

Exo70p mediates the secretion of specific exocytic vesicles at early stages of the cell cycle for polarized cell growth

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In budding yeast, two classes of post-Golgi secretory vesicles carrying different sets of cargoes typified by Bgl2p and invertase are delivered to the plasma membrane for secretion. The exocyst is implicated in tethering these vesicles to the daughter cell membrane for exocytosis. In this study, we report that mutations in the exocyst component Exo70p predominantly block secretion of the Bgl2p vesicles. Furthermore, a defect in invertase vesicle trafficking caused by *vps1Δ* or *pep12Δ* in the *exo70* mutant

background is detrimental to the cell. The secretion defect in *exo70* mutants was most pronounced during the early budding stage, which affected daughter cell growth. The selective secretion block does not occur at the vesicle formation or sorting stage because the exocytic vesicles are properly generated and protein processing is normal in the *exo70* mutants. Our study suggests that Exo70p functions primarily at early stages of the cell cycle in Bgl2p vesicle secretion, which is critical for polarized cell growth.

Introduction

The budding yeast *Saccharomyces cerevisiae* undergoes polarized growth and provides an excellent model system for the study of cell polarity. Yeast cells are surrounded by a rigid cell wall, which protects the cells from the environment but also physically restrains membrane expansion. For budding, cells not only need to deliver proteins and lipids to the bud membrane but also need to secrete enzymes to remodel the cell wall in order to allow surface expansion. Harsay and Bretscher (1995) discovered that yeast cells have two distinct classes of exocytic vesicles carrying different sets of cargoes for secretion. One class carries plasma membrane proteins and cell wall modification enzymes such as Bgl2p (hereafter referred to as Bgl2p vesicles), whereas the other class carries proteins such as the periplasmic enzyme invertase (hereafter referred to as invertase vesicles). Several proteins are implicated in the generation of specific vesicles (David et al., 1998; Gall et al., 2002). However, whether the tethering or docking of these vesicles at the plasma membrane is differently regulated is unknown.

The exocyst is an octameric protein complex composed of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p.

These proteins are localized to the bud tip, the site of active exocytosis and cell surface expansion, where they tether the post-Golgi secretory vesicles to the plasma membrane before fusion (for reviews see Guo et al., 2000; Hsu et al., 2004). In the present study, we report that Exo70p functions primarily at early stages of the cell cycle to regulate the secretion of specific vesicles that are critical for polarized cell growth.

Results and discussion

The *exo70* mutants accumulate post-Golgi secretory vesicles

Exo70p was initially identified through purification of the exocyst complex from yeast lysates (TerBush and Novick, 1995). To study the function of Exo70p, we generated *exo70* mutant strains. The *exo70-35* mutant has a growth defect that is more pronounced at temperatures $\leq 25^{\circ}\text{C}$, whereas *exo70-38* is a temperature-sensitive mutant that grows normally at 25°C but fails to grow above 35°C (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200606134/DC1>). To study secretion in these mutants, we examined the cells that were shifted to their restrictive temperatures by thin-section EM. As shown in Fig. 1 A, the *exo70-35* and *exo70-38* cells accumulated post-Golgi vesicles that were 80–100 nm in diameter. These vesicles were preferentially distributed in the daughter cells. Consistent with the EM result, immunofluorescence staining of Sec4p, the Rab

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Abbreviations used in this paper: 5-FOA, 5-fluoroorotic acid; CPY, carboxypeptidase Y; SC, synthetic complete media; YPD, yeast extract/peptone/glucose.

The online version of this article contains supplemental material.

protein residing on the post-Golgi vesicles, was polarized to the bud in both mutants (Fig. 1 B). These results indicate that the polarized delivery of exocytic vesicles is not affected in the *exo70* mutants, which is consistent with previous studies indicating that the exocyst functions at the vesicle-tethering step after vesicles are transported to the daughter cells (for reviews see Guo et al., 2000; Hsu et al., 2004).

The *exo70* mutants are primarily defective in the secretion of Bgl2p vesicles

All of the previously identified exocyst mutants were defective in invertase secretion (Novick et al., 1980; Zhang et al., 2005b). Therefore, we examined invertase secretion in the *exo70* mutants. Surprisingly, the *exo70-35* and *exo70-38* mutants were able to secrete invertase at levels that were close to the wild-type cells (Fig. 2 A). As controls, all of the other exocyst mutants showed substantial invertase secretion defects at the restrictive temperature (Fig. 2 A). We next examined the secretion of Bgl2p in these mutants. As shown in Fig. 2 B, *exo70-35* accumulated Bgl2p at both 25 and 34°C, and *exo70-38* accumulated Bgl2p at the restrictive temperature of 37°C. The newly accumulated Bgl2p in *exo70-38* after shifting to 37°C was comparable with other temperature-sensitive exocyst mutants. The growth defect of *exo70-38* is also similar to other exocyst mutants (Fig. S1 B). To better analyze the secretion of these cargoes, we performed pulse-chase experiments. As shown in Fig. 2 C, the wild-type cells secreted >90% of the newly synthesized Bgl2p within 30 min at 37°C, whereas *exo70-38* only secreted ~40% of Bgl2p. *sec10-2* also showed the Bgl2p secretion defect, which was less severe than that in *exo70-38*. On the other hand, *exo70-38* secreted >80% of the newly synthesized invertase within 30 min at 37°C, whereas *sec10-2* only secreted ~30% of invertase under the same condition (Fig. 2 D).

To further investigate the selectivity of vesicle block in *exo70* mutants, we separated the vesicles by density gradients as previously described (Harsay and Bretscher, 1995; Harsay and

Schekman, 2002). As shown in Fig. 2 (E–G), both *exo70-35* and *sec6-4* accumulated Bgl2p vesicles, whereas only a diminutive amount of invertase vesicles was detected in *exo70-35*. Our results confirmed an early prediction (Harsay and Bretscher, 1995; Harsay and Schekman, 2002) that certain secretory mutants may be specifically defective in one branch of the exocytic pathways. To date, all of the other *sec* mutants have been characterized by their block of both classes of vesicles (Novick et al., 1980; Zhang et al., 2005b; unpublished data). Thus, the *exo70* mutants are unique in their selectivity in Bgl2p vesicle block. We do not totally exclude the possibility that new *exo70* mutant alleles that have more detectable invertase secretion defects can be identified. However, analyses of the two *exo70* mutant alleles that are different in nature (Fig. S1) strongly suggest that Exo70p primarily mediates the secretion of Bgl2p vesicles.

Cargoes in the invertase vesicles travel through endosomal compartments before reaching the plasma membrane. Vps1p is a dynamin implicated in the formation of vesicles that are destined to the endosomes from the TGN (Nothwehr et al., 1995). Pep12p is a SNARE protein involved in the fusion of Golgi or early endosomal vesicles with the late endosomes (Gerrard et al., 2000). In the *vps1Δ* or *pep12Δ* cells, invertase is delivered to the cell surface by routing through the Bgl2p pathway (Gurunathan et al., 2002; Harsay and Schekman, 2002). We found that *exo70-35* was synthetic lethal with *vps1Δ* or *pep12Δ* (Fig. 2 H). This result suggests that in the *exo70-35* cells that are constitutively defective in Bgl2p vesicle secretion, further disruption of the other exocytic route is detrimental to the cell.

We have also analyzed carboxypeptidase Y (CPY) processing and found that this protein is correctly sorted, and there is no kinetic delay for CPY traffic in the *exo70* mutant (Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200606134/DC1>). The normal processing of CPY together with the evident accumulation of post-Golgi secretory vesicles suggest that protein sorting to vacuole and vesicle formation at the TGN is

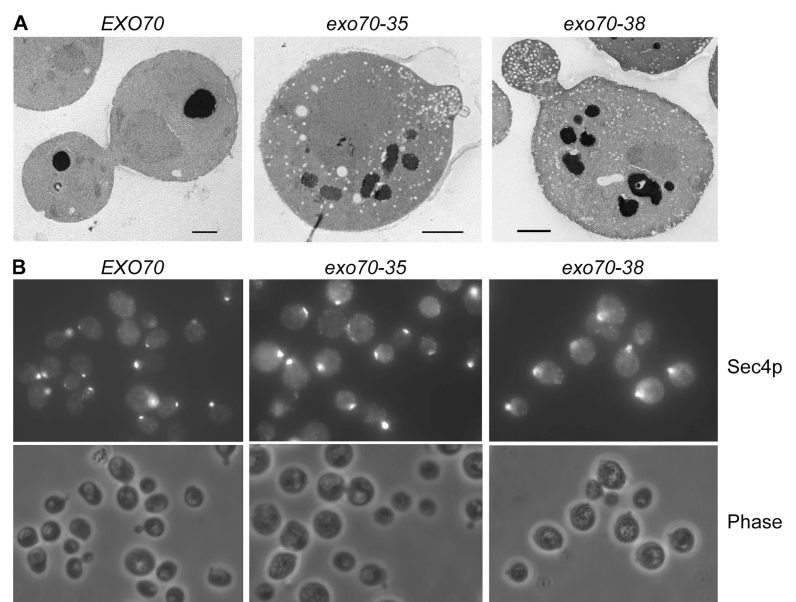


Figure 1. The *exo70* mutant cells accumulate post-Golgi secretory vesicles. (A) Thin-section transmission EM of wild-type, *exo70-35*, and *exo70-38* cells. Bars, 1 μ m. (B) Sec4p is polarized in the wild-type and *exo70* mutants as revealed by immunofluorescence. For the EM and immunofluorescence experiments, the wild-type and *exo70-38* cells were shifted from 25 to 37°C for 2 h; the *exo70-35* cells were shifted from 34 to 25°C for 3 h.

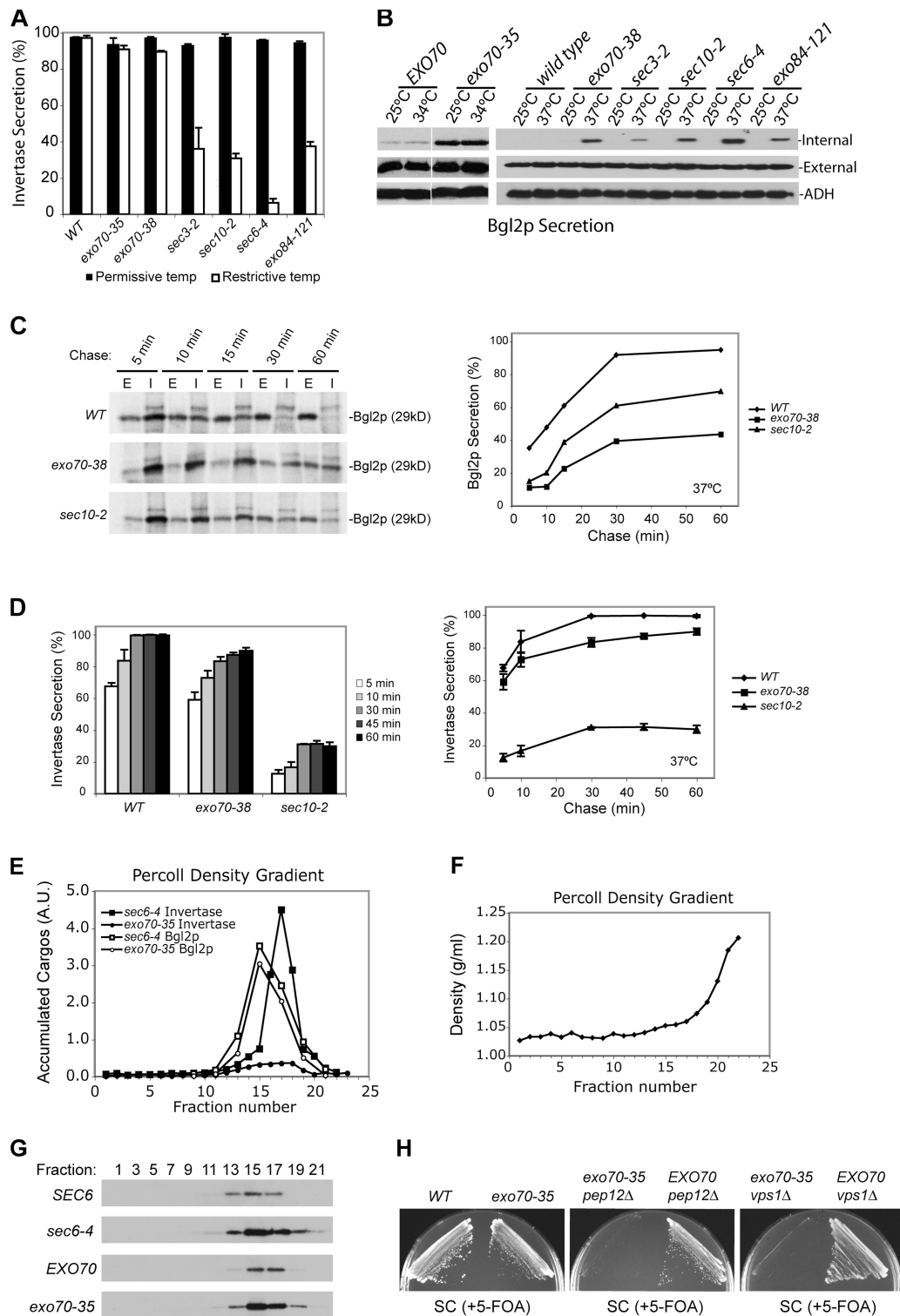
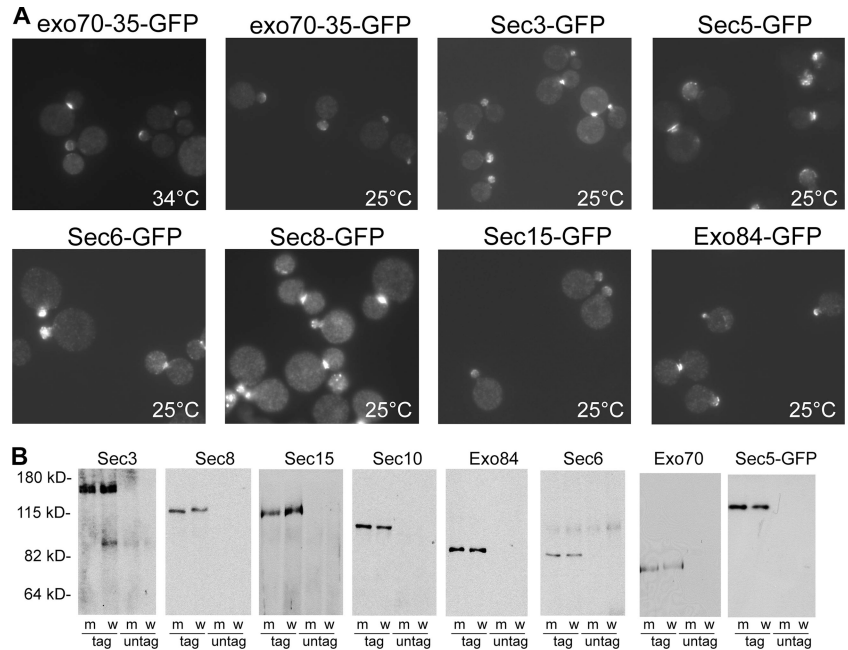


Figure 2. The secretion of Bgl2p vesicles but not invertase vesicles was blocked in *exo70* mutant cells. (A) Invertase secretion in wild-type, *exo70*, and other exocyst mutants ($n = 3$). (B) Western blot analysis of the internal and external pools of Bgl2p in wild-type and mutant cells. Alcohol dehydrogenase (ADH) was used as a control to show that the same amounts of proteins were loaded. (C and D) Pulse-chase analysis of Bgl2p (C) or invertase (D) secretion in wild-type (WT), *exo70-38*, and *sec10-2* cells. The pulse-chase analyses were performed as described in Materials and methods. The secretion of newly synthesized Bgl2p or invertase was quantified and is plotted on the right. E, external; I, internal. (E–G) The *exo70-35* cells accumulate secretory vesicles containing Bgl2p but not invertase. Secretory vesicles were separated from *sec6-4* and *exo70-35* cells by Percoll density gradients. The amounts of invertase and Bgl2p for each fraction (E) and the density profile of the Percoll gradients (F) are shown in the plots. The internal Bgl2p in the wild-type and mutant cells fractionated on Percoll gradients was analyzed by Western blotting (G). (H) *exo70-35* is synthetic lethal with *vps1Δ* or *pep12Δ*. Endogenous *EXO70* in wild-type, *vps1Δ*, and *pep12Δ* strains was disrupted by *HIS3* and supplemented with a *CEN URA3 EXO70* plasmid and *exo70-35* plasmid. The *vps1Δ* and *pep12Δ* strains cannot survive when the *EXO70* plasmid was lost on 5-FOA plates. Error bars represent SD.

Figure 3. **Localization and structural integrity of the exocyst complex in *exo70* mutant cells.** (A) GFP-tagged exocyst components are polarized in the *exo70-35* cells at the restrictive temperature of 25°C. (B) The exocyst was immunoprecipitated by anti-GFP antibody from the Sec5 GFP-tagged strain. The untagged strain was used as a negative control. Comparison of the exocyst components isolated from the wild-type and *exo70-35* cells by Western blotting. W, wild type; m, *exo70-35* mutant; tag, Sec5-GFP-tagged strain; untag, untagged strain.



not defective in *exo70* mutants. Rather, the selective block of Bgl2p vesicle exocytosis is likely imposed at the plasma membrane stage, which suggests that different exocytic pathways can be specifically regulated at the target membrane as well as at the TGN.

Cellular localization and structural integrity of the exocyst complex in *exo70* mutants

Using fluorescence microscopy, we examined exocyst localization in the *exo70* mutants. As shown in Fig. 3 A, all of the exocyst subunits were polarized, including the *exo70-35* mutant protein itself. We also found that the immunoprecipitated exocyst complex was intact, and the *exo70-35* protein was able to associate with other exocyst proteins in the complex (Fig. 3 B). Similar results were obtained from the *exo70-38* cells (Fig. S2 B). Because the localization and composition of the exocyst complex are mostly unaffected in the *exo70* mutants, the secretion defects are most likely caused by the malfunction of Exo70p itself.

The *exo70* mutant cells accumulate vesicles at early stages of the cell cycle

During our EM analyses of the *exo70* cells, we noted that vesicle accumulation was much more prominent in small-budded cells than in large-budded cells. The correlation between bud size and vesicle number suggests that the secretion block in *exo70* mutants mostly occurs during early stages of budding. In contrast, the other exocyst mutants accumulate secretory vesicles at various stages of the cell cycle (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200606134/DC1>). Failure in secretion results in an ~5% increase of cell density, which allows separation of the *sec* mutant cells and wild-type cells by density gradients (Novick et al., 1980). In the present study, wild-type and mutant cells were subjected to Percoll density gradients. As shown in Fig. 4 A, the wild-type cells were

distributed to a single low density fraction, and the *sec6-4* cells were accumulated at a single high density fraction. However, the *exo70-35* cells were distributed in both the low and high density fractions. We found that most of the large-budded cells (93%; $n = 250$) were present in the lighter fraction. In contrast, the denser fraction contained very few large-budded cells; instead, most were small- or medium-budded cells corresponding to the vesicle-accumulating population observed by EM. The *exo70-35* cells with lower density were not caused by incomplete phenotypic penetration or spontaneous reversions because cells collected from the two density peaks were equally defective in growth on plates (unpublished data). Besides *sec6-4* and *exo70-35*, we have also analyzed *exo70-38* and other exocyst mutants, including *sec3-2*, *sec10-2*, and *exo84-121*. Although the *exo70-38* cells were distributed to both heavy and light fractions, the other exocyst mutants were all distributed to a single high density fraction (Fig. 4 B, top). Further microscopic analysis of the cells obtained from the *exo70-38* heavy fraction demonstrated that most of these cells were in their small- or medium-budded stage, whereas cells obtained from the other mutants were heterogeneous in budding stages (Fig. 4 B, bottom). This study clearly indicates that *exo70* mutants block secretion primarily at early stages of the cell cycle.

It has been proposed that the two types of secretory vesicles play distinct roles in yeast cells (Harsay and Bretscher, 1995; Harsay and Schekman, 2002). The invertase vesicles carry proteins such as invertase, acid phosphatase, and possibly the general amino acid permease (Roberg et al., 1997), which are secreted under certain physiological conditions. The Bgl2p vesicles contain materials for plasma membrane expansion and cell wall remodeling. We examined whether block of the Bgl2p vesicle pathway in *exo70* mutants could affect daughter cell growth. Budding of the wild-type and *exo70-35* cells was compared at various time points after release from α -factor arrest at G₁. As shown in Fig. 5 A, by 60 min, the wild-type cells

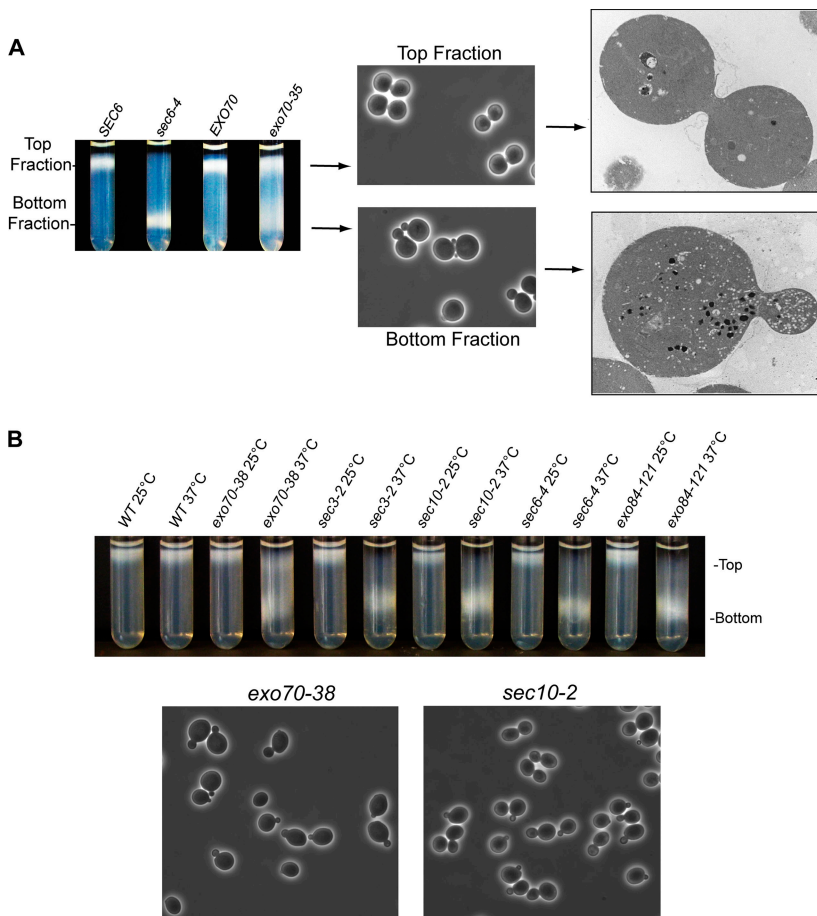


Figure 4. The secretion defect of *exo70* mutants is most prominent at early stages of the cell cycle. (A) *SEC6*, *sec6-4*, *EXO70*, and *exo70-35* cells were subjected to Percoll density gradients. The top and bottom fractions of the *exo70-35* cells were analyzed by microscopy. EM samples are shown on the right. (B) Percoll density gradient analysis of *exo70-38*, *sec3-2*, *sec6-4*, *sec10-2*, and *exo84-121*. The cells obtained from the heavy fraction (bottom band) of *exo70-38* and *sec10-2* were compared by microscopy (bottom panel). WT, wild type.

exhibited much larger bud sizes than the mutant cells (1.5-fold; $n = 150$). Clearly, block of the Bgl2p vesicle alone is sufficient to cause a delay in daughter cell growth. Furthermore, because secretion in the *exo70* mutants is primarily blocked at early stages of the cell cycle, we speculated that growth defects of the mutant cells would be more prominent during early budding. To test this, we examined the growth of the temperature-sensitive *exo70-38* mutant synchronized at different cell cycle stages. We first assessed the growth of cells at the early budding stage by examining bud formation from cells released from G₁ at 37°C. As shown in Fig. 5 B, the majority of the wild-type cells were capable of generating buds after 60 min. In contrast, most of the *exo70-38* cells were either unbudded or at the tiny-budded stage. We then tested whether growth of the cells at later stages of the cell cycle was affected in *exo70-38*. Cells released from G₁ were first incubated at 25°C for 90 min to generate large-budded cells and were shifted to 37°C for various periods of time. We found that both the wild-type and *exo70-38* cells were able to complete cytokinesis after 30 min of incubation at 37°C, as revealed by the disconnection of the cytoplasm between the mother and daughter cells. However, after cytokinesis, the *exo70-38* cells were arrested at the next budding stage, whereas the wild-type cells were able to proceed to new rounds of the cell cycle (Fig. 5 C). On the other hand, the *sec10-2* cells were defective in both budding and later stages of cell growth (Fig. 5, B and C).

The selective block of Bgl2p vesicles primarily during budding in *exo70* mutants suggests that the function of Exo70p in exocytosis is spatially and temporally coupled to polarized cell growth. The exocyst complex is under the control of Rho GTPases (Novick and Guo, 2002). It was previously reported that a Cdc42p mutant, *cdc42-6*, specifically accumulated Bgl2p vesicles at early stages of the cell cycle (Adamo et al., 2001). The phenotypical similarity between *cdc42-6* and the *exo70* mutants suggests that Cdc42p and Exo70p function together during polarized cell growth. One possibility is that Cdc42p activates Exo70p during yeast budding. We have examined the interaction between Exo70p and Cdc42p in vitro using recombinant fusion proteins. However, the interaction was very weak, and there was no clear nucleotide dependence in the binding (unpublished data). Nevertheless, Cdc42p may regulate Exo70p function via intermediate molecules.

Exo70p functions in concert with the other exocyst components, which mediate the secretion of both the invertase and Bgl2p vesicles. Future studies may likely identify specific mutant alleles in those subunits that also preferentially affect Bgl2p secretion. Overall, characterization of the *exo70* mutants revealed the intimate coupling between exocytosis and polarized cell growth. Future studies of the *exo70* mutants will help us understand the nature of the Bgl2p vesicle pathway and how this pathway is linked to polarized cell growth and cell cycle progression.

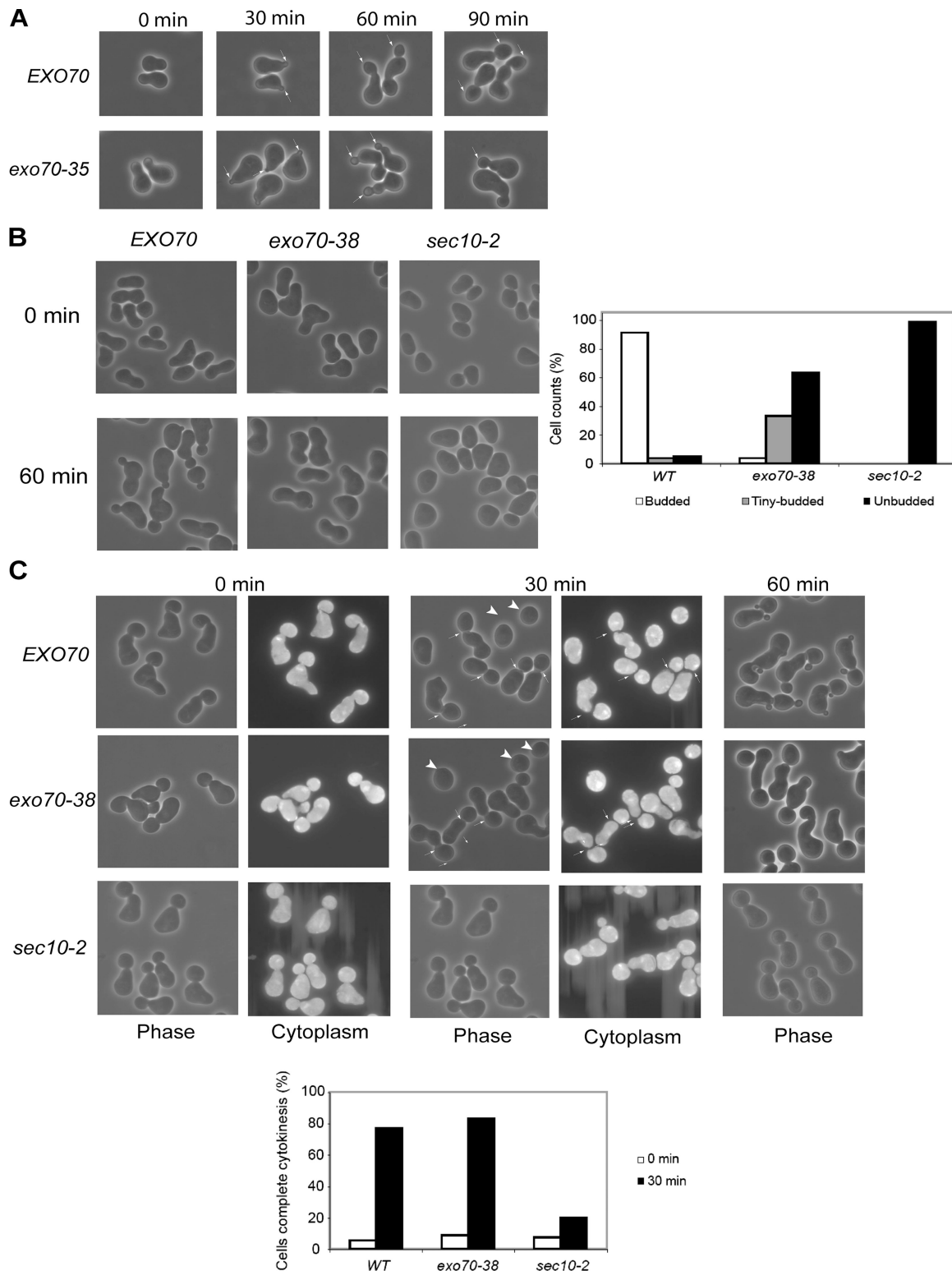


Figure 5. **The *exo70* mutants affect daughter cell growth primarily at the early budding stage.** (A) Wild-type and *exo70-35* cells were synchronized by α factor and were released into fresh media. The samples after various times of release were collected for microscopic analysis of daughter cell growth. Arrows indicate the buds. (B) Wild-type (*WT*), *exo70-38*, and *sec10-2* cells were synchronized by α factor and released into fresh media at 37°C for analysis of daughter cell growth. The left panels show the samples collected before or after 60-min release. The percentage of budded, tiny-budded, or unbudded cells after 60-min release was quantified (right; $n = 200$). (C) Wild-type, *exo70-38*, and *sec10-2* cells were synchronized by α factor and released into fresh media at 25°C for 90 min to generate large buds. The cells were then shifted to 37°C for various times and collected for phase-contrast microscopy (left). The completion of cytokinesis was monitored by staining of the cytoplasm with a high concentration of DAPI (right). Arrows indicate the separation of mother and daughter cells. Arrowheads indicate the newly released daughter cells. The percentage of cells that completed cytokinesis before or after a 30-min shift to 37°C was quantified (bottom; $n = 200$).

Materials and methods

Yeast strains and procedures

Standard methods were used for yeast media, growth, and genetic manipulations. The yeast strains used in this study are listed in Table S1 (available at <http://www.jcb.org/cgi/content/full/jcb.200606134/DC1>). The *exo70-38* mutant was isolated by random mutagenesis. For generation of the *exo70-35* mutant, site-directed mutagenesis on the C terminus of *Exo70* was performed on a *CEN TRP1 EXO70* plasmid. The mutant alleles were introduced into *GY2810*, in which the endogenous *EXO70* gene was disrupted by *HIS3* and supplemented with a *CEN URA3 EXO70* plasmid. The transformants were replicated onto synthetic complete media (SC) plates containing 5-fluoroorotic acid (5-FOA) to select for the loss of the *CEN URA3 EXO70* plasmid. To generate *exo70-35 vps1Δ* and *exo70-35 pep12Δ* double mutants, *vps1Δ* and *pep12Δ* strains in which the endogenous *EXO70* was disrupted by *HIS3* and supplemented with *CEN URA3 EXO70* were transformed with a *CEN LEU2 EXO70* or *CEN LEU2 exo70-35* plasmid. The transformants were replicated on SC plates containing 5-FOA to select for the loss of the *CEN URA3 EXO70* plasmid.

EM and light microscopy

Wild-type and *exo70-35* cells were grown at 34°C in yeast extract/peptone/glucose (YPD) medium to early log phase and were shifted to 25°C for 3 h. The *exo70-38* and *sec10-2* cells were grown at 25°C and shifted to 37°C for 2 h. Cells were processed for thin-section transmission EM using a transmission electron microscope (model 1010; JEOL) at 100,000× as previously described (Zhang et al., 2005a). Fluorescence microscopy of the GFP-tagged exocyst and immunofluorescence observation of Sec4p were performed as previously described (Zhang et al., 2005b). The images were captured by a fluorescence microscope (DM IRB; Leica) using a 100× objective and a high resolution CCD camera (ORCA-ER; Hamamatsu) and were analyzed by OpenLab 3.1.4 software (Improvision).

Invertase and Bgl2p secretion assay

Invertase assays were performed as described previously (Zhang et al., 2005a). For *exo70-38* and other exocyst mutants, the cells were first shifted to 37°C for 1 h and then were grown in low glucose medium (0.1% glucose) at the same temperature for 1 h. For *exo70-35*, cells were started at 34°C and were transferred to 25°C for 1 h before shifting to low glucose medium for 1 h and processing for the assay. Bgl2p assays were performed as described previously (Adamo et al., 2001). For *exo70-38* and other exocyst mutants, the cells were shifted to 37°C for 2 h. For *exo70-35*, cells were started at 34°C and were transferred to 25°C for 2 h before being processed for the Bgl2p assay.

To analyze the secretion of newly synthesized Bgl2p, cells were cultured to early log phase at 25°C and were shifted to SC lacking methionine/cysteine at 37°C for 15 min. The cells were then labeled with [³⁵S]methionine/cysteine for 10 min and chased for various times at 37°C. Bgl2p from external and internal fractions were immunoprecipitated using anti-Bgl2p polyclonal antibody and were analyzed by SDS-PAGE and autoradiography. Accordingly, to analyze the secretion of newly synthesized invertase, the wild-type, *exo70-38*, and *sec10-2* cells were cultured to early log phase at 25°C and shifted to 37°C for 15 min. Cells were then shifted to low glucose medium for 10 min to derepress the invertase expression (pulse). The chase was initiated by adding 2% glucose to the culture to repress invertase expression. The secretion of newly synthesized invertase at various chase times was determined by invertase enzymatic assay.

Separation of vesicles by density gradients

Vesicle fractionation was performed as previously described (Harsay and Schekman, 2002). Yeast cells (100-ml culture per gradient) were grown in YPD medium to early log phase and shifted to YP plus 0.1% glucose at the restrictive temperatures to induce invertase expression. Cells were spheroplasted, lysed, and subjected to differential centrifugation. The pellets from the 100,000-g spin were fractionated on 20–55% Percoll step gradients (4 ml in TLA100.3 tubes [Beckman Coulter]), and 160-μl fractions were collected for invertase and Bgl2p assays.

Percoll density gradient separation of the cells

The wild-type and *exo70-35* cells were grown at 34°C to early log phase and were shifted to 18°C for 3 h. The cells were washed and resuspended in 0.2 ml of synthetic minimal media. The cells were then layered on a 70% Percoll/synthetic minimal medium mixture and centrifuged for 20 min at 25,000 g in TLA100.3 tubes. For *exo70-38* and other exocyst mutants, the cells were shifted for 2 h at 37°C before centrifugation.

Synchronization of cells by α-factor arrest

For synchronization of *exo70-35* cells, early log-phase cells were arrested at G₁ phase using 5 μM α factor for 2.5 h at 34°C. The cells were then released into fresh YPD at 25°C for various periods of time and were collected for light microscopy. To examine the growth of *exo70-38* cells at different cell cycle stages, cells were treated with α factor for 2.5 h at 25°C and either directly released into fresh medium at 37°C or first released at 25°C for 90 min to generate large buds followed by a shift to 37°C for various times. Cells were then collected for light microscopy observation. The completion of cytokinesis was determined by staining with 1 mg/ml DAPI.

Online supplemental material

Table S1 lists the major yeast strains used in this study. Fig. S1 shows the growth properties of the *exo70* mutants and the mutation sites on the protein. Fig. S2 A shows CPY processing in *exo70-35* by pulse-chase assay. Fig. S2 B is the immunoprecipitation experiment using radiolabeled yeast lysate showing that the isolated exocyst complex is mostly intact in the *exo70-38* mutant. Fig. S3 displays the EM images of representative *exo70-35*, *exo70-38*, and *sec10-2* cells at their small-budded and large-budded stages. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200606134/DC1>.

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