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The Critical Role of Exo84p in the Organization and Polarized Localization of the Exocyst Complex*[S]

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The exocyst complex plays an essential role in tethering secretory vesicles to specific domains of the plasma membrane for exocytosis. However, how the exocyst complex is assembled and targeted to sites of secretion is unclear. Here, we have investigated the role of the exocyst component Exo84p in these processes. We have generated an array of temperature-sensitive yeast *exo84* mutants. Electron microscopy and cargo protein traffic analyses of these mutants indicated that Exo84p is specifically involved in the post-Golgi stage of secretion. Using various yeast mutants, we systematically studied the localization of Exo84p and other exocyst proteins by fluorescence microscopy. We found that pre-Golgi traffic and polarized actin organization are required for Exo84p localization. However, none of the exocyst proteins controls Exo84p polarization. In addition, Sec3p is not responsible for the polarization of Exo84p or any other exocyst component to the daughter cell. On the other hand, several exocyst members, including Sec10p, Sec15p, and Exo70p, clearly require Exo84p for their polarization. Biochemical analyses of the exocyst composition indicated that the assembly of Sec10p, Sec15p, and Exo70p with the rest of the complex requires Exo84p. We propose that there are at least two distinct regulatory mechanisms for exocyst polarization, one for Sec3p and one for the other members, including Exo84p. Exo84p plays a critical role in both the assembly of the exocyst and its targeting to sites of secretion.

Polarized exocytosis is essential for cell growth and morphogenesis and has been implicated in a wide range of physiological functions such as neurotransmission and embryogenesis. Studies using different model systems and cell types have begun to reveal evolutionarily conserved mechanisms underlying polarized exocytosis (1–3). The exocyst is an evolutionarily conserved multiprotein complex that plays an essential role in tethering post-Golgi secretory vesicles to specific domains of the plasma membrane for exocytosis (4–6). The exocyst was first identified in the budding yeast *Saccharomyces cerevisiae* (7). It contains Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (8–10), all of which are specifically localized to regions of active exocytosis and cell-surface expansion: the sites of bud emergence, the tips of small daughter cells, and the mother-daughter junction of the dividing cells (8, 11–13). This pattern of localization is in contrast to that of the yeast membrane fusion machinery, the t-SNAREs[1] (14), which are evenly distributed along the entire yeast plasma membrane (15, 16). Genetic and cell biological analyses indicate that the exocyst functions downstream of the Rab GTPase Sec4p and upstream of the SNAREs (12, 15, 17, 18). The exocyst component Sec15p can associate with secretory vesicles and interact with the GTP-bound form of Sec4p (12). Sec4p is required for the efficient assembly of the exocyst complex and the correct targeting of Sec15p to sites of active exocytosis at the plasma membrane (12, 19).

Although all of the exocyst components appear to have the same pattern of cellular localization, the dynamics and regulatory mechanisms for their bud tip localization seem to be very different (11, 13, 19, 20). The polarization of Sec3p is independent of the secretory pathway and actin cytoskeleton (11, 19, 20). It was therefore proposed that Sec3p is the exocyst component most proximal to the plasma membrane and may serve as a spatial landmark defining the sites of secretion (11, 21). In contrast to Sec3p, several other components, including Sec8p and Sec15p, were shown to rely on polarized actin cables for their targeting to the bud tip marked by Sec3p (11, 19, 20, 22, 23).

To understand the role of the exocyst complex in vesicle tethering, we first need to investigate how the exocyst components are assembled and how they are targeted to the sites of secretion. Here, we characterized the exocyst component Exo84p using an array of temperature-sensitive *exo84* mutants. Electron microscopy and enzyme secretion assays of these mutants indicated that Exo84p is specifically involved in the post-Golgi stage of exocytosis. Systematic analyses of the localization of Exo84p and the other members of the exocyst in various yeast mutants revealed distinctive regulatory mechanisms for the targeting of individual exocyst components to sites of secretion. Combining the results from fluorescence imaging and biochemical analyses, we conclude that Exo84p plays a critical role in both the assembly of the exocyst and the targeting of this complex to specific sites of the plasma membrane for exocytosis.

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Fig. S1 and Table S1.

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[1] The abbreviations used are: SNAREs, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors; SC, synthetic complete; CPY, carboxypeptidase Y; GFP, green fluorescent protein; ER, endoplasmic reticulum.

TABLE I
Yeast strains used in this study

ABY strains were from the laboratory of A. Bretscher; BHY strains were from the laboratory of B. K. Haarer; DTY strains were from the laboratory of D. TerBush; and NY strains were from the laboratory of P. Novick.

Strains	Genotype
NY20	MAT**a** *his4-619 ,ura3-52, sec6-4*
NY179	*MAT***a** *ura3-52, leu2-3,112*
NY2449	*MATα ura3-52, leu2-3,112, his3Δ300*
DTY595	*MATα ura3-52, leu2-3,112, his3Δ200, trp1-1, exo70Δ::HIS3, GAL+ (EXO70, TRP, CEN), LA+*
NY27	*MATα ura3-52, sec2-59*
BHY43	*MAT***a** *ura3-52, leu2-3, his3Δ200, sec3-101*
NY772	*MAT***a** *ura3-52, leu2-3,112, sec3-2*
NY2450	*MAT***a** *ura3-52, leu2-3,112, his3Δ300, sec3ΔkanMX*
NY774	*MATα ura3-52, leu2-3,112, sec4-8*
NY776	*MATα ura3-52, leu2-3,112, sec5-24*
NY778	*MATα ura3-52, leu2-3,112, sec6-4*
NY760	*MATα ura3-52, leu2-3,112, sec7-1*
NY780	*MATα ura3-52, leu2-3,112, sec8-9*
NY784	*MAT***a** *ura3-52, leu2-3,112, sec10-2*
NY786	*MATα ura3-52, leu2-3,112, sec15-1*
NY1218	*MATα ura3-52, leu2-3,112, sec18-1*
NY1213	*MATα ura3-52, leu2-3,112, sec19-1*
NY1426	*MATα ura3-52, leu2-3,112, sec22-3*
NY1490	*MAT***a** *leu2-3,112, ura3-52, trp1, his3Δ200, LA-*
DTY622	*MATα ura3-52, leu2-3,112, his3Δ200, trp1, exo70Δ::HIS, GAL+ (exo70-38, TRP, CEN), LA+*
ABY973	*MAT***a**/*α tpm2Δ::HIS3/tpm2Δ::HIS3, his3Δ-200/his3Δ-200, leu2-3,112/leu2-3, 112, lys2-801/lys2-801, trp1-1/trp1-1, ura3-52/ura3-52*
ABY971	*MAT***a**/*α tpm1-2::LEU2/tpm1-2::LEU2, tpm2Δ::HIS3/tpm2Δ::HIS3, his3Δ200/his3Δ-200, leu2-3,112/leu2-3, 112, lys2-801/lys2-801, trp1-1/trp1-1, ura3-52/ura3-52*
GY1231	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (exo84-102, LEU2, CEN, pRS315)*
GY1232	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (exo84-110, LEU2, CEN, pRS315)*
GY1233	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (exo84-112, LEU2, CEN, pRS315)*
GY1234	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (exo84-113, LEU2, CEN, pRS315)*
GY1235	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (exo84-117, LEU2, CEN, pRS315)*
GY1236	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (exo84-121, LEU2, CEN, pRS315)*
GY1237	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (exo84-122, LEU2, CEN, pRS315)*
GY1238	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (exo84-125, LEU2, CEN, pRS315)*
GY1264	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (TEF-Exo84, URA3, CEN)*
GY1265	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84Δ::HIS3 (EXO84, LEU2, CEN, pRS315)*
GY2478	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84-112*
GY2479	*MAT***a** *ura3-52, leu2-3,112, his3Δ200, trp1, LA-, exo84-121*

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—Standard methods were used for yeast media, growth, and genetic manipulations (24). The major yeast strains used in the study are listed in Table I.

Generation of exo84 Mutants—Plasmid *pRS315*-PTExo84, containing the 303-bp promoter and the 343-bp terminator sequence of *EXO84*, was constructed for "gap repair" of the *EXO84* gene from yeast. The resulting plasmid is *pRS315-Exo84* (*CEN*, *LEU2*). Plasmid *pRS315-HIS3*, containing the original promoter and terminator of *EXO84* but with the *EXO84* open reading frame sequence replaced by *HIS3*, was used for disruption of the *EXO84* gene from the yeast chromosome. To generate *exo84* mutants, mutagenesis was performed by "error-prone" PCR using *pRS315*-Exo84 as a template (25). The PCR products containing random mutations in the *EXO84* gene were mixed with equal amounts of *pRS315*-PTExo84 linearized at the promoter-terminator junction. This mixture of DNA was used transformation into the yeast strain GY1264 (*exo84Δ::HIS3*, p416TEF-Exo84, *CEN*, *URA*) to allow homologous recombination between the PCR products and the linearized plasmid. Transformants were selected on Leu/His-deficient synthetic complete (SC) medium plates at 25 °C and then replicated onto Leu/His-deficient SC medium plates containing 1 mg/ml 5-fluoroorotic acid (Zymo Research Corp.) to select for yeast cells that lost the balancer plasmid p416TEF-Exo84. The selected colonies were replicated onto two sets of Leu/His-deficient SC medium plates and incubated at 25 and 37 °C, respectively, for identification of mutants that could not survive at 37 °C. Candidate mutants were confirmed by retransformation of isolated plasmids containing mutant *exo84* back into the host strain. For *exo84-112* and *exo84-121*, we integrated the mutant DNA into the *EXO84* locus in the chromosome. Briefly, plasmids containing the open reading frames of *exo84-112* and *exo84-121* flanked by the *EXO84* promoter and terminator were constructed, and the sequences containing the *exo84* mutants were isolated by enzymatic digestion. These linearized DNA fragments were integrated into a yeast strain (GY1264) in which the *EXO84* locus was disrupted by *HIS3* and supplemented by a *URA3 CEN EXO84* plasmid. Transformants were replicated onto SC medium plates containing 1 mg/ml 5-fluoroorotic acid to select for the loss of the *CEN URA3 EXO84* plasmid. The correctly integrated transformants from surviving colonies were identified by PCR and further confirmed by retesting their temperature sensitivity.

Invertase Assays—Measurement of internal and external invertase activities was performed on wild-type and *exo84* mutant cells as described previously (26). Cells were grown overnight at 25 °C in yeast extract peptone glucose medium to early log phase, and 4.0 A_{600} units of cells were collected. For each sample, 1 unit of cells was immediately pelleted, resuspended in 1 ml of ice-cold 10 mM NaN_3, and stored on ice as a 0-min control. The remaining samples were incubated in yeast extract peptone plus 0.1% glucose for 1 h at 37 °C for invertase induction. Each experiment was performed in triplicate. Internal and external invertase activities were measured at the beginning and end of the shift. The percentage of total invertase secretion was calculated as Δexternal/(Δexternal + Δinternal).

Media Secretion Assay—For collection and staining of media glycoproteins, wild-type and *exo84* mutant cells were grown overnight at 25 °C from stationary cell culture and harvested before the A_{600} reached 1.0. About 24 A_{600} units of cells were resuspended in 40 ml of prewarmed medium and incubated in a 37 °C shaker for 1 h. The supernatant was centrifuged twice, and 30 ml of the supernatant were transferred to a new tube containing 120 μl of 2% (w/v) sodium deoxycholate and incubated for 30 min on ice. Samples were precipitated with 1.9 ml of 100% (w/v) trichloroacetic acid, followed by a 1-h incubation on ice. The mixtures were spun down at 3600 rpm for 30 min with brake off to avoid disturbing the pellet. The pellets and 1 ml of the remaining supernatant were transferred to new 2-ml screw cap tubes and spun at high speed for 10 min to thoroughly pellet the precipitated protein. The trichloroacetic acid-precipitated proteins were then washed twice with acetone, air-dried, and dissolved into 34 μl of loading buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% (v/v) glycerol, and 5% 2-mercaptoethanol) for SDS-PAGE. The gel was first fixed in 10% acetic acid and 40% ethanol for 30 min and incubated in 7.5% acetic acid for 10 min and then in 1% periodic acid for 15 min. After six washes with distilled water, the gel was incubated in Schiff reagent (27) for 15 min. The gel was washed with water briefly and then three times for 10 min with freshly pre-

pared 0.5% sodium metabisulfate. Finally, the gel was washed with water to remove excess staining. The profile of the glycoproteins upon SDS-PAGE was similar to that obtained by the method using metabolically labeled cells (28) (data not shown).

Carboxypeptidase Y (CPY) Assay—To analyze the transport and maturation of CPY (29), yeast cells were grown at 25 °C in synthetic medium supplemented with amino acids to an A_{600} of 0.5–1.0. Cells were harvested, resuspended at a concentration of 5 A_{600} units/ml in SC medium, and shifted to the experimental temperature of 25 or 37 °C for 30 min. Cultures were preincubated at the appropriate experimental temperature for 5 min and then labeled with 100 μCi of [^{35}S]methionine/cell suspension. After labeling, cultures were chased by the addition of 5 mM methionine, 1 mM cysteine, and 0.2% yeast extract. After appropriate chase periods, samples were harvested and precipitated by the addition of trichloroacetic acid to 10% final concentration. Whole cell lysates were generated by glass bead disruption in buffer containing 50 mM Tris (pH 7.5), 1 mM EDTA, and 1% SDS, followed by boiling at 95 °C for 3 min. 1 ml of buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, and 0.5% Tween 20 was added to the heated samples for immunoprecipitation with anti-CPY antibody. Immunoprecipitated proteins were separated by SDS-PAGE and analyzed by autoradiography.

Electron Microscopy—Cells were grown to early log phase and shifted to 25 or 37 °C for 1 h. The cells were then collected by vacuum filtration using a 0.45-μm nitrocellulose membrane and fixed for 1 h at room temperature in 0.1 M cacodylate buffer (pH 7.4) containing 3% formaldehyde, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. The cells were processed further as described by Mulholland *et al.* (30). After dehydration and embedding in Spurr (Polysciences, Inc.), thin sections were cut out, transferred onto 600-mesh uncoated copper grids (Ernest F. Fullam, Inc.), and stained with uranyl acetate and lead citrate. Cells were observed on a JEOL 1010 transmission electron microscope.

Fluorescence Microscopy—Chromosomal tagging of the exocyst by green fluorescent protein (GFP) was performed as described previously (9, 11, 19). GFP tagging of the yeast exocyst components does not affect the functions of these proteins, as there is no difference in cell growth under all of the conditions tested when the GFP-tagged version is the sole copy of the exocyst in the genome (9, 11, 13, 31). For microscopy examination, cells were grown to early log phase (A_{600} = 0.6) in uracil-deficient SC medium containing 2% dextrose and fixed as described previously (19). GFP was scored as mislocalized when it appeared diffused or in multiple patches in the mother cells. Protein localization in yeast cells released from G_0 phase was examined as described previously (22). Immunostaining of endogenous Sec4p in yeast cells was performed as described previously (32). Anti-Sec4p polyclonal antibody was used at 1:1000 dilution.

Isolation of the Exocyst Complex—Immunoisolation of the exocyst complex from [^{35}S]cysteine/methionine-labeled yeast cells was carried out as described previously (8). The proteins were separated by 4–12% SDS-PAGE and detected by autoradiography.

RESULTS

exo84 Mutants Are Specifically Defective in Post-Golgi Secretion—EXO84 is an essential gene that encodes a protein that migrates at 90 kDa upon SDS-PAGE (9). To facilitate our study of Exo84p and the exocyst complex by genetic and biochemical methods, we carried out experiments to generate temperature-sensitive yeast strains carrying mutations in the *EXO84* gene. Eight temperature-sensitive *exo84* mutants were obtained by PCR-based random mutagenesis of the full-length *EXO84* open reading frame as described under "Materials and Methods." Sequence analyses indicated that most of the mutations are positioned at the C terminus of Exo84p (data not shown). We reintroduced the isolated plasmids bearing the mutated *exo84* DNA into a host strain in which the *EXO84* gene was deleted and replaced with *HIS3*. These strains are viable at 25 °C but not at 37 °C, indicating that these temperature-sensitive mutants were indeed caused by mutations in *EXO84*. These temperature-sensitive *exo84* mutants are subsequently referred to as *exo84-102*, *exo84-110*, *exo84-112*, *exo84-113*, *exo84-117*, *exo84-121*, *exo84-122*, and *exo84-125*.

We first examined the secretory properties of the *exo84* mutants using the invertase assay. Invertase follows the secretory pathway and is secreted to the periplasmic space. It has been

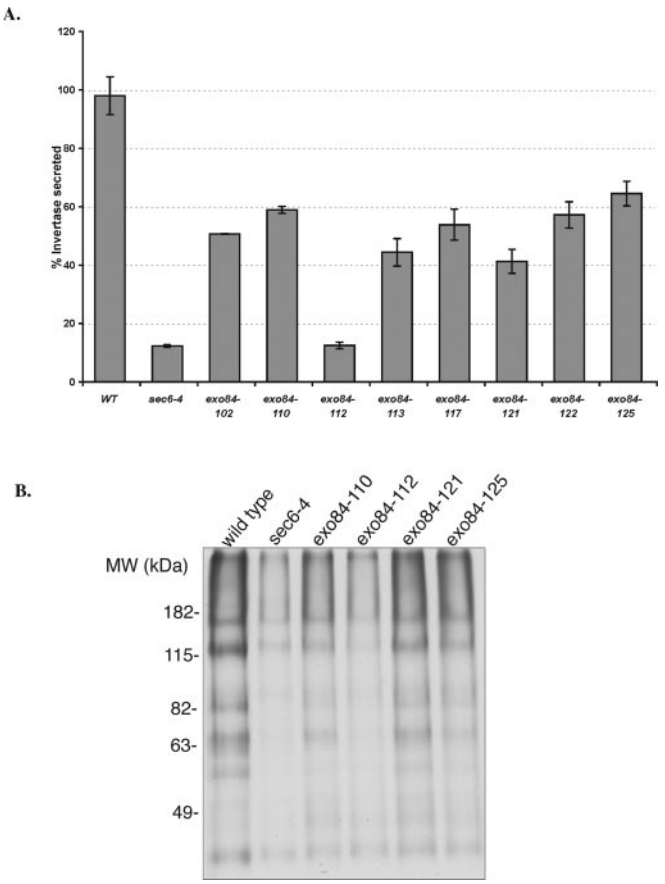

FIG. 1. **Secretion defects in temperature-sensitive *exo84* mutants.** *A*, *exo84* mutants are defective in invertase secretion. Different alleles of *exo84* mutants were tested for their invertase secretion after shifting to the restrictive temperature of 37 °C for 1 h. The wild-type (*WT*) and *sec6-4* mutant strains were used as controls in the assay. Different alleles of *exo84* mutants showed different degrees of secretion defects. *B*, *exo84* mutants are defective in media protein secretion. Different alleles of *exo84* mutants were tested for secretion of glycoproteins into the medium after shifting to the restrictive temperature of 37 °C for 1 h. The wild-type and *sec6-4* mutant strains were used as controls in the assay. Different alleles of *exo84* mutants showed different degrees of secretion defects.

used as an enzymatic marker for exocytosis (26). Cells were shifted to low glucose medium to induce the expression of invertase and grown at the restrictive temperature of 37 °C for 1 h. We found that all of the mutant strains showed different degrees of invertase secretion defects (Fig. 1*A*). In particular, the *exo84-112* strain secreted only 10% of the total invertase produced into the medium. The wild-type strain (used as a control) secreted 98% of the total invertase produced.

A previous study suggests that there are several classes of vesicles designated to the plasma membrane for exocytosis (33). To examine the general secretion profile of these *exo84* mutants, we adapted a method to stain the glycoproteins secreted into the medium by the yeast cells (27). Wild-type and *exo84-110*, *exo84-112*, *exo84-121*, and *exo84-125* cells were shifted from 25 to 37 °C for 1 h. Glycoproteins secreted into the medium were collected by trichloroacetic acid precipitation and subjected to SDS-PAGE. The resulting gel was stained with periodic acid-Schiff, which recognized all of the glycoproteins. The intensity of the stained proteins reflects the amounts of glycoproteins secreted into the medium. Wild-type and *sec6-4* (a known late *sec* mutant) cells were used as controls. As shown in Fig. 1*B*, *sec6-4* had very faint staining compared with the wild-type strain, as expected. *exo84-112* and *sec6-4* had similar staining patterns. The other three *exo84* mutants had stronger

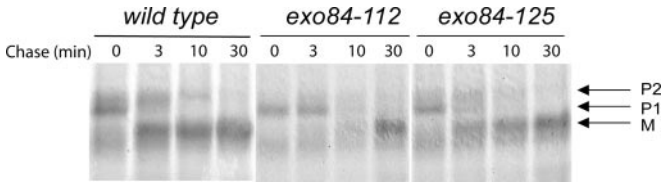

FIG. 2. **CPY traffic is not affected in *exo84* mutants.** CPY processing was tested in *exo84-112* and *exo84-125* mutants at 37 °C for 30 min by ^{35}S-PRO-MIX pulse-chase and immunoprecipitation. *P1*, the ER form of CPY; *P2*, the Golgi form of CPY; *M*, the mature vacuolar form of CPY.

staining compared with *exo84-112*, although they were still much weaker compared with the wild-type strain. Combining the results from the invertase secretion and media protein secretion assays, we conclude that Exo84p plays an important role in the yeast secretory pathway.

To determine whether other stages of the secretory pathway are blocked in the *exo84* mutant cells, we examined the processing of CPY in wild-type and mutant strains. CPY is a vacuolar enzyme that is transported from the late Golgi compartment to vacuoles, where it is cleaved into its mature form (34). Because it is not secreted to the plasma membrane, post-Golgi secretory mutants do not affect CPY traffic. Cells were labeled with [^{35}S]methionine and chased with fresh medium at 25 or 37 °C for 30 min. Whole cell lysates were prepared from wild-type, *exo84-112*, and *exo84-125* strains and immunoprecipitated by anti-CPY antibody. The immunoprecipitated CPY was subjected to SDS-PAGE and autoradiography. The results demonstrate that the *exo84* mutant was able to process CPY efficiently at both the endoplasmic reticulum (ER)-to-Golgi stage (Fig. 2, *P2*) and the Golgi-to-vacuole stage (*M*) at 37 °C. Therefore, the secretion defect observed in the *exo84* mutants is specific to the Golgi-plasma membrane stage.

To visualize the secretion defects in the *exo84* mutants, we used electron microscopy to examine the internal membrane structures in the *exo84* mutant cells. We chose the *exo84-112* (GY2478) and *exo84-121* (GY2479) mutant strains for this study because they have different degrees of media glycoprotein secretion defects. Cells were grown at 25 °C, and half of the cells were then shifted to 37 °C for 1 h. The cells were processed for thin section electron microscopy. As shown in Fig. 3*A*, shifting to the restrictive temperature of 37 °C for 1 h led to accumulation of a large number of vesicles in both *exo84-112* (at 25 °C, 13 ± 10/section, $n = 7$; and at 37 °C, 145 ± 43/section, $n = 9$) and *exo84-121* (at 25 °C, 3 ± 2/section, $n = 9$; and at 37 °C, 72 ± 15/section, $n = 12$). More vesicles accumulated in *exo84-112* than in *exo84-121*, consistent with the invertase assay and media glycoprotein secretion assay results (Fig. 1). The vesicles were 80–100 nm in diameter, typical of post-Golgi stage secretory vesicles (26). In addition, we did not observe any defects in other intracellular organelles. The vesicles seemed to be preferentially distributed to the bud and mother-daughter junction regions. However, the thin sectioning of the samples in the electron microscopy procedure made it difficult to draw definitive conclusion. We therefore examined the localization of Sec4p (which resides on the post-Golgi vesicles) in the mutants by immunofluorescence. As shown in Fig. 3*B*, although not as concentrated as the signal in the wild-type cells, the Sec4p signal generally remained polarized. This result suggests that vesicles can be transported along actin cables in a polarized fashion to the buds in *exo84* mutants. However, these vesicles cannot efficiently fuse with the plasma membrane as a consequence of the loss of Exo84p function. Similar observations have been reported for the other exocyst mutants (32).

Exo84p Polarization Requires Actin Cables—Polarized actin cables are necessary for the delivery of secretory vesicles to the

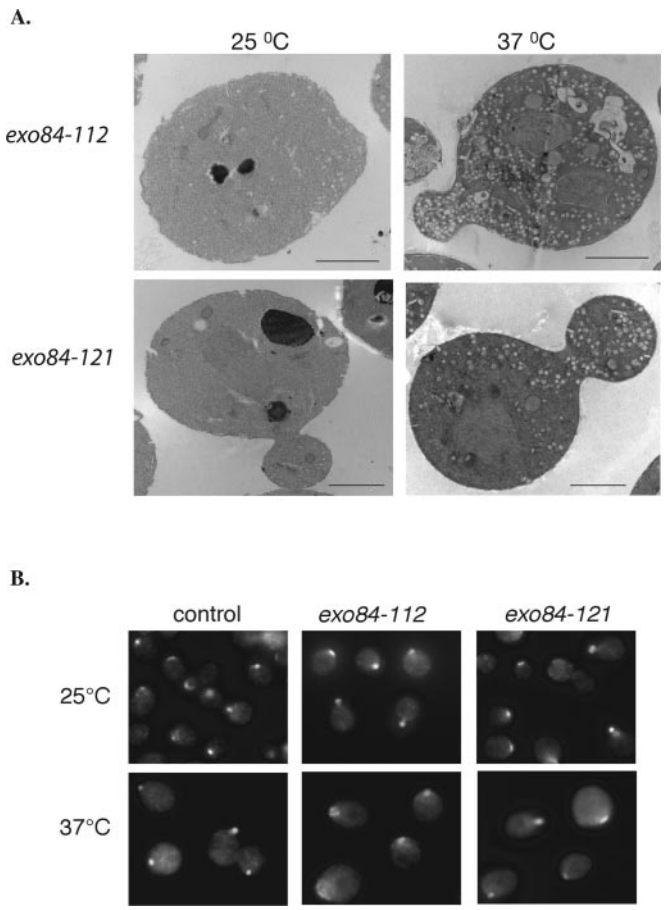

FIG. 3. ***exo84* mutants accumulate secretory vesicles at the restrictive temperature.** *A*, *exo84-112* (*upper panels*) and *exo84-121* (*lower panels*) mutant cells were grown at 25 °C (*left panels*) or shifted to 37 °C (*right panels*) for 1 h and then processed for electron microscopy. *Scale bars* = 1 μm. *B*, Sec4p remained polarized in the *exo84-112* and *exo84-121* mutants as revealed by immunofluorescence.

plasma membrane. To determine whether Exo84p polarization requires actin cables, we expressed Exo84-GFP in *tpm1-2 tpm2*Δ cells, in which actin cables are rapidly disassembled when shifted to 34.5 °C because of mutations in the tropomyosin gene (23). The *tpm1-2 tpm2*Δ and *TPM1 tpm2*Δ (control strain) cells expressing Exo84-GFP were grown to log phase and then shifted to 34.5 °C for periods of 10 and 90 min. As shown in Fig. 4, the control strain had clear polarization of Exo84-GFP to the tips of small buds at both 25 and 34.5 °C. The *tpm1-2 tpm2*Δ cells could also polarize Exo84-GFP to bud tips at 25 °C. However, after 10 min at 34.5 °C, the number of cells with polarized Exo84-GFP decreased. After 90 min, Exo84-GFP was almost completely depolarized. We further studied the role of actin cables in the initial targeting of Exo84p to the bud tip by monitoring a homogeneous population of unbudded cells released from G_0 phase. We found that the control strain formed buds with polarized Exo84-GFP at both 25 and 34.5 °C. The *tpm1-2 tpm2*Δ cells could also polarize Exo84-GFP to bud tips when they were released from G_0 phase at 25 °C. However, Exo84-GFP could not polarize in *tpm* mutant cells released at 34.5 °C. Overall, the results indicate that Exo84-GFP is dependent on actin cables for its initial targeting to and maintenance at the bud tip.

Exo84p Polarization Requires an Intact Early Secretory Pathway but Is Independent of the Other Exocyst Members—It was previously shown that Sec3p localization to the bud tip is independent of the functional secretory pathway (11). Here, we examined the polarization of Exo84p in mutants defective in

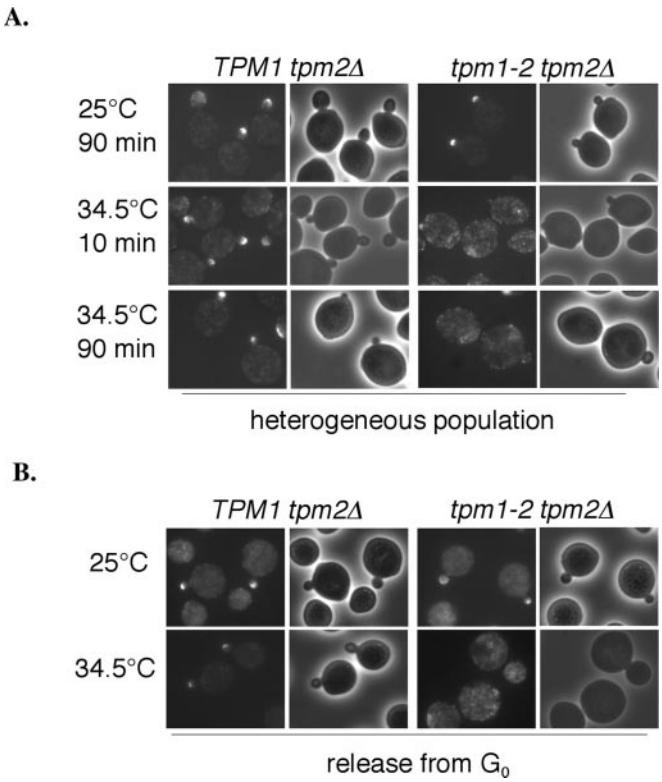

FIG. 4. **Exo84p is mislocalized in the tropomyosin mutant *tpm1-2 tpm2Δ*.** *A*, Exo84-GFP was mislocalized in the *tpm1-2 tpm2Δ* mutants shifted to 34.5 °C for 10 and 90 min. *B*, the initial targeting of Exo84-GFP was blocked in the *tpm1-2 tpm2Δ* mutant. Cells were arrested at G_0 phase and then released in fresh medium for 90 min at 25 or 34.5 °C. Cells were fixed in methanol/acetone for microscopy.

various stages of membrane traffic. We expressed Exo84-GFP in *sec18-1* and *sec22-3*, which affect ER-to-Golgi transport; *sec19-1*, which is defective in recycling of all of the Rab GTPases from the membranes and which affects primarily ER-to-Golgi transport; and *sec7-1*, which affects budding of vesicles from the Golgi compartment. *sec18-1*, *sec22-3*, and *sec19-1* have a growth defect at 34 °C, whereas *sec7-1* is defective at 37 °C. As shown in Fig. 5, all of the yeast strains had a bright crescent of Exo84-GFP localized to the tips of small buds at 25 °C. The wild-type strain maintained this clear polarization to the tips of the buds after shifting to 34 or 37 °C. In contrast, the temperature-sensitive mutants all lost the ability to polarize Exo84-GFP to the bud tip at the restrictive temperatures. This result suggests that Exo84p cannot be polarized in mutants that affect the steps before the generation of post-Golgi vesicles. Next, we investigated Exo84p polarization in the *sec2-59* mutant, which is defective in the guanine exchange factor Sec2p. Post-Golgi vesicles can be generated in the *sec2-59* mutant, but they accumulate randomly in the cells (32). As shown in Fig. 5, Exo84p was mislocalized.

We also examined Exo84-GFP localization in exocyst mutants to determine whether the proper function of a member(s) of the exocyst is necessary for Exo84p polarization. Exo84-GFP was expressed under the control of its endogenous promoter in *sec5-24*, *sec6-4*, *sec8-9*, *sec10-2*, *sec15-1*, *exo70-38*, and several *sec3* mutants. Cells were grown to log phase in SC medium and then shifted to their restrictive temperatures for periods of 10 min (data not shown) and 90 min (*sec5-24*, *sec6-4*, *sec10-2*, and *sec15-1* to 34 °C and *sec8-9*, *exo70-38*, and *exo84-117* to 37 °C). As shown in Fig. 5, Exo84-GFP was well polarized in all of these mutants at 25 °C. After shifting to the restrictive temperatures for 90 min, *sec5-24*, *sec6-4*, *sec8-9*, *sec10-2*, and *sec15-1* maintained a bright patch of Exo84-GFP in the bud comparable with the localization pattern in the wild-type cells. We have previously shown that Exo84-GFP is affected in the *sec5-24* mutant at 37 °C (9). We found that *sec5-24* could not survive even at 34 °C (Supplemental Fig. S1*A*). The invertase secretion assay indicated that this mutant allele has severe secretion defects at 34 °C comparable with those at 37 °C (Supplemental Fig. S1*B*). We examined the localization of Exo84-GFP in the *sec5-24* strain at 34 °C and found that Exo84-GFP was clearly polarized. The mutant that slightly affected Exo84p localization was *exo70-38*. Even though Exo84-GFP remained polarized to the bud, its localization within the bud was affected. In contrast to the other strains, in which Exo84-GFP was confined as a "tight" dot in the emerging bud, Exo84-GFP in *exo70-38* was slightly diffused in the area of bud emergence. In the small bud stage, instead of having a crescent shape at the bud tip, the Exo84-GFP signal filled the entire bud, and some of it projected into the mother cell. Overall, we found that Exo84p remained polarized in mutants defective in all of the other seven exocyst components. It has been shown that post-Golgi secretory vesicles accumulate preferentially in the daughter cells in these mutants (26, 32). The failure of the vesicles to be processed for subsequent fusion does not prevent the polarization of Exo84p to the daughter cells.

Polarization of Exo84p to the Bud Tip Is Independent of Sec3p—Sec3p was found to be independent of actin and other exocyst components for its polarization and was therefore proposed to be a spatial landmark defining sites of secretion (11, 21). Here, we examined the polarization of Exo84-GFP in two *sec3* mutants as well as in a strain lacking Sec3p (*sec3Δ*).

The *sec3-2* strain harbors mutations in *SEC3* that lead to secretion defects at or above 37 °C (21, 26). We found that Exo84-GFP was polarized in *sec3-2* at both 25 and 37 °C (Fig. 6). To further determine whether the physical association of Sec3p with the exocyst is required for recruiting Exo84p, we examined the localization of Exo84-GFP in the *sec3-101* mutant strain. The *sec3-101* mutant lacks its C terminus (35), which is responsible for interacting with the other exocyst components (13). As shown in Fig. 6, Exo84p remained polarized in *sec3-101*. To further confirm this conclusion, we also tested the localization of Exo84p in the *sec3Δ* strain. The *sec3Δ* strain is viable at 25 °C in SC medium but is inviable or grows extremely slowly at 34 and 37 °C (36). The *sec3Δ* cells had rounder buds than wild-type cells even at 25 °C, but Exo84-GFP was still polarized to the tips of the buds. Although Sec3p is not involved in the targeting of Exo84p to the bud, it may be involved in "fine-tuning" Exo84p localization to the bud tip at elevated temperatures. As shown in Fig. 6, at 34 °C, the Exo84-GFP signal became less concentrated in the buds in *sec3Δ* cells. At 37 °C, Exo84-GFP was completely mislocalized. A summary of the localization of Exo84p in various mutants is presented in Table II.

Similar to Exo84p, a previous study also demonstrated a similar localization pattern for Sec8p in *sec3Δ* cells (36). We therefore examined the localization of all of the other exocyst proteins in the *sec3Δ* strain. We found that all of these exocyst components were, like Exo84p, polarized in the *sec3Δ* cells at 25 °C (data not shown).

Exo84p Is Required for Polarized Localization of the Exocyst Components—We have shown that Exo84p is independent of the other exocyst members for its polarization to the bud tip. Next, we examined whether Exo84p is required for the polarization of the other exocyst members. We choose *exo84-117* and *exo84-121* for the analysis, as they are tight alleles that show secretion defects only after shifting to 37 °C. The most defective allele, *exo84-112*, was not chosen because it is slow in growth even at 25 °C and is severely defective and even dead at 37 °C.

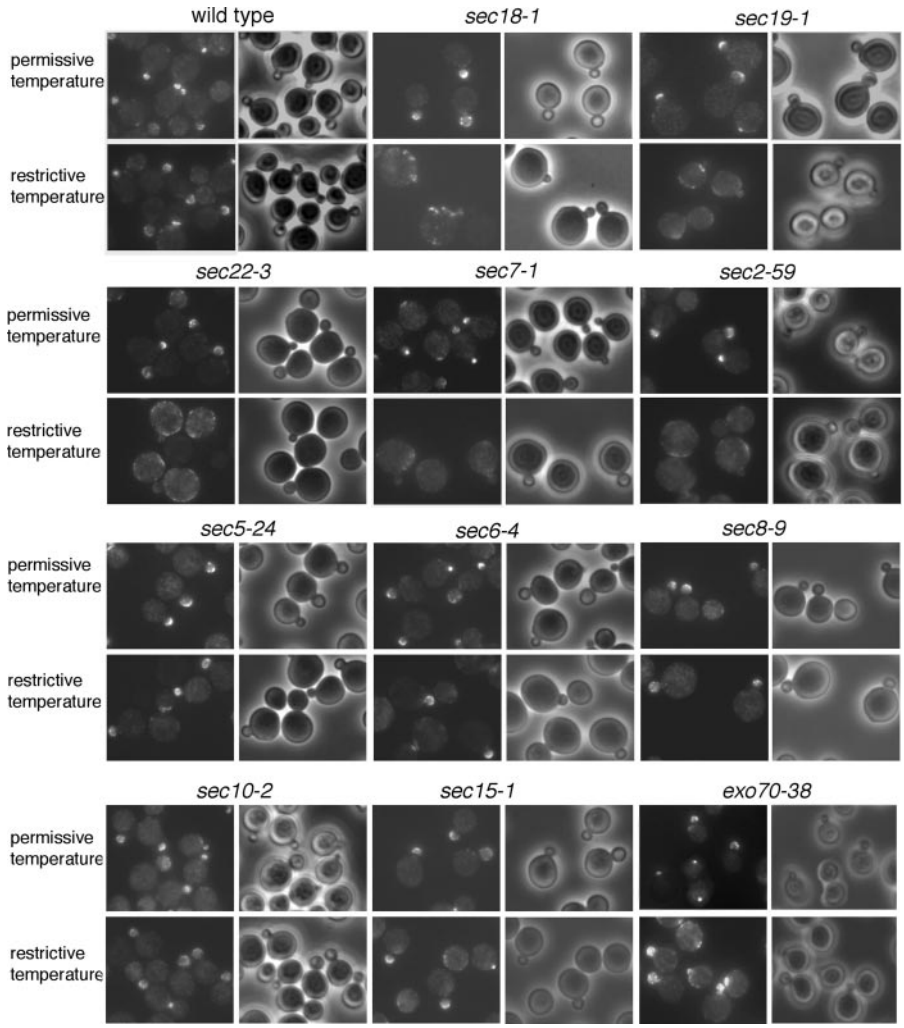

FIG. 5. **Polarization of Exo84p is dependent on the upstream secretory pathway but not on other members of the exocyst.** Exo84-GFP was expressed in the wild-type cells; the *sec18-1* (defective in SNARE disassembly and ER-to-Golgi traffic), *sec19-1* (defective in guanine nucleotide dissocaiation inhibitor protein and multiple traffic stages), *sec22-3* (defective in ER-to-Golgi traffic), and *sec7-1* (defective in Golgi traffic) mutant cells *sec2-59* (defective in activating Sec4p); and all of the exocyst mutant cells, including *sec3-2* (shown in Fig. 6), *sec5-24*, *sec6-4*, *sec8-9*, *sec10-2*, *sec15-1*, and *exo70-38*. The cells were grown to log phase at 25 °C and then shifted to their restrictive temperatures (37 °C for *sec7-1 and exo70-38* and 34 °C for the rest) for 90 min before microscopy.

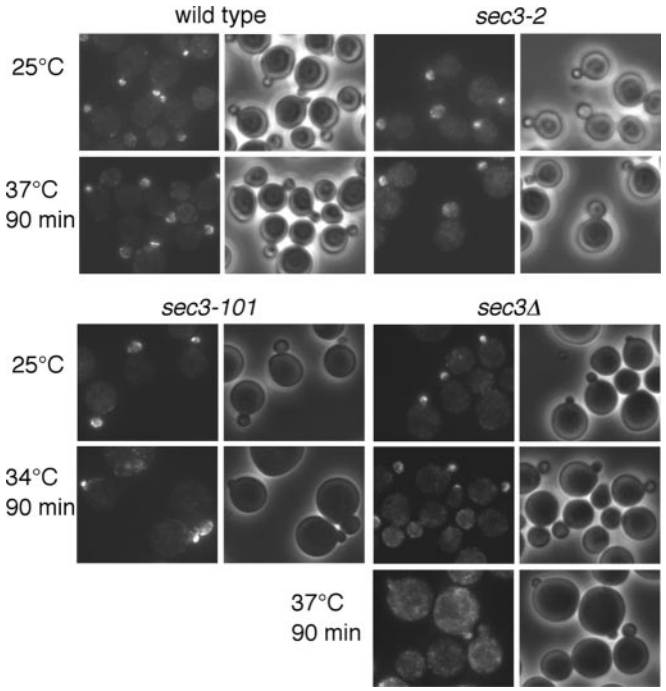

FIG. 6. **Exo84-GFP polarization is independent of Sec3p.** Wild-type, *sec3-2*, *sec3-101*, and *sec3*Δ cells expressing Exo84-GFP were grown at 25 °C to log phase and then shifted to the restrictive temperatures for 90 min. The cells were processed for microscopy.

The effects observed using this allele may be difficult to interpret. All of the exocyst components were GFP-tagged and expressed under the control of their own promoters in the *exo84* mutants. The same results were obtained for both *exo84-117* and *exo84-121*. Here, only the images obtained using *exo84-117* are shown in Fig. 7.

At 25 °C, all of the exocyst proteins were clearly polarized to the tips of the buds in both the wild-type and *exo84-117* mutant strains. However, at 37 °C, the localization patterns of these proteins changed (Fig. 7). The extent of the mislocalization varied among the exocyst components, but they can be separated into three groups. Group I includes Sec5p and Sec6p. These proteins remained polarized to the daughter cells in the mutant. However, they spread all over the daughter cells and "leaked" through the bud neck into the mother cells. Group II includes Sec3p and Sec8p. 27.3% of the cells had depolarized Sec3p, and 34.7% of the cells had depolarized Sec8p. In the rest of the cells, Sec3p and Sec8p were polarized, but many had the "spreading" pattern in the buds. Group III includes Sec10p, Sec15p, and Exo70p. These proteins were almost completely depolarized (depolarized in >80% of the cells). Clearly, Sec10p, Sec15p, and Exo70p rely on Exo84p for their polarization.

We also GFP-tagged the *exo84-117* mutant by chromosomal integration. The expression level of the GFP-tagged mutant protein was the same as that of wild-type Exo84-GFP (data not shown). We found that the mutant protein itself was diffused in the cells at 37 °C (Fig. 7). We examined the localization of Sec4p in the mutant cells by immunofluorescence with anti-

TABLE II
Summary of Exo84-GFP localization indifferent mutants
Boldface type indicates that the localization pattern was affected. +, polarized; −, depolarized.

Strain	25 °C	10 min at restrictive temperature	90 min at restrictive temperature	Restrictive temperature
				°C
Wild-type	+	+	+	34
Wild-type	+	+	+	37
TPM1 tpm2Δ	+	+	+	34.5
tpm1-2 tpm2Δ	+	+	**−**	34.5
sec18-1	+	**+/−**	**−**	34
sec22-3	+	**+/−**	**−**	34
sec7-1	+	**−**	**−**	37
sec19-1	+	+	**−**	34
sec2-59	+	**−**	**−**	34
sec3-2	+	+	+	37
sec3-101	+	+	+	34
sec3Δ	+	**+/−**	+	34
sec5-24	+	+	+	34
sec6-4	+	**+/−**	+	34
sec8-9	+	**+/−**	+	37
sec10-2	+	+	+	34
sec15-1	+	+	+	34
exo70-38	+	**+, some fluorescence signal appears in mother cells**	**+, signal all over bud and some spreads into mother**	37
exo84-117-GFP	+	**−**	**−**	37

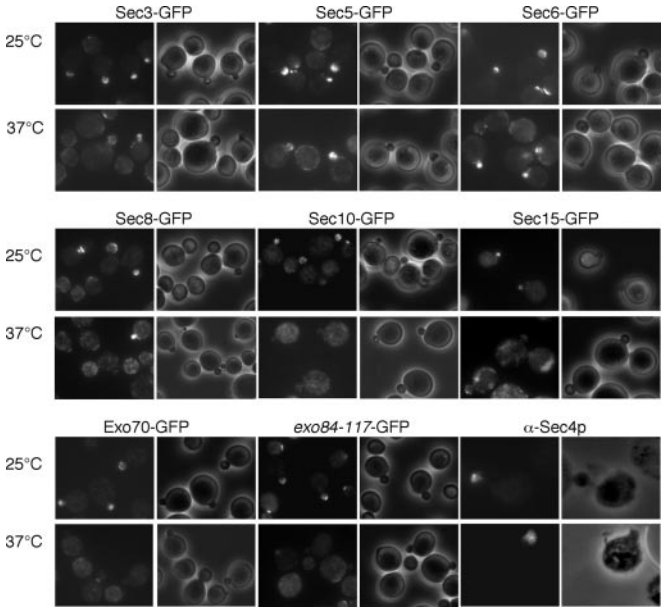

FIG. 7. **Exo84p is necessary for the proper localization of the exocyst components.** *exo84-117* mutant cells expressing the GFP-tagged exocyst components under the control of their endogenous promoters were grown to log phase at 25 °C and then shifted to the restrictive temperature of 37 °C for 2 h. Cells were fixed in methanol/acetone for microscopy. *exo84-117* cells were also grown and shifted in the same way and then fixed in 3.7% formaldehyde for staining with anti-Sec4p antibody (*α-Sec4p*). GFP-tagged Sec3p, Sec5p, Sec6p, and Sec8p remained polarized to the bud tip but had a change in the location of GFP in the bud. GFP-tagged Sec10p, Sec15p, and Exo70p were no longer polarized after 2 h at 37 °C. *exo84-117*-GFP itself was also mislocalized; however, Sec4p remained polarized in the *exo84-117* mutant.

Sec4p antibody. Sec4p was well polarized in the *exo84-117* cells at all temperatures, consistent with the idea that the Rab protein functions upstream of the exocyst (12, 18). A summary of these observations is presented in Table III.

Assembly of the Exocyst Complex Is Disrupted in exo84 Mutants—To understand the role of Exo84p in the assembly of the exocyst complex, we purified the exocyst from the *exo84* mutant cells. We first tagged Sec8p at its C terminus with triple c-Myc epitope sequences by chromosomal integration. Cells were

TABLE III
Localization of the exocyst components in the exo84-117 mutant at 37 °C
The cells were scored (n = 50) after shifting to the restrictive temperature of 37 °C for 2 h.

	Polarized	Polarized/affected[a]	Depolarized
	%	%	%
Sec5-GFP	2	90	8.0
Sec6-GFP	9.4	86.8	3.8
Sec3-GFP	10.9	61.8	27.3
Sec8-GFP	6.1	59.2	34.7
Sec10-GFP	5.6	11.1	83.3
Sec15-GFP	2	6.3	91.7
Exo70-GFP	5.5	12.7	81.8
exo84-117-GFP	17.5	7	75.5

[a] "Polarized/affected" indicates that, although the proteins were still polarized in the daughter cells, they were not concentrated at the bud tips as in the wild-type cells. Frequently, the GFP signals spread in the entire bud and sometimes even leaked through the neck to the mother cells.

grown in medium containing ^{35}S-PRO-MIX, and lysates were prepared for immunoprecipitation using anti-c-Myc monoclonal antibody 9E10 as described previously (7). The purified complex was analyzed by SDS-PAGE and autoradiography. For comparison, the wild-type strain with its Sec8p c-Myc-tagged was used. As a negative control, an untagged strain was included in the same procedure. As shown in Fig. 8, Sec3p, Sec5p, and Sec6p co-purified with Sec8p at almost identical levels in the *exo84* mutant cells compared with the wild-type cells. However, greatly reduced amounts of Sec10p (24%), Sec15p (28%), and Exo70p (<5%) co-purified with Sec8p in the mutant. Exo84p migrated at almost the identical position (~90 kDa) upon SDS-PAGE as the C-terminal degradation product of Sec3p (referred to as Sec3pCT), similar to previous observations (7, 8). Although it was difficult to differentiate these two peptides upon SDS-PAGE, the intensity of the corresponding band at 90 kDa was clearly reduced. Because full-length Sec3p remained unchanged in the mutant, it is very unlikely that the reduced intensity of the 90-kDa band(s) was caused by a reduction of Sec3pCT. Instead, we believe that the mutant form of Exo84p was either absent from or greatly reduced in the complex. From the analysis of the composition of the exocyst complex, we conclude that Exo84p is important for the recruitment

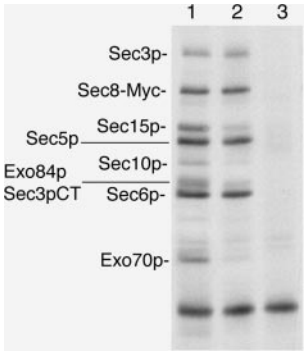

FIG. 8. **Exocyst complex assembly is disrupted in *exo84-117*.** Sec8p was epitope-tagged with triple c-Myc sequences at its C terminus by chromosomal integration. Cells were grown in medium containing ^{35}S-PRO-MIX, and lysates were prepared for immunoprecipitation using anti-c-Myc monoclonal antibody 9E10. The purified complex was analyzed by SDS-PAGE and autoradiography. The partially assembled exocyst complex purified from the *exo84* mutant strain is shown (*lane 2*). For comparison, the wild-type strain with its Sec8p c-Myc-tagged was used (*lane 1*). An untagged strain was included as a negative control in the same procedure (*lane 3*). The purified components are labeled to the left. Exo70p, Sec10p, and Sec15p were absent or greatly reduced in the complex. Sec3pCT is the C-terminal degradation product of Sec3p. It migrated at almost the identical position as Exo84p, as observed previously (8).

and/or stable association of Sec10p, Sec15p, and Exo70p with the complex. It is interesting to note that GFP-tagged Sec10p, Sec15p, and Exo70p belong to Group III and were completely depolarized in the *exo84* mutants.

DISCUSSION

The exocyst complex has been implicated in tethering post-Golgi secretory vesicles to specific domains of the plasma membrane for exocytosis (6, 37–40). To elucidate the molecular mechanisms of exocyst function in tethering, it is essential to investigate how the individual subunits are assembled and how the exocyst is targeted to the sites of secretion. Addressing these critical questions is facilitated in yeast cells because all of the eight exocyst components are specifically targeted to the daughter cells at early stages of the cell cycle, a pattern that can be easily monitored by microscopy. In addition, GFP tagging does not affect the function of any of the exocyst proteins (9, 11, 13, 31). In this study, we have characterized Exo84p function in the secretory pathway and analyzed the targeting and assembly of Exo84p and other exocyst proteins using an array of yeast mutants. Our results established the role of Exo84p in post-Golgi traffic. The results revealed complicated regulatory mechanisms for the targeting of different exocyst components to sites of secretion.

The yeast Exo84p was first identified via a database search using the mammalian Exo84 protein sequence. Additional biochemical analyses have confirmed that Exo84p is an integral member of the yeast exocyst complex (9). Here, to better analyze Exo84p function and its role in the targeting of and assembly with the other members of the exocyst, we generated a number of temperature-sensitive *exo84* mutant alleles. Previous experiments demonstrated that Exo84p depletion not only leads to accumulation of post-Golgi vesicles, but also results in defects in other intracellular structures (9). However, chronic depletion of this essential gene product by galactose over a long period of time may indirectly affect overall protein synthesis and membrane traffic (41). Here, using the temperature-sensitive *exo84* mutants, we have demonstrated that Exo84p is specifically involved in the post-Golgi stage of membrane traffic. Our analysis of CPY traffic indicated that there are no kinetic delays for ER-to-Golgi or Golgi-to-vacuole traffic in *exo84* mutants. Furthermore, electron microscopy revealed specific accumulation of post-Golgi vesicles. Overall, the data established that Exo84p is essential for exocytosis of post-Golgi vesicles at the plasma membrane.

A key feature of the exocyst is its specific localization to sites of exocytosis and active membrane expansion (7, 11, 13, 42, 43). Study of the targeting mechanisms of the exocyst is important for our understanding of the molecular basis of polarized exocytosis. It was previously proposed that Sec3p is a spatial landmark for exocytosis at the plasma membrane (11). Sec3p is localized to sites of exocytosis independently of actin (11). In addition, it directly interacts through its N terminus with Rho1 and Cdc42, members of the Rho family of small GTPases (13, 31). Cdc42 and Rho1 compete for binding to this region, and disruption of the binding (Sec3ΔN) results in mislocalization of Sec3p (13, 31). Although Sec3p is a spatial landmark for exocytosis, it is unlikely to be a "recruiter" for the other exocyst components to sites of secretion. First, we found that Exo84p remained polarized in the *sec3-2* mutant at the restrictive temperature. We also found that Exo84p remained polarized in *sec3-101*, which is missing its C-terminal exocyst-binding region (13, 35). Finally, we found that Exo84p could be polarized even when Sec3p was deleted. In fact, all of the exocyst components were polarized in *sec3*Δ cells at 25 °C. In a previous study (13), we found that Sec3ΔN, which cannot bind Cdc42 and Rho1, failed to polarize in the cells, whereas full-length Sec3p, expressed at similar or higher levels, still retained its polarization. However, when *sec3ΔN* replaced the endogenous copy of *SEC3*, the other exocyst components remained in the daughter cells, and a small portion of the Sec3ΔN protein became polarized (13).[2] We speculate that the portion of the Sec3ΔN protein that appeared in the bud was probably retained through its interaction with the rest of the exocyst complex.

Why is the exocyst localization affected in the *sec3* deletion strain at 37 °C? The *sec3*Δ cells are severely defective in secretion at elevated temperatures (36), which may affect the localization of some of the exocyst proteins indirectly through the general secretion defect. However, the secretion defect alone cannot be solely responsible for the localization defect of Exo84p because Exo84-GFP was well polarized in some of the *sec* mutants at the restrictive temperatures. We speculate that, although Sec3p does not mediate the targeting of the other exocyst proteins to the daughter cells, it may be implicated in the organization of the complex or fine-tuning the distribution of the complex at the daughter cell membrane.

If Sec3p is not the recruiter, there must be pathways that target different exocyst components to sites of active secretion. Here, we systematically analyzed the localization of Exo84p in various yeast mutants. First, we found that actin plays an important role in the targeting of Exo84p, as disruption of actin cables directed toward the daughter cells in the tropomyosin mutant resulted in Exo84p mislocalization. This mislocalization is similar to that of Sec8p and Sec15p (11, 19, 22, 23) but is in contrast to that of Sec3p, which is independent of actin for its polarization (11, 19). It is possible that the targeting of Exo84p and several other members of the exocyst relies on the arrival of post-Golgi secretory vesicles to the buds. If this were the case, cells with disruption of pre-Golgi stages of traffic would not be able to generate post-Golgi vesicles, and therefore, Exo84p would not be delivered to the daughter cells. Indeed, we found that Exo84p failed to localize to the buds in *sec* mutants with disruption of ER-to-Golgi traffic (*sec18-1*, *sec19-1*, and *sec22-3*) or Golgi budding (*sec7-1*) (Fig. 5). It was previously observed in *sec2* mutants (in which the guanine exchange fac-

[2] W. Guo, unpublished data.

tor for Sec4p is defective) that, even though post-Golgi vesicles can be produced, they accumulate in a randomized fashion in the cells and that Sec4p is delocalized from the bud (32). We found that Exo84p was mislocalized in *sec2-59* cells (Fig. 5). On the other hand, in all of the exocyst mutants, in which vesicles accumulate preferentially in the daughter cells (26), we found that Exo84p was polarized in the buds.

Overall, the results indicate that Exo84p relies on polarized actin cables for its targeting to the bud. Exo84p and several other exocyst components could be directly transported via the secretory vesicles to the bud or could be recruited from the cytosol to the bud upon arrival of the secretory vesicles. On the other hand, Sec3p is targeted via a distinctive mechanism. Sec3p may join Exo84p and the other exocyst proteins at the daughter cell membrane upon the arrival of the secretory vesicles. Our results are consistent with the recent study of Boyd *et al.* (20), who showed that Sec3-GFP can recover its bud tip signal after photobleaching when the actin assembly is disrupted, whereas the other components, including Exo84-GFP, cannot. They also showed that Exo70-GFP had biphasic photobleaching recovery and proposed that Exo70p may partially rely on actin for its transport to the bud. Our results suggest that the stable association of Exo70p with the rest of the complex at the bud membrane and/or the actin-dependent branch for Exo70p targeting relies on Exo84p as discussed below.

We found that three members of the exocyst (Sec10p, Sec15p, and Exo70p) clearly depend on Exo84p for their polarization. The delocalization of these components in the *exo84* mutant is probably a consequence of the delocalization of the Exo84p itself, as we observed GFP-tagged *exo84-117* diffused in the cells (Fig. 7). Biochemical analysis of the composition of the exocyst complex in *exo84* mutants indicated that the ability of Sec10p, Sec15p, and Exo70p to associate with the rest of the complex was selectively disrupted when Exo84p was mutated (Fig. 8). Previous results indicate that Sec15p associates with the secretory vesicles and interacts with Sec4p (12). The mislocalization of Sec15p in the *exo84* mutants suggests that, once Sec15p arrives at the plasma membrane, it cannot be maintained there when Exo84p is defective. Recently, White and co-workers (44) investigated the exocyst complex organization in mammalian cells. Using available antibodies against Sec5p, Sec6p, Sec10p, and Exo84p, they were able to show that Sec10p, Sec15p, and Exo84p form a subcomplex separable from other members of the complex. Our localization analyses and biochemical results are consistent with this observation and suggest that the organization of the exocyst complex has been well conserved during evolution.

Because the exocyst functions in a step prior to SNARE assembly and membrane fusion, the individual exocyst components are ideal targets of cellular regulators that spatially and temporally control exocytosis (4–6, 37, 40). The assembly of the exocyst complex may integrate various sources of cellular information to ensure that exocytosis occurs at the right time and place. Future studies will involve the identification of proteins that regulate the function of the exocyst. These studies will help us not only to elucidate the mechanisms of vesicle tethering, but also to understand the molecular basis for polarized exocytosis.

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