec5-24* phenotype and only mildly enhanced the *sec6-4 ts* phenotype. Both of these genes encode exocyst subunits. Therefore, *exo70ΔdC* is synthetically lethal with *ts* mutations in genes of the secretory machinery that include most, but not all, of the exocyst components.

Disruption of the Interaction between Rho3p and Exo70p Produces No Deleterious Phenotype

Deletion of domain C of Exo70p removes the site of interaction between Exo70p and Rho3p. Although it is unlikely that loss of this interaction is the sole cause of the synthetic effects seen above, we had not definitively ruled out this possibility. Therefore, to specifically disrupt the Exo70p-Rho3p interaction, we mutated multiple surface-exposed charged residues to alanine within this domain of Exo70p to produce mutants that lost or showed reduced binding to Rho3p. In parallel, these mutant constructs were integrated into yeast to test if they produced any phenotypes. Of 15 individual residues or clusters of residues mutated (Figure 4A), only six could be tested for in vitro binding to Rho3p because many of the point mutations caused the protein to form either inclusion bodies in *Escherichia coli* or aggregates upon purification. Both *exo70K354A*, *K355A*, and *exo70E370A* led to reduced binding of Exo70p to GTP-bound Rho3p (Figure 4B). However, when *exo70K354A*, *K355A*, and *exo70E370A* were integrated into yeast, neither mutation produced any growth defects under various growth conditions. In addition, the mutant proteins were able to localize to sites of secretion in the presence of LatA, unlike *exo70ΔdC* (data not shown). The other point mutants were also integrated into yeast, yet none of them produced a growth phenotype (data not shown). These results suggest that the Exo70p-Rho3p interaction is not essential and that loss of this interaction does not produce any obvious growth defect.

exo70K354A, *K355A*, and *exo70E370A* Show Synthetic Effects with *SEC3*

The *exo70ΔdC* mutation removes a significant portion of Exo70p and in addition to blocking its interaction with Rho3p, it could potentially prevent its interaction with other exocyst subunits. Previous results showed that this domain is required for an interaction with Sec6p (Dong *et al.*, 2005).

We tested the point mutants that showed reduced binding to Rho3p for synthetic effects with secretory pathway mutants. The point mutants *exo70K354A*, *K355A*, and *exo70E370A* were combined with the same set of *ts* mutations that showed synthetic phenotypes with *exo70ΔdC*. Both *exo70K354A K355A* and *exo70E370A* were synthetically lethal with *sec3Δ* and enhanced the phenotype of *sec3-2*, but did not enhance any of the other alleles tested (Table 2). Several of the other point mutants were also tested for synthetic effects on *sec8-9* and *sec10-4* mutants but they failed to show any enhancement (data not shown). Therefore, *exo70ΔdC* affects a function(s) of Exo70p that is likely to extend beyond its interaction with Rho3p.

Rho3-1 Is Synthetically Lethal with Several Exocyst ts Mutants

Previous studies have shown that Rho3p is involved in secretion and several *RHO3* mutations result in an accumulation of secretory vesicles (Imai *et al.*, 1996; Adamo *et al.*, 1999; Robinson *et al.*, 1999). To test whether disruption of Rho3p function produces the same synthetic effects as the *exo70ΔdC* mutation, we crossed the *ts RHO3* mutant, *rho3-1*, to the same set of *ts* mutations that were synthetically lethal with *exo70ΔdC*. We found that *sec3-2*, *sec10-1*, and *sec15-1* were synthetically lethal with *rho3-1* (Table 2). Furthermore, *rho3-1* enhanced the *ts* phenotype of *sec9-4*, consistent with previous results that demonstrated a role of Rho3p in SNARE function (Lehman *et al.*, 1999).

Exo70ΔdC Is Synthetically Lethal with sec3ΔN

The exocyst component Sec3p shares properties in common with Exo70p: it can localize to sites of secretion independent of the actin cytoskeleton, and it interacts with members of the Rho GTPase family (Finger *et al.*, 1998; Guo *et al.*, 2001; Zhang *et al.*, 2001; Boyd *et al.*, 2004). The interaction of Sec3p with either Rho1p or Cdc42p, much like the interaction of Rho3p with Exo70p, is not essential, and there is no growth defect associated with loss of the Rho binding, amino-terminal domain of Sec3p, (*Sec3ΔN*), just as there is no growth defect associated with loss of Exo70 domain C; yet, both mutations block actin-independent localization to exocytic sites. It has also been reported that the double mutant *sec3ΔN rho3V51* (a mutation in *rho3* that blocks the interaction with Exo70p) is viable and does not mislocalize Exo70p or the exocyst, which demonstrates that Rho proteins are not

Figure 4. Point mutations in the region between residues 346 and 515 of Exo70p disrupt its interaction with Rho3p. (A) Amino acid sequence of Exo70p between residues 346 and 515. Residues in gray and underlined in gray have been mutated to alanine. The numbers below the mutated residues indicate each mutation that was created. Some of the mutations changed two adjacent charged residues (mutants 1, 2, 3, 7, 10, 13, and 15) or two charged residues separated by an uncharged residue (mutants 11 and 12) to alanine simultaneously. The residues underlined in black, K354, K355 (mutant 3) and E370 (mutant 4), respectively, disrupt the interaction of Exo70p with Rho3p when changed to alanine as tested in an in vitro binding assay shown in B. (B) In vitro binding of Exo70(K354A, K355A)p, Exo70(E370A)p, and Exo70(E499A)p to either GTP γ S- or GDP-bound GST-Rho3p. (C) Structure of domain C depicting residues that disrupt the binding of Rho3p (highlighted in yellow). Those labeled with white font were identified in this study, whereas those labeled with orange font were previously identified (He *et al.*, 2007). The structure of domain C was rendered using Cn3D 4.1 (NCBI) and PDB file 2PFV (Moore *et al.*, 2007). (D), dissection of EXO70/*exo70 Δ dC*; *SEC3/sec3 Δ N*; [2μ *SEC1*] diploid cells. *exo70 Δ dC* is marked with circles, *sec3 Δ N* is marked with squares, and 2μ *SEC1* is marked with diamonds. Circles or squares with dashed lines indicate comigration with the 2μ *SEC1* plasmid. Eleven double mutants are predicted of which six should cosegregate with the 2μ *SEC1* plasmid (based on the frequency of 15 of the 29 viable spores being *SEC1*⁺). None of the predicted double mutants are viable.

required for exocyst localization (Roumanie *et al.*, 2005). However, to determine whether there are redundant functions performed by the Rho binding domains of Sec3p and Exo70p, *sec3 Δ N* was crossed with *exo70 Δ dC* and the resulting diploids dissected. Unexpectedly, tetrad analysis resulted in a pattern of spore growth indicative of the inviability of *sec3 Δ N* *exo70 Δ dC* double mutants. All the point mutations that were created (Figure 4A) between residues 346 and 515 were also tested to see whether they were synthetically lethal with *sec3 Δ N* but none were, including *exo70K354A*, *K355A*, and *exo70E370A* (Table 2; data not shown). Therefore, the combination of *sec3 Δ N* and *exo70 Δ dC* is synthetically lethal, and the likely cause of this lethality is due to more than just the loss of the Rho3p-Exo70p interaction.

Overexpression of *SEC1*, *SEC4*, *SRO7*, or Exocyst Genes Fails to Rescue the Lethality of the *sec3 Δ N* *exo70 Δ dC* Double Mutant

Previously published results have shown that the conditional lethal or lethal phenotypes of *sec3 Δ* , *sec5 Δ* , and *exo70 Δ* can be bypassed by overexpression of either *SEC1* or *SEC4* (Wiederkehr *et al.*, 2004). Although the combination of *sec3 Δ N* and *exo70 Δ dC* is lethal, the major portions of both proteins are still present and therefore overexpression of either *SEC1* or *SEC4* might be expected to rescue the lethality of the double mutant. Dissection of several diploids containing a multi-copy plasmid construct overexpressing either *SEC1* or *SEC4* still produced a pattern of spore growth indicative of the inviability of the double mutants. The dissected tetrads in Figure 4D yielded 11 predicted double mutants of which at least six should have been carrying the 2μ plasmid that results in overexpression of *SEC1*. Yet, none of the 11 double mutants was viable. From dissections of tetrads containing 2μ *SEC4*, the results were similar: none of the 10 predicted double mutants was viable, although five were expected to carry the *SEC4* plasmid. *SRO7*, an effector of Sec4p, has been shown previously to rescue several exocyst deletion mutants when overexpressed (Grosshans *et al.*, 2006). However, a 2μ *SRO7* plasmid was unable to rescue the *sec3 Δ N* *exo70 Δ dC* double mutant when analyzed by tetrad dissection (10 predicted double mutants, 0 of 6 viable that should have been carrying the 2μ *SRO7* plasmid). Thus, the overexpression of *SEC1*, *SEC4*, or *SRO7* fails to rescue the lethality of the *sec3 Δ N* *exo70 Δ dC* double mutant (Figure 4D). Similarly, overexpression of various individual exocyst proteins (*SEC5*, *SEC6*, *SEC8*, *SEC10*, and *SEC15*) was also unable to rescue the double mutant (data not shown). The simplest explanation for these data is that the amino terminal domain of Sec3p and domain C of Exo70p play an essential yet redundant role that cannot be bypassed by multi-copy suppressors that can bypass a complete deletion of either single gene.

Exo70 Δ dC Leads to Decreased Assembly of Certain Exocyst Components

The synthetic effects observed between *exo70 Δ dC* and various *ts* secretory mutants might reflect the role of Exo70p in exocyst assembly. In the absence of domain C of Exo70p, the assembly of the exocyst may be partially compromised and an additional insult, in the form of a mutation in another exocyst component, could reduce exocyst function to the point of lethality. If exocyst assembly is disrupted by *exo70 Δ dC*, the exocyst complex isolated from this mutant background should exhibit differences in subunit composition relative to a wild-type strain. To test this hypothesis, pull-downs of exocyst proteins were performed from lysates of wild-type or *exo70 Δ dC* cells and the resulting exocyst complexes probed for their assembly state. When either

Figure 5. Assembly of several exocyst subunits is reduced in a *exo70ΔdC* background. In all of the panels, the negative control lane (NEG) is a pull-down from a strain containing either 3xHA-tagged Sec5p or 13xmyc-tagged Sec6p but no TAP-tagged exocyst component. (A) Western blots to detect Sec5p (3xHA tagged) being pulled down by either Sec8-TAP or Sec10-TAP from lysates of *wt* or *exo70ΔdC* cells. Input represents 1% of total protein. (B) Western blots detecting Sec6p (13xmyc tagged) being pulled down by Sec15-TAP from lysates of *wt* or *exo70ΔdC* cells. Input represents 1% of total protein probed with antibodies to either Sec6 (anti-myc antibodies) or Sec15 (anti-rabbit IgG antibodies). (C) Western blots detecting Sec6p (13xmyc tagged) being pulled down by either Sec8-TAP or Exo84-TAP from lysates of *wt* or *exo70ΔdC* cells. (D) Western blots detecting Sec5p (3xHA tagged) being pulled down by Sec10-TAP from lysates of *wt*, *exo70ΔdC*, *exo70(K354A,K355A)*, or *exo70(E370A)* cells. (E) Western blots detecting Sec5p (3xHA tagged) pulled down with Sec8-TAP from lysates of *wt*, *exo70ΔdC*, or *rho3-1* cells. In *rho3-1* cells, they were grown at either 25 or 37°C for 2 h before being harvested for analysis.

Sec8p or Sec10p was the epitope-tagged construct used for the pull-down, significantly less Sec5p was present in the isolated exocyst complexes from *exo70ΔdC* cells compared with wild-type cells (Figure 5A). By densitometry, 59 and 52% of Sec5p was pulled down by Sec8p or Sec10p, respectively, from *exo70ΔdC* relative to *wt* cells. Similarly, when Sec15p was used for the pull down, less Sec6p (39.7 ± 1% as measured by densitometry) was isolated from *exo70ΔdC* cells relative to wild-type cells (Figure 5B). The effect on assembly caused by *exo70ΔdC* seems to be specific for either Sec5p, when precipitating Sec8p and Sec10p, or Sec6p when pulled down using Sec15p. Pull-downs of either Sec8p or Exo84p did not show any decrease in the level of Sec6p in the exocyst complex in the mutant background (Figure 5C). To determine whether these changes were caused by loss of Rho3p signaling, we examined assembly in *exo70* mutants that fail to bind Rho3p and in a *rho3* mutant. In strains containing either *exo70K354A*, *K355A* or *exo70E370A*, Sec10p pull-downs showed no reduction in the amount of Sec5p assembled in the exocyst, unlike cells containing *exo70ΔdC* (Figure 5D). Sec8p pull-downs were performed in a *rho3-1* background after incubation at the permissive (25°C) or

restrictive (37°C) temperature. There was no difference in the amount of assembled Sec5p in exocyst complexes isolated from *rho3-1* cells grown at the permissive versus the restrictive temperature (Figure 5E). The densitometric ratio of the Sec5p band to the Sec8p band was 0.52 for cells grown at 25°C and 0.5 for cells grown at 37°C. Together, these results suggest that the loss of Rho3p signaling is not the cause of the defect in exocyst assembly observed in *exo70ΔdC* cells.

These results suggest that Sec5p and Sec6p reside in different exocyst subcomplexes with Exo70p serving as a link between them. This proposal is consistent with the observation that residues 346-515 of Exo70p are important for its interaction with Sec6p (Dong *et al.*, 2005). To determine the composition of exocyst subunits interacting with Exo70p, complete TAPs of the exocyst with TAP-tagged Exo70p were performed, and the resulting complexes were analyzed. Silver staining of the exocyst complex retrieved using either Exo70-TAP or Exo70ΔdC-TAP showed no significant differences in the overall composition of the exocyst (Figure 6A). Western blots showed no differences in the amounts of Sec3p, Sec5p, Sec8p, Sec10p, and Sec15p that were isolated by pulling down Exo70p versus Exo70ΔdCp (Figure 6B). However, as in the data described above, there was a significant reduction in the amount of Sec6p pulled down by Exo70ΔdCp (43% of wild type, as measured by densitometry) compared with Exo70p (Figure 6B). The reduction in the amount of Sec6p bound to

Figure 6. TAP tag pull-downs of the exocyst by using either Exo70-TAP or Exo70ΔdC-TAP show reduced levels of Sec6p in the assembled complex. The negative control lane (NEG) is a pull-down from a strain containing 3xHA-tagged Sec5p but no Exo70-TAP or Exo70ΔdC-TAP. (A) Silver-stained gel of the isolated exocyst complex from either *wt* (EXO70) cells or *exo70ΔdC* (ΔdC) cells. Exo70-TAP or Exo70ΔdC-TAP was pulled down, and the resulting complexes treated with TEV protease to release the exocyst complex from the IgG beads. The released exocyst complexes were then isolated on calmodulin beads and separated by SDS-polyacrylamide gel electrophoresis for silver staining. Exo70-CBP and *exo70* ΔdC -CBP indicate Exo70p and Exo70 ΔdC p, respectively, with the calmodulin binding peptide after TEV cleavage. (B) Western blots detecting different exocyst components in exocyst complexes isolated from Exo70-TAP and Exo70ΔdC-TAP pull-downs. The input lanes represent 1% of total protein, and the blot was probed with anti-HA antibodies to 3xHA-tagged Sec5p.

Figure 7. Domain C of Exo70p and the N terminus of Sec3p are required for proper localization of the exocyst. The localization of Sec5-GFP is shown in *sec3ΔN;exo70ΔdC;[MET17p-EXO70, CEN]* (*EXO70* under the inducible *MET17* promoter) cells repressed for 24 h in 1 mM methionine (C and D) or subsequently induced in 0 mM methionine for 4 h (E and F). As a control, the localization of Sec5-GFP is shown in *wt* cells grown in 1 mM methionine (A and B), the same conditions that repress expression of *EXO70* in the above-mentioned strain. (G) Quantitation of Sec5-GFP localization in *sec3ΔN;exo70ΔdC;[MET17p-EXO70, CEN]* with or without 1 mM methionine or *wt* cells treated with 1 mM methionine. At least 100 cells of each condition were counted. The error bars represent $\pm$ SD.

Exo70ΔdCp versus Exo70p was very similar to the difference seen when using Sec15-TAP to pull down the exocyst in an *EXO70* versus *exo70ΔdC* background.

Loss of *exo70ΔdC* and *sec3ΔN* Leads to Mislocalization of Sec5-GFP

Domain C of Exo70p functions to localize the protein to sites of secretion independent of actin and mediates the incorporation of Sec5p and Sec6p into the assembled exocyst complex. This domain is also redundant with the N terminus of Sec3p in performing an essential but uncharacterized function. To assess the connection between the redundant domains of Exo70p and Sec3p with respect to exocyst localization and assembly, we constructed a *sec3ΔN exo70ΔdC* strain carrying full-length *EXO70* behind the inducible *MET17* promoter. This strain also contains *SEC5* engineered with a C-terminal GFP tag as a reporter for exocyst localization. The expression of Exo70p is required to suppress the lethal phenotype caused by combining *sec3ΔN* and *exo70ΔdC*. In the presence of 1 mM methionine, the expression of Exo70p is repressed, whereas if methionine is

absent from the growth media, Exo70p expression is induced. To test the combinatorial effect of *sec3ΔN* and *exo70ΔdC* on exocyst localization, the above strain was grown in 1 mM methionine for 24 h. After repression, these cells were observed under the microscope and Sec5-GFPp was mislocalized and seemed diffuse within cells (Figure 7, C and D). Cells were generally more circular than *wt* cells, which suggested a loss of polarization. On induction of *EXO70* expression in growth media free of methionine for 4 h, Sec5p was now polarized at bud tips and bud necks (Figure 7, E and F). Wild-type cells expressing Sec5-GFPp grown in 1 mM methionine did not show the loss of localization seen in the mutant cells (Figure 7, A and B). These results show that domain C of Exo70p and the N terminus of Sec3p both act to mediate localization of the exocyst to sites of secretion.

DISCUSSION

We find here that three of the four domains of Exo70p are essential for function. Deletion of the C-terminal domain D

results in a recessive lethal phenotype. This domain contains an evolutionarily conserved patch of positively charged residues (Dong *et al.*, 2005) that bind to phosphatidylinositol biphosphate (PIP₂) on the plasma membrane and is essential for viability (He *et al.*, 2007; Liu *et al.*, 2007). Deletion of the amino terminal domain A also results in a recessive lethal phenotype, but no specific function can yet be assigned to this domain. Deletion of the internal domain B results in a dominant lethal phenotype, even at low levels of expression. This toxicity is probably caused by the truncated protein binding to an Exo70 interaction partner, without fulfilling the normal Exo70p function.

Deletion of domain C produced surprising results: cells grew normally and showed no obvious phenotypes, despite the loss of more than a quarter of this essential subunit. Unlike full length Exo70p, the mutant protein mislocalized in the presence of LatA, a drug that depolymerizes actin filaments. Therefore, the ability of Exo70p to localize to sites of secretion independent of the actin cables upon which secretory vesicles travel, requires domain C. We speculate that the interaction of Exo70ΔdCp with other exocyst subunits allows the mutant protein to localize by traveling to exocytic sites on secretory vesicles, an actin-dependent process.

The *exo70ΔdC* mutant is synthetically lethal with *sec3ΔN*. This result is particularly striking because neither mutation alone significantly affects growth under a variety of conditions. Furthermore, the double mutant cannot be rescued by overexpression of *SEC1*, *SEC4*, or *SRO7*, any of which can rescue a complete deletion of *EXO70* or *SEC3* (Wiederkehr *et al.*, 2004). These two domains of Exo70p and Sec3p perform an important function in localizing the exocyst. In an *exo70ΔdC sec3ΔN* double mutant rescued by *EXO70* under a *MET17* promoter, localization of Sec5-GFP is lost if *EXO70* expression is repressed. Sec5-GFP localization is restored upon induction of *EXO70* expression. The simplest explanation for these results is that domain C of Exo70 and the amino terminal domain of Sec3 share an essential, yet redundant function that cannot be readily bypassed. Exo70 and Sec3 are unique among exocyst subunits in their ability to localize to exocytic sites by an actin-independent pathway that probably reflects a direct interaction with components of the plasma membrane. The amino-terminal domain of Sec3p binds PIP₂ together with either Rho1-GTP or CDC42-GTP (Guo *et al.*, 2001; Zhang *et al.*, 2001, 2008). Domain C of Exo70p binds Rho3-GTP (Dong *et al.*, 2005); however, this interaction does not seem to be important for localization (see below). Therefore, we anticipate that domain C is important for binding some other component of the plasma membrane. Moreover, our data suggest that at least one subunit of the exocyst must make contact with the plasma membrane to fulfill its role in tethering secretory vesicles to exocytic sites.

Exo70ΔdC also exhibits synthetic lethality with *ts* mutations in all exocyst genes except *SEC5* as well as with *sec4-8*, *sec2-41*, and *sec9-4*; *ts* mutations in genes encoding the Rab protein required for secretion, its associated guanine nucleotide exchange factor, and the plasma membrane t-SNARE homologue of SNAP-25, respectively. Although there are no direct interactions reported between Exo70p and either Sec4p or Sec9p, Sec15p is a known effector of Sec4p (Guo *et al.*, 1999b), and Sec6p has been shown to interact with Sec9p (Sivaram *et al.*, 2005). These genetic interactions are likely to result from a loss of exocyst assembly. Biochemical analyses verified that the *exo70ΔdC* mutation reduces levels of Sec5p and Sec6p within the assembled exocyst. Our previous studies have demonstrated that domain C of Exo70p mediates an interaction with Sec6p (Dong *et al.*, 2005), which explains the

Figure 8. Model of the interactions between exocyst subunits mediated by Exo70p. Without residues 346-515 (domain C), the interactions between Sec5p and either Sec8p or Sec10p and Sec6p with Sec15p are reduced (indicated by dashed lines). Domain C does not seem to affect the interaction of Exo70p with the remaining exocyst components (indicated by solid lines). The *exo70ΔdC* mutant may reveal the function of Exo70p in bridging different exocyst subcomplexes.

reduced incorporation of Sec6p in the exocyst. Some Sec6p is still incorporated, presumably through interactions with other subunits such as Sec8p, which is thought to bind Sec6p (TerBush and Novick, 1995). The reduction in the incorporation of Sec5p or Sec6p is only revealed when certain exocyst subunits are used for the pull-down. This suggests that there may be subpopulations of exocyst components and Exo70p may bridge them or itself be contained within a subcomplex. In the absence of domain C, Sec5p and Sec6p are no longer stably assembled in their appropriate subcomplexes, which leads to their reduced incorporation in the exocyst. Analysis of *exo84* mutants also predicts a subcomplex containing Sec5p and Sec6p (Zhang *et al.*, 2005). Our data suggest that these proteins are likely to be peripheral components of the exocyst, a model consistent with the observed interaction of Sec6p with the plasma membrane t-SNARE Sec9p (Sivaram *et al.*, 2005). A schematic diagram of the interactions between exocyst components based on the above-mentioned results (Figure 8) depicts possible subcomplexes.

Within domain C of Exo70p, we have identified a site of interaction with the Rho3p GTPase. The interaction between Exo70p and GTPγS-bound Rho3p was reduced by changing both residues K354 and K355, or E370 alone, to alanine. Nonetheless, these mutations do not produce a phenotype in vivo unless combined with a deletion of *SEC3* or with the *ts* allele *sec3-2*. These results further support a functional relationship between Exo70p and Sec3p not shared by other subunits of the exocyst. Although both of these exocyst subunits interact with Rho proteins, the Exo70p-Rho3p interaction is not critical for exocyst function. In addition to the point mutations we have identified that disrupt the Exo70p-Rho3p interaction, recent mutagenesis data indicates another nearby interaction site within this domain (He *et al.*, 2007). Although all of these mutations disrupt the Exo70p-Rho3p interaction, not one of them is synthetically lethal with *sec3ΔN*, unlike *exo70ΔdC*, and none of those tested show the same synthetic effects as *exo70ΔdC* with mutants of the secretory pathway. *Rho3-1* does not show the same synthetic effects as *exo70ΔdC*, although it is synthetically lethal with *sec3-2*, *sec10-2*, and *sec15-1*. The synthetic effects of *rho3-1* are more severe than those of the *exo70* point mutations described above, and this probably reflects the role of other Rho3p effectors, such as Myo2p, that function in secretion (Robinson *et al.*, 1999). Furthermore, pull-downs of the exocyst performed in a *rho3-1* mutant background did

not show the same defect in exocyst assembly as an *exo70ΔdC* mutant. Therefore, the interaction of Rho3p with Exo70p does not seem to play a major role in exocyst assembly or function.

The synthetic effects and loss of actin-independent localization of Exo70p upon removal of domain C highlight the importance of this domain. Actin-independent localization may rely on the interaction of domain C with a component of the plasma membrane other than Rho3p. If the other exocyst proteins are extended rods, like Exo70p, the functional domains of each protein may need to align in a specific orientation with respect to the vesicle and plasma membrane. Exo70p and Sec3p, through their interactions with Rho GTPases, phosphoinositides and additional components of the plasma membrane, may provide an essential foundation for this process.

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