Sec6p Anchors the Assembled Exocyst Complex at Sites of Secretion

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The exocyst is an essential protein complex required for targeting and fusion of secretory vesicles to sites of exocytosis at the plasma membrane. To study the function of the exocyst complex, we performed a structure-based mutational analysis of the *Saccharomyces cerevisiae* exocyst subunit Sec6p. Two “patches” of highly conserved residues are present on the surface of Sec6p; mutation of either patch does not compromise protein stability. Nevertheless, replacement of SEC6 with the patch mutants results in severe temperature-sensitive growth and secretion defects. At nonpermissive conditions, although trafficking of secretory vesicles to the plasma membrane is unimpaired, none of the exocyst subunits are polarized. This is consistent with data from other exocyst temperature-sensitive mutants, which disrupt the integrity of the complex. Surprisingly, however, these patch mutations result in mislocalized exocyst complexes that remain intact. Our results indicate that assembly and polarization of the exocyst are functionally separable events, and that Sec6p is required to anchor exocyst complexes at sites of secretion.

INTRODUCTION

In eukaryotic cells, membrane-bound vesicles are required for carrying protein and membrane cargo between functionally distinct organelles and to the plasma membrane for exocytosis. The precise spatial and temporal regulation of vesicle fusion is achieved by a number of essential proteins (Wickner and Schekman, 2008 and references therein). Specific SNARE proteins, present on both the vesicle (v-SNARE) and the target (t-SNARE) membranes, are critical for membrane fusion. Tethering proteins, which are proposed to bridge the vesicle and target membranes at a distance, are either dimeric coiled-coil proteins or components of multisubunit protein complexes (Sztul and Lupashin, 2006). These proteins interact with the donor and target membranes through specific interactions with small Ras superfamily GTPases and phospholipids (Wu *et al.*, 2008). Many tethering proteins bind directly to SNAREs and the regulatory Sec1/Munc18 family proteins and likely play crucial roles in the temporal and spatial regulation of SNARE complex assembly before membrane fusion (Cai *et al.*, 2007; Kummel and Heinemann, 2008).

The exocyst is a conserved multisubunit protein complex required for tethering and fusion of vesicles at specific sites of polarized secretion on the plasma membrane (TerBush *et al.*, 1996; Guo *et al.*, 1999; Munson and Novick, 2006; Wu *et al.*, 2008). This complex is distantly related to tethering factors found in other trafficking pathways, including the GARP (Golgi-associated retrograde protein), COG (con-

served oligomeric Golgi), and Dsl1 complexes (Whyte and Munro, 2002; Koumandou *et al.*, 2007). The exocyst is comprised of eight subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p. These proteins localize to regions of exocytosis and membrane growth in eukaryotic cells, where their activity is regulated by small Rab, Rho, and Ral GTPases (Wu *et al.*, 2008). Temperature-sensitive (ts) yeast exocyst mutants accumulate secretory vesicles at sites of secretion (bud tips and mother-bud necks) at a stage before exocytic SNARE complex assembly. The exocyst also plays critical roles in *Drosophila*, mammals and plants, not just in exocytosis, but also in endocytic recycling and cytokinesis (Gromley *et al.*, 2005; Sommer *et al.*, 2005; Oztan *et al.*, 2007; Hala *et al.*, 2008).

Elucidation of the molecular function of the exocyst requires detailed knowledge of each of the subunits and their assembly into functional complexes. High-resolution structures of four exocyst subunits have been determined: the C-terminal domain of *Drosophila melanogaster* Sec15 (Wu *et al.*, 2005), the nearly full-length yeast Exo70p (Dong *et al.*, 2005; Hamburger *et al.*, 2006; Moore *et al.*, 2007), the C-terminal domain of yeast Exo84p (Dong *et al.*, 2005), and the C-terminal domain of yeast Sec6p (Sivaram *et al.*, 2006). They fold into remarkably similar elongated helical bundle structures with diverse surface features (Munson and Novick, 2006; Sivaram *et al.*, 2006). Protein–protein interactions within the exocyst complex and with small GTPases have been identified genetically, by qualitative in vitro binding studies and by yeast two-hybrid analyses (reviewed in Munson and Novick, 2006). The exocyst associates with secretory vesicles through the interaction of Sec15p with the Sec4p GTPase (Guo *et al.*, 1999), and most of its subunits appear to traffic with secretory vesicles to sites of secretion (Boyd *et al.*, 2004). Exocyst function is regulated by interactions of Sec3p and Exo70p with membrane-anchored Rho GTPases and phosphoinositides (Adamo *et al.*, 1999; Guo *et al.*, 2001; Zhang *et al.*, 2001, 2008; He *et al.*, 2007), as well as interactions with the yeast lethal giant larvae (Lgl) homologues Sro7p and Sro77p (Lehman *et al.*, 1999; Zhang *et al.*, 2005a;

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Abbreviations used: SC, synthetic complete; ts, temperature sensitive.

Table 1. Yeast strains used in this study

Strain	Relevant genotype
MMY251	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 (LEU2 CEN SEC6)</i>
MMY252	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-49)</i>
MMY253	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-54)</i>
NY778	<i>MATα leu2-3,112 ura3-52 sec6-4 (Novick et al., 1980)</i>
MMY275	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 (LEU2 CEN SEC6-HA₃)</i>
MMY276	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-49-HA₃)</i>
MMY335	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-54-HA₃)</i>
MMY204	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 (URA3 CEN SEC6)</i>
MMY246	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 SEC3-GFP(HIS3) (URA3 CEN SEC6)</i>
MMY238	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 SEC5-GFP(HIS3) (URA3 CEN SEC6)</i>
MMY239	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 SEC8-GFP(HIS3) (URA3 CEN SEC6)</i>
MMY247	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 SEC10-GFP(HIS3) (URA3 CEN SEC6)</i>
MMY248	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 SEC15-GFP(HIS3) (URA3 CEN SEC6)</i>
MMY249	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 EXO70-GFP(HIS3) (URA3 CEN SEC6)</i>
MMY250	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 EXO84-GFP(HIS3) (URA3 CEN SEC6)</i>
MMY443	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec2-41 (URA3 CEN SEC6)</i>
MMY279	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec3-2 (URA3 CEN SEC6)</i>
MMY444	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec4-8 (URA3 CEN SEC6)</i>
MMY387	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec5-24 (URA3 CEN SEC6)</i>
MMY414	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec8-9 (URA3 CEN SEC6)</i>
MMY623	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec9-4 (URA3 CEN SEC6)</i>
MMY446	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec9-7 (URA3 CEN SEC6)</i>
MMY415	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec10-2 (URA3 CEN SEC6)</i>
MMY278	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sec15-1 (URA3 CEN SEC6)</i>
MMY416	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 exo70Δ::HIS3 (TRP1 CEN exo70-38) (URA3 CEN SEC6)</i>
MMY448	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 sso1Δ::HIS sso2-1 (URA3 CEN SEC6)</i>
MMY441	<i>MATα sec6Δ::KanMX-6 hisΔ1 leu2Δ0 ura3Δ0 (LEU2 CEN SEC6-HA₃) (URA3 SEC8-MYC₃)</i>

Grosshans *et al.*, 2006; Hattendorf *et al.*, 2007). Most of the subunits are essential in yeast, except Sec3p, although *sec3Δ* cells grow quite slowly (Wiederkehr *et al.*, 2003). In addition, the lethality of *sec5Δ* and *exo70Δ* can be bypassed by overexpression of either *SEC1* or *SEC4* (Wiederkehr *et al.*, 2004). These structural, biochemical, and genetic studies indicate that each of the subunits plays diverse roles in exocyst function; however, many questions remain about the specific function(s) of the exocyst and its subunits.

Here, we focus on mutational studies of the yeast exocyst subunit Sec6p, which forms a direct link between the exocyst complex and the exocytic plasma membrane SNARE protein Sec9p and has been proposed to regulate SNARE complex assembly (Sivaram *et al.*, 2005). Qualitative protein–protein interaction studies also indicated that Sec6p can dimerize in vitro (Sivaram *et al.*, 2005), interact with the exocyst subunits Sec10p, Sec8p, and Exo70p (Dong *et al.*, 2005; Sivaram *et al.*, 2006), and bind Rtn1p, an interaction that is important for cortical ER inheritance (De Craene *et al.*, 2006). We identified and mutated two patches of residues on the surface of Sec6p that are highly conserved from yeast to humans and show that these mutants have ts growth and secretory defects in yeast. These phenotypes are due to a loss of polarized localization of the exocyst subunits at nonpermissive conditions. However, unlike other exocyst ts mutants, exocyst complexes are intact at the nonpermissive conditions, indicating that the Sec6p patch residues are necessary to anchor functional, assembled exocyst complexes at sites of secretion. The conservation of these patch residues suggests an anchoring role for Sec6p in all eukaryotes.

MATERIALS AND METHODS

Plasmid and Strain Construction and Invertase Secretion

Mutations (*sec6-49*: L418A, Y422A, W433A, Q470A, and Q474A; *sec6-54*: D607A, T632A, E635R, Y636A, and D639R) were introduced into the *SEC6*

gene by PCR, and cloned into the NdeI and BamHI sites of pET15b (Novagen, Madison, WI) containing an N-terminal His₆ tag. For analysis in yeast, constructs were placed into the BamHI and NotI sites of pRS315 (*LEU2 CEN*) including the endogenous promoter of *SEC6* (0.5 kb of flanking 5' and 3' genomic DNA). A C-terminal triple hemagglutinin (HA) tag was added to the pRS315 constructs for use in indirect immunofluorescence and immunoprecipitations. All clones were confirmed by sequencing. The *sec6 LEU2 CEN* plasmids were introduced as the sole copy of *SEC6* in yeast by plasmid shuffling the wild-type *SEC6 URA3 CEN* plasmid out of MMY204 (Table 1). For green fluorescent protein (GFP) and synthetic lethal analyses, MMY204 was mated with strains containing either a single C-terminally GFP-tagged exocyst subunit (Huh *et al.*, 2003) or a single ts mutation (gifts from P. Novick, Yale University; W. Guo, University of Pennsylvania; and J. Jäntti, University of Helsinki). Diploid strains were sporulated and dissected, and the mutant plasmids were introduced into the GFP⁺ *sec* haploids as described above. Media, growth conditions, and genetic methods for yeast were described previously (Munson *et al.*, 2000). Yeast strains used in this study are listed in Table 1. Invertase secretion was performed as in Adamo *et al.* (1999).

Protein Expression, Purification, and Characterization

His₆-Sec6p, His₆-Sec6-49, and His₆-Sec6-54 were expressed and purified as described for His₆-Sec6p (Sivaram *et al.*, 2005), with the following change for the His₆-Sec6-49 anion-exchange buffers: 10 mM HEPES, pH 7.4, and 1 mM DTT ± 1 M NaCl. Protein concentrations were determined by ninhydrin protein assay (Rosen, 1957). Circular dichroism spectroscopy was performed as described (Sivaram *et al.*, 2005). Maltose binding protein (MBP) pulldown binding assays were performed as described (Sivaram *et al.*, 2006) using a 20 mM HEPES, pH 7, 100 mM NaCl, 1 mM EDTA, 0.1% NP-40, and 1 mM DTT binding buffer. For experiments with MBP-Exo84CT, the soluble C-terminal domain (residues 523-753; Dong *et al.*, 2005) was subcloned into pMALc2X (New England Biolabs, Beverly, MA). MBP-Exo84CT was expressed at 20°C for 3 h after induction with 0.1 mM IPTG and purified by amylose resin affinity chromatography.

Indirect Immunofluorescence

Strains containing wild-type or mutant Sec6p with C-terminal HA₃ tags were grown to log phase in synthetic complete (SC) media at 25°C and either fixed or shifted to nonpermissive conditions (prewarmed YPD at 37°C for 1 h) and then fixed with 37% formaldehyde. Cells were spheroplasted, permeabilized in HS/SDS buffer (0.1 M HEPES, pH 7.4, 1.0 M sorbitol, and 0.5% SDS), and washed twice in HS buffer. Permeabilized cells were placed on slides (Electron Microscopy Sciences, Fort Washington, PA) prepared with 0.1% polylysine. A 1:400 dilution of α-HA-AlexaFluor 488 (Molecular Probes, Eugene,

Figure 1. Mutations of highly conserved surface residues do not disrupt the structure or stability of yeast Sec6p. (A) Structure of the C-terminal domain of Sec6p, showing the location of the conserved patch amino acids. The Patch-1 residues L418, Y422, W433, Q470, Q474, and V478 were mutated to Ala (Sec6-49). In Patch-2, residues D607, T632, and Y636 were mutated to Ala, and residues E635 and D639 mutated to Arg, to create Sec6-54. Structure-based sequence alignment of the relevant regions of the C-terminal domain of Sec6p (*S. cerevisiae*; S.c.; Sivaram *et al.*, 2006) are shown aligned with five species: *Kluyveromyces lactis*, K.l.; *Schizosaccharomyces pombe*, S.p.; *Drosophila melanogaster*, D.m.; *Danio rerio*, D.r.; and *Homo sapiens*, H.s. The yellow L indicates the position of the ts L633P mutation in the *sec6-4* strain. Patch residues on the surface of Sec6p are indicated in cyan (mutated in Sec6-49) and red (mutated in Sec6-54). (B) Purified recombinant full-length Sec6p patch mutants are folded and thermally stable. Circular dichroism wavelength scans of the recombinant mutant proteins show similar α -helicity to the wild-type Sec6p at both 4 and 37°C. At temperatures >40°C, however, the wild-type and mutant proteins precipitate, precluding detailed thermodynamic analyses.

OR) was added to visualize Sec6p-HA₃. For Sec4p, a 1:500 dilution of α -Sec4 antibody (P. Brennwald, University of North Carolina) was used, followed by washing and incubation with 1:1000 goat α -mouse-AF488 (Molecular Probes). Differential interference contrast (DIC) and fluorescent images were obtained at room temperature using an Axioskop2 plus epifluorescent microscope (Zeiss, Thornwood, NY) fitted with a 100 $\times$ Plan-NEOFLUAR (Zeiss 1.30 NA oil immersion) objective lens. Images were collected using a Diagnostic Instruments camera (Sterling Heights, MI; model 2.1.1) and 3rd Party Interface Advanced (ver. 3.5.4 for MacOS) software. Immunofluorescent images were adjusted for total contrast in Adobe Photoshop (San Jose, CA).

GFP Fluorescence

Strains containing C-terminal GFP-tagged exocyst subunits and either wild-type or mutant Sec6p were grown to log phase in SC media at 25°C and shifted to nonpermissive conditions for 1 h. Samples were immediately resuspended in PBS containing 10% glycerol, fixed with 37% formaldehyde for 10 min, washed, and resuspended in PBS with 10% glycerol. Cells were viewed as described above. Localization of the GFP-tagged proteins was quantitated in Adobe Photoshop by counting cells with localized or mislocalized exocyst subunits. For each strain and condition, $n > 100$ cells were counted for two replicates each.

Immunoprecipitation

Strains were grown to log phase in SC media at 25°C or shifted to nonpermissive conditions for 1 h. One hundred OD units of cells were pelleted and washed with 10 mM Tris, pH 7.4, and 10 mM Na₂S₂O₃ at either 25 or 37°C. Supernatants were removed and pellets were resuspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 0.5% NP-40, 1 mM DTT, and 1 mM PMSF). Cells were lysed by bead beating, and the lysates cleared by centrifugation and normalized for total protein concentration by Bradford assay (Bio-Rad, Hercules, CA). The supernatants were added to prewashed protein A beads (GE Healthcare Bio-Sciences, Piscataway, NJ) and mixed for 30 min at 4°C.

Input samples were collected, the remaining supernatants were placed in fresh tubes, and 1:50 dilutions of HA antibody (Roche) were added. Samples were incubated at 4°C for 1 h. Lysate-antibody mixes were added to fresh, prewashed protein A beads. These mixtures were incubated at 4°C for 30 min, the supernatants were removed, and the beads were washed with lysis buffer. Input and bound fractions were analyzed by 10% SDS-PAGE gels and Western blotting. Exocyst subunits were detected using antisera raised against C-terminal constructs of Exo70p, Exo84p, and Sec10p (Pocono Rabbit Farm and Laboratory, Canadensis, PA) or antibodies against Sec3p, Sec5p, and Sec15p (generous gifts from P. Brennwald, University of North Carolina), followed by HRP-conjugated goat α -rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL (GE Healthcare Bio-Sciences), and visualized using a luminescent image analyzer (Fuji Film, Tokyo, Japan; LAS-3000).

RESULTS

Mutation of Conserved Sec6p Surface Residues Leads to Growth and Secretion Defects

We previously determined the x-ray crystal structure of the C-terminal domain of yeast Sec6p, residues 411–805 (Sivaram *et al.*, 2006). This domain is sufficient for interactions with the exocyst subunits Exo70p and Sec10p, but not Sec8p or the t-SNARE Sec9p (Dong *et al.*, 2005; Sivaram *et al.*, 2006). Structure-based sequence alignments revealed two highly conserved surface “patches,” which we hypothesize are important sites for protein–protein interactions in all eukaryotes (Figure 1A; Sivaram *et al.*, 2006). These patches are composed of the following amino acids: L418, Y422, W433,

Figure 2. Mutation of the full-length Sec6p patches leads to ts- and rich-media-sensitive growth and secretion defects in vivo. (A) 10-fold serial dilutions of yeast containing a *CEN* plasmid copy of either wild-type *SEC6*, *sec6-49*, or *sec6-54* as the sole copy of *SEC6* were plated onto either SC-leu or YPD plates and incubated at the indicated temperatures. (B) The patch mutants also show a defect in the secretion of invertase, similar to that of the *sec6-4* allele. % Invertase secretion = (secreted invertase)/(secreted + internal invertase)*100.

Q470, Q474, and V478 in Patch-1, and D607, T632, E635, Y636, and D639 in Patch-2. Patch-2 overlaps a conserved acidic region, as well as being in close proximity to the original *sec6-4* ts mutation (L633P; Novick *et al.*, 1980; Lamping *et al.*, 2005; Sivaram *et al.*, 2006). When these patch residues were individually mutagenized to alanine in the context of the full-length Sec6p and substituted for the wild-type in yeast, no apparent effects on growth rates were observed (data not shown). We reasoned that this lack of phenotype could be due to tight protein-protein interactions at these surfaces and that single alanine perturbations would be insufficient to disrupt them. Therefore, we combined mutations in each patch (Figure 1A). All the residues in Patch-1 were mutated to Ala (called *sec6-49*). For Patch-2, residues 607, 632, and 636 were mutated to Ala and residues 635 and 639 to Arg, causing a reversal of charge at these positions (called *sec6-54*). These mutations were anticipated to severely disrupt protein-protein interactions in these regions.

Because these are surface mutations, they should not significantly destabilize the α -helical structure of Sec6p. To test this, we cloned and purified the recombinant Sec6p mutant proteins from *Escherichia coli* and analyzed their secondary structure and stability using circular dichroism. As expected, the patch mutations did not decrease the stability of the proteins. Both of the mutant proteins showed the same characteristic α -helical signal as the wild-type Sec6p (~50% helicity) and also had similar thermal stabilities to wild-type at 37°C (Figure 1B). In contrast, the protein containing the ts mutant allele *sec6-4* must be considerably destabilized, because we were unable to produce any soluble recombinant protein in *E. coli* (data not shown).

To test the function of these mutant proteins as the sole copy of Sec6p in yeast, we performed a plasmid shuffle experiment to replace the wild-type *SEC6* with the mutants. The mutants were expressed under control of the endogenous promoter from a low-copy (*CEN*) plasmid and had equivalent expression levels to the endogenous Sec6p at both 25 and 37°C (data not shown). Both patch mutants were able to replace the wild-type *SEC6* *URA3* plasmid when grown on SC plates containing 5-fluoro-orotic acid (5-FOA) at 25°C. However, on SC plates at higher temperatures, both mutants exhibited ts growth defects (Figure 2A). In addition, both *sec6-49* and *sec6-54* showed severe growth inhibition when plated on rich YPD medium, even at temperatures that were permissive for growth on SC medium (Figure 2A). The growth rates of the mutants in SC liquid cultures were

similar to wild-type at 25°C and were two- to threefold slower than wild-type at 37°C, and the mutants did not grow in liquid YPD at 37°C (data not shown). The slow growth on rich YPD media may be explained by the higher rate of exocytosis compared with synthetic media, which would exacerbate the secretory defect. Similar results have been observed for other exocyst mutants (Haarer *et al.*, 1996; Wiederkehr *et al.*, 2003). Consistent with their growth phenotypes, the mutant strains were defective for secretion of the marker protein invertase at the nonpermissive conditions (Figure 2B). Because the recombinant mutant proteins were stable both in vitro and in vivo, we conclude that Sec6p's function, rather than its stability, is compromised by mutation of residues in these conserved patches.

Exocyst Subunits Are Mislocalized in Mutant *sec6* Strains

To determine the defect(s) caused by mutations of the conserved patch regions, we examined the phenotypes of the mutant yeast strains in more detail. First, we monitored the localization of the Sec6p mutants at the permissive and the restrictive conditions. The mutants could not be visualized using a GFP tag, because placing a GFP tag at either the N- or C-terminal end resulted in slow growth, even at conditions permissive for growth of the untagged mutant (data not shown). However, when we tagged the mutants at their C-termini with a triple HA epitope tag, these strains grew similarly to the untagged strains (data not shown). Immunofluorescence experiments demonstrated that the patch mutants were properly polarized to tips of the growing bud and mother-bud necks at the permissive conditions (Figure 3). After a shift to the nonpermissive conditions (YPD at 37°C for 1 h), the mutant proteins were completely mislocalized (Figure 3), suggesting that the mutations disrupt the ability of Sec6p to localize to, or remain localized at, sites of secretion. Similarly, the *sec6-4* protein was not polarized at the nonpermissive temperature (Lamping *et al.*, 2005).

To rule out that mislocalization of the Sec6p mutants was caused by a general disruption of the secretory pathway, we monitored Sec4p localization by immunofluorescence. Sec4p is the Rab family GTPase associated with secretory vesicles; its localization is sensitive to perturbations of the secretory pathway (Ayscough *et al.*, 1997; Walch-Solimena *et al.*, 1997; Roumanie *et al.*, 2005). We found that Sec4p localization was unperturbed in the *sec6-49* and *sec6-54* mutant cells after 1 h at nonpermissive conditions (Figure 3), as was previously observed for *sec6-4* and other exocyst ts mutants (Walch-Solimena *et al.*, 1997; Roumanie *et al.*, 2005). Therefore, al-

Figure 3. Sec6p patch mutants are mislocalized at the nonpermissive conditions, but vesicle trafficking is unimpaired. Immunofluorescence of HA-tagged Sec6p and the patch mutants at the permissive and restrictive conditions (left). These cells show normal trafficking of secretory vesicles, as monitored by immunofluorescence of the Rab GTPase, Sec4p (right).

though the Sec6p patch mutant proteins are mislocalized at the nonpermissive conditions, the *sec6* mutant cells appear fully competent in localizing secretory vesicles to sites of polarized secretion. Moreover, consistent with the observation that Sec4p trafficking is normal, we observed no obvious morphological defects in the mutant cells (data not shown).

We next tested the localization of the other exocyst subunits at the nonpermissive conditions to investigate whether mislocalization of Sec6p mutants would also affect exocyst complex localization. On the basis of previous results with *sec6-4* and other exocyst ts mutants (Finger *et al.*, 1998; Roumanie *et al.*, 2005; Zhang *et al.*, 2005b; He *et al.*, 2007), we expected to find mislocalization of most of the subunits due to disruption of complexes at the nonpermissive conditions. The exceptions to this would be subunits proposed to localize and/or stabilize the complex at sites of secretion: Sec3p, Exo70p, and Exo84p. To visualize each of the exocyst subunits individually, we constructed strains in which the genomic copy of one of the exocyst subunits was C-terminally GFP tagged (Huh *et al.*, 2003), and the only copy of *SEC6* was either the wild-type or one of the patch mutants (Table 1). We observed significant mislocalization of all the exocyst subunits at the nonpermissive conditions (75–90%, except Sec3-GFP, which was ~50%; Figure 4, A and B). As a control, we also observed similar mislocalization of each of the exocyst subunits in *sec6-4* cells (data not shown), consistent with previous results (Roumanie *et al.*, 2005). This suggested two possibilities: 1) the exocyst complex was being disassembled and dispersed from sites of secretion; or 2) interactions with a factor(s) at sites of secretion were destabilized, and hence the subunits became mislocalized.

Exocyst Complexes Are Assembled at Nonpermissive Conditions

We reasoned that the conserved patches on Sec6p are important for interactions with other exocyst subunits. If true, mutation of these patches would therefore cause global destabilization and disassembly of the exocyst, resulting in dispersal of the subunits. This phenotype has been previously reported for the *sec6-4* ts allele, as well as for ts alleles

of several other exocyst subunits (TerBush and Novick, 1995). To examine whether the exocyst complex is disassembled at the nonpermissive conditions, we examined the state of the complex by coimmunoprecipitation analyses. For these experiments, we immunoprecipitated the wild-type and mutant Sec6p-HA proteins with α -HA antibodies, and assayed for the presence of the other exocyst subunits by Western blot analyses. Surprisingly, we found that, even after 1 h at nonpermissive conditions, similar amounts of the exocyst subunits were coimmunoprecipitated with wild-type and the Sec6p mutants—indicating that the complexes were still assembled (Figure 5A).

The presence of assembled exocyst complexes at nonpermissive conditions is clearly distinct from similar immunoprecipitation experiments using the *sec6-4* ts strain; the complexes in *sec6-4* cells were completely disassembled and/or degraded at the nonpermissive temperature (TerBush and Novick, 1995). To demonstrate that the exocyst is capable of disassembly under our experimental conditions, we immunoprecipitated wild-type Sec6p-HA in a strain containing the *sec5-24* ts allele as the sole copy of *SEC5*. We used *sec5-24* because degradation of the *sec6-4* protein precluded the use of the Sec6-4-HA for immunoprecipitations. We found, at nonpermissive conditions, nearly complete loss of the other exocyst subunits bound to Sec6p (Figure 5B). Another possibility is that exocyst complexes are in a dynamic equilibrium and can reassemble after lysis during the course of our immunoprecipitation experiments. To exclude this possibility, we performed a lysate mixing experiment using two strains with differently tagged subunits (Figure 5C). The first strain contained *SEC8-GFP* and untagged *SEC6* (Huh *et al.*, 2003); the second had *SEC8-MYC₃* and *SEC6-HA₃*. The strains were lysed, either kept separate or mixed in a 1:1 ratio, and then the Sec6p-HA was immunoprecipitated with α -HA antibodies. The Myc-tagged Sec8p coimmunoprecipitated with Sec6p-HA (from the same strain), but the GFP-tagged Sec8p did not bind Sec6p-HA (from the mixed lysates), indicating that exocyst complexes do not form during the immunoprecipitation experiments. Therefore, we conclude that exocyst complexes remain intact in the patch mutant strains, even at nonpermissive conditions where mislocalization occurs.

Mutant Sec6p Proteins Bind to Known Partners

The finding that the exocyst remains assembled at nonpermissive conditions suggests that the conserved patches are not required for Sec6p to interact with other subunits of the exocyst complex. We have previously shown that the C-terminal domain of Sec6p is sufficient for binding both Exo70p and Sec10p (Sivaram *et al.*, 2006). In contrast to the full-length Sec6p, however, this domain is not sufficient for binding Sec8p and the t-SNARE Sec9p, nor for dimerization (Sivaram *et al.*, 2005, 2006). Here, we examined the interactions between the full-length Sec6p patch mutants and Exo70p, Sec10p, and Sec8p using qualitative pulldown assays and found that the Sec6p mutants bound with affinities similar to that of wild type (Figure 5D). As a negative control, we found that neither the wild-type nor the mutant Sec6p proteins interacted above background with the C-terminal domain of Exo84p (residues 523–753; Dong *et al.*, 2005). Furthermore, as expected from our previous data, gel filtration experiments demonstrated that the mutants were still dimeric and capable of binding Sec9p (data not shown). Together, these binding results corroborate the immunoprecipitation results, and indicate that the binding partner(s) for the conserved patches is not one of the exocyst subunits, nor Sec9p.

Figure 4. Conserved patch residues are required for proper polarization of exocyst subunits. (A) Genomically GFP-tagged exocyst subunits were crossed into strains in which the sole copy of *SEC6* was either wild-type or one of the patch mutants. After 1 h at the nonpermissive condition, all of the GFP-exocyst subunits were mislocalized. (B) The amount of cells with either polarized or mislocalized exocyst subunits were counted, and the percentage of cells with mislocalized exocyst subunits is shown. Cells were considered polarized if a compact area of fluorescence was observed at the bud tip or mother-daughter neck. More than one hundred cells were counted for two independent replicates. Error bars, the range of values for the two measurements.

Figure 5. Exocyst complexes are assembled at nonpermissive conditions. (A) The exocyst subunits coimmunoprecipitate at nonpermissive conditions. After incubation at permissive (25°C) or nonpermissive conditions (37°C), the cells were lysed, and *Sec6-HA*, *sec6-49HA*, or *sec6-54HA* were immunoprecipitated with α -HA antibody. One percent of each input and 10% of each bound sample were loaded on SDS-PAGE gels for analysis. Coimmunoprecipitation of the exocyst was determined by Western blotting for the individual components. No binding of the exocyst subunits to the α -HA beads was observed in the untagged control *SEC6* strain. Samples for each blot were run on the same gel, although some differences in mobility between the input and bound protein lanes were observed. Data are representative of four independent experiments. (B) The exocyst disassembles in a strain containing the *sec5-24* ts allele as the sole copy of *SEC5*. After incubation at permissive (25°C) or nonpermissive conditions (37°C), the cells were lysed, and *Sec6-HA* was immunoprecipitated. One percent of the input and 10% of the bound sample were examined by Western blot analysis as described in A. No binding of the exocyst subunits to the α -HA beads was observed in the untagged control *SEC6* strain. (C) The exocyst does not reassemble after cell lysis. Strains containing either *Sec8-GFP/Sec6p* or *Sec8-Myc/Sec6-HA* were lysed. The lysates were either used individually or mixed, for immunoprecipitation with α -HA antibody. In the mixed lysate, *Sec6-HA* coimmunoprecipitates *Sec8-Myc* (from the same strain), but not *Sec8-GFP* (from the other strain). (D) Binding studies using recombinant *Sec6p*, *Sec6-49*, and *Sec6-54* proteins demonstrate that the *Sec6p* patch mutants bind *Sec8p*, *Sec10p*, and *Exo70p* (residues 63-623). *Exo84p* (residues 523-753) does not bind to *Sec6p* or either of the mutants above background levels. For *Sec10p* and *Sec8p* binding, MBP-tagged wild-type and mutant *Sec6p* proteins were used to bind His₆-tagged wild-type and mutant *Sec6p* proteins was analyzed. (E) 2 μ *SSO1*, *SEC9* and *SNC2* overexpression suppresses the ts *sec6* patch mutants. 10-fold serial dilutions of *SEC6*, *sec6-49*, and *sec6-54* cells containing pRS426 (2 μ *URA3*), *SEC9*-2 μ *URA3*, *SSO1*-2 μ *URA3*, *open1-SSO1*-2 μ *URA3*, or *SNC2*-2 μ *URA3* were plated onto SC-ura and incubated at the indicated temperatures.

To test this possibility further, we examined genetic interactions between the mutants and the other exocyst subunits. We first tested for suppression of the growth phenotypes by overexpression of the other exocyst subunits, as well as *SEC4*, *SEC1*, and *SEC9*, the other plasma membrane t-SNARE, *SSO1*, and the secretory vesicle v-SNARE, *SNC2*, from 2 μ plasmids. We also tested for suppression by overexpression of an "open" mutant of *Sso1p* (Munson and Hughson, 2002). None of the exocyst subunits or *SEC4* or

SEC1 suppressed the *sec6* mutants (data not shown). These results support the idea that the *Sec6p* binding partner(s) is not a subunit of the exocyst complex. In contrast, overexpression of *SEC9*, *SSO1*, and *SNC2* all suppressed the *sec6-49* and *-54* ts phenotypes (Figure 5E). The likely explanation is that loss of assembled exocyst complexes at sites of secretion leads to a decrease in SNARE complex assembly, which can be rescued by overexpression of the plasma membrane SNAREs. We also expected that the open *Sso1p* mutant

Table 2. Summary of synthetic lethal interactions between *sec6* alleles and other exocyst genes

Mutants	<i>sec6-49</i> ^a	<i>sec6-54</i>	<i>sec6-4</i> ^b
<i>sec2-41</i>	–	–	+ / –
<i>sec3-2</i>	–	–	+
<i>sec4-8</i>	–	–	+ / –
<i>sec5-24</i>	+ / –	–	+ / –
<i>sec8-9</i>	–	–	–
<i>sec9-4</i>	+ / –	+ / –	+ / –
<i>sec9-7</i>	–	–	ND
<i>sec10-2</i>	+ / –	–	+ / –
<i>sec15-1</i>	+ / –	–	+ / –
<i>exo70-38</i>	+ / –	–	ND
<i>sso2-1</i>	+	+	ND

^a +, similar growth to single *sec6* allele; + / –, slower growth than single *sec6* allele (or growth at lower temperature); –, double mutant inviable at 25°C.

^b From Finger and Novick (2000) and references therein. ND, not done.

would rescue this defect, because it readily forms SNARE complexes. However, similarly to the synthetic defects observed in combination with other exocyst ts mutants, this mutant failed to suppress the *sec6-49* and *-54* ts phenotypes (Figure 5E), likely because of a detrimental overaccumulation of SNARE complexes (Munson and Hughson, 2002). In contrast, the *sec6-4* allele, in which the exocyst complex is completely disassembled (TerBush and Novick, 1995), has a more severe block in SNARE complex assembly (Grote *et al.*, 2000) that cannot be suppressed by overexpression of *SSO1* and *SEC9* (Aalto *et al.*, 1993). Additionally, unlike the *sec6-4* allele, the *sec6-49* and *-54* mutants were synthetically lethal when crossed with most of the exocyst ts strains (Table 2). The differences in synthetic lethality between our *sec6* mutants and *sec6-4* (Finger and Novick, 2000) may reflect the severity of the ts defects in these cells, especially *sec6-54*, rather than specific functional differences. However, the combination of destabilization of the exocyst by the *sec* ts mutants, together with partial mislocalization of the complex by the *sec6-49* and *-54* mutations at 25°C, could result in more severe defects. Together, the biochemical, phenotypic, and genetic interactions observed for both of the patch mutants indicate that *sec6-49* is very similar, albeit weaker, to the *sec6-54* allele. In support of this idea, we found that combining the *sec6-49* and *-54* mutations together resulted in similar ts and YPD growth defects as the individual mutants (data not shown). The patch residues appear to comprise a single binding site for an as yet unknown factor, which is not an exocyst complex subunit or Sec9p.

DISCUSSION

We have identified a novel class of Sec6p mutants that disrupt the polarized localization of the exocyst complex at nonpermissive conditions, yet do not affect complex assembly. We show that mutation of conserved surface residues on the C-terminal helical bundle domain of Sec6p do not destabilize full-length Sec6p (Figure 1), yet result in severe temperature- and media-sensitive growth defects (Figure 2). At the nonpermissive conditions, vesicle trafficking to sites of secretion appears unimpaired, whereas the Sec6p patch mutants are completely mislocalized (Figure 3). Consequently, all of the other exocyst subunits are also mislocal-

ized (Figure 4). Previous studies of exocyst temperature sensitive mutants showed that this mislocalization is caused by destabilization and disruption of the exocyst complex. However, we show that exocyst complexes remain intact in the *sec6* patch mutant strains, even at the nonpermissive conditions (Figure 5).

Unexpectedly, the extensive exocyst mislocalization observed in our *sec6-49* and *-54* strains at the nonpermissive conditions included Sec3p, which was proposed to be the spatial landmark for exocyst localization (Finger *et al.*, 1998). Sec3p-GFP appeared polarized in yeast cells in the absence of ongoing exocytosis and in other exocyst ts mutants (Finger *et al.*, 1998; Zhang *et al.*, 2005b). However, localization of the endogenous Sec3p by immunofluorescence indicated that the GFP tag may artificially stabilize Sec3p-GFP at sites of secretion (Roumanie *et al.*, 2005), although this was recently disputed (Zhang *et al.*, 2008). Localization of the endogenous Sec3p in *sec6-4* cells (Roumanie *et al.*, 2005) was similar to our results with Sec3-GFP in the *sec6-4* strain. Several variations between our studies and previous results could account for these discrepancies: the use of plasmid-borne, instead of genomic GFP-tagged Sec3p; the testing of Sec3-GFP in the presence of the endogenous Sec3p; differences in strain backgrounds; and the fact that our construct contains a linker sequence (9 a.a.) between the C-terminus of Sec3p and the GFP (Huh *et al.*, 2003). Our data, combined with the observations that Sec3p is not an essential gene and that the other exocyst subunits are polarized in *sec3Δ* cells at 25°C (Wiederkehr *et al.*, 2003; Zhang *et al.*, 2005b), suggest that Sec3p is not the spatial landmark for the exocyst complex.

Other candidates for targeting the exocyst to sites of secretion are Exo70p and Exo84p (Boyd *et al.*, 2004; He *et al.*, 2007). However, we found that Exo70p-GFP was mislocalized in the *sec6-49* and *-54* strains at the nonpermissive conditions (Figure 4). Similarly, although the localization of Exo84-GFP was proposed to be independent of the exocyst subunits (e.g., not mislocalized in previous ts strains; Zhang *et al.*, 2005b), Exo84-GFP was also mislocalized in the *sec6-49* and *-54* strains (Figure 4). Therefore, we conclude that the conserved surface patches on Sec6p are required for localization and/or stability of the exocyst complex at sites of secretion and that Sec3p, Exo70p, and Exo84p are not sufficient to localize the complex in these *sec6* mutant strains. Nevertheless, the presence of a landmark is not strictly necessary, because actin cables are polarized independently of the exocyst, bringing vesicles to the correct sites of exocytosis (Walch-Solimena *et al.*, 1997). Therefore, we propose that exocyst subunits travel to sites of secretion where they assemble together, and that Sec6p is required to anchor the assembled exocyst complexes at these sites. Perhaps Sec6p acts in cooperation with Sec3p, Exo70p, and/or Exo84p for stabilization of the exocyst complexes on the plasma membrane or perhaps their interactions (e.g., with Rho proteins and phosphoinositides) are more regulatory in nature (Roumanie *et al.*, 2005; Wu *et al.*, 2008). It may be that either Exo70p or Sec3p are sufficient to localize the exocyst, when wild-type Sec6p is present to stabilize the complex at sites of secretion (Hutagalung *et al.*, 2008).

The spatial and temporal regulation of exocyst assembly and disassembly is currently unclear. Most of the exocyst subunits appear to traffic to the plasma membrane with secretory vesicles (Boyd *et al.*, 2004), although it is unknown whether they traffic as preassembled complexes or they assemble upon vesicle arrival. Assembly upon arrival at sites of secretion, which was suggested by FRAP data (Boyd *et al.*, 2004), is compelling, because the interactions between

subunits on the vesicle with those on the plasma membrane could tether the membranes together. If so, then the patch residues of wild-type Sec6p would be necessary to anchor the complex at these sites after vesicle arrival. In the case of the patch mutants, Sec4p would need to hydrolyze the bound GTP, perhaps upon exocyst assembly, to release Sec15p and therefore the rest of the exocyst complex. On the other hand, it is possible that the exocyst assembles at sites of vesicle formation and budding; the patch residues of Sec6p would be required for proper trafficking of the complex to sites of secretion. This could only be the case if vesicles are capable of transport in the absence of assembled exocyst complexes, because trafficking of vesicles containing Sec4p is unimpaired in the patch mutant strains (as well as in the *sec6-4* and other exocytic *sec* mutants). This situation would require that Sec4p on the budding vesicle hydrolyze the bound GTP to release the complex. Further mutational and GFP localization studies will be necessary to distinguish between these scenarios.

Regardless of where assembly takes place, the finding that the complex remains assembled despite a block in exocytosis and in the absence of polarized accumulation suggests two possibilities. First, the complex cannot disassemble, because disassembly is triggered by a factor(s) at sites of secretion or by membrane fusion. If the complex is locked into an assembled conformation in the *sec6-49* and *-54* mutants, then it may be unable to be recycled for subsequent rounds of vesicle tethering and fusion, thus contributing to the ts phenotype. Alternatively, the complex is assembled in these mutants because it normally does not disassemble unless destabilized. In this case, mislocalization of the assembled exocyst complexes at nonpermissive conditions would lower the concentration of active exocyst complexes that are available to function at sites of secretion. This would ultimately result in decreased SNARE complex assembly and membrane fusion.

Conservation of these Sec6p patch residues suggests an important anchoring function for Sec6p in all eukaryotes. Our analyses indicate that the binding partner for the patches on Sec6p is not a subunit of the exocyst complex, nor is it the t-SNARE Sec9p (Figure 5). Therefore, further genetic and biochemical experiments will be necessary to identify the anchoring factor and analyze its role in polarizing Sec6p and the exocyst complex at sites of secretion.

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