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Sec3p Is Involved in Secretion and Morphogenesis in *Saccharomyces cerevisiae*

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Two new temperature-sensitive alleles of *SEC3*, 1 of 10 late-acting *SEC* genes required for targeting or fusion of post-Golgi secretory vesicles to the plasma membrane in *Saccharomyces cerevisiae*, were isolated in a screen for temperature-sensitive secretory mutants that are synthetically lethal with *sec4–8*. The new *sec3* alleles affect early as well as late stages of secretion. Cloning and sequencing of the *SEC3* gene revealed that it is identical to *profilin synthetic lethal 1* (*PSL1*). The *SEC3* gene is not essential because cells depleted of Sec3p are viable although slow growing and temperature sensitive. All of the *sec3* alleles genetically interact with a profilin mutation, *pfy1–111*. The *SEC3* gene in high copy suppresses *pfy1–111* and *sec5–24* and causes synthetic growth defects with *ypt1*, *sec8–9*, *sec10–2*, and *sec15–1*. Actin structure is only perturbed in conditions of chronic loss of Sec3p function, implying that Sec3p does not directly regulate actin. All alleles of *sec3* cause bud site selection defects in homozygous diploids, as do *sec4–8* and *sec9–4*. This suggests that *SEC* gene products are involved in determining the bud site and is consistent with a role for Sec3p in determining the correct site of exocytosis.

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae* is polarized, making it a useful model system for studies of polarized growth and secretion. The bud site on the mother cell is selected according to haploid- or diploid-specific programs (Chant and Pringle, 1995); haploid cells bud axially at sites adjacent to the previous bud site, whereas diploid cells bud in a bipolar pattern, either adjacent to the previous bud site or at the opposite pole of the cell. These patterns can be identified by visualizing the chitinous bud scars on the mother cell. The cytoskeleton and the secretory pathway orient to direct growth toward the bud site. Budding can therefore be seen as a process of polarized secretion. Studies on the incorporation of cell wall components such as cell wall-bound acid phosphatase (Field and Schekman, 1980), mannan (Farkas *et al.*, 1974), and glucan (Tkacz and Lampen, 1972) have shown that during most of the budding process, secretion is directed to the tip of the growing bud. When the daughter cell is about two-thirds the size of the mother cell, secretion becomes isotropic over the entire surface of the bud. Very little growth or secretion occurs in the mother cell. Following nuclear division the secretory pathway reorients to the mother-bud neck, resulting in cytokinesis and septation (Byers, 1981). This implies a need for a spatial signal to direct secretory components to the site of exocytosis.

Mutations affecting actin or proteins, such as profilin, that regulate actin assembly and proteins that regulate bud emergence, such as the polarity establishment proteins Cdc24p, Cdc42p, Bem1p, and Bem2p, result in isotropic secretion over the surface of the mother cell, shown by chitin deposition over the entire cell surface rather than only at bud scars. The resulting cells are large, round, and multinucleate (Sloat and Pringle, 1978; Adams *et al.*, 1990; Bender and Pringle, 1991). In the temperature-sensitive mutant *cdc24*, secretion of acid phosphatase at the restrictive temperature occurs at levels similar to that of wild-type cells, but is directed over the entire cell surface, and buds are not formed (Field and Schekman, 1980). The roles of four of these polarity establishment proteins have been defined. Cdc42p is a member of the Rho/Rac family of small GTPases (Johnson and Pringle, 1990). Cdc24p is the guanyl nucleotide exchange protein and *BEM3*, a suppressor

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of the temperature sensitivity of *bem2* mutants, encodes the GTPase-activating protein for the Cdc42p GTPase (Zheng *et al.*, 1994). Bem2p is the GTPase-activating protein for the Rho1p GTPase (Peterson *et al.*, 1994). Rho/Rac GTPases have been shown to regulate actin assembly (Li *et al.*, 1995). Actin mutants have been shown to accumulate post-Golgi secretory vesicles (Novick and Botstein, 1985), indicating that in addition to being required for polarity of secretion, the actin cytoskeleton facilitates the exocytic process itself.

A number of gene products are only required for targeting and/or fusion of post-Golgi secretory vesicles with the plasma membrane. These gene products are encoded by *SEC1, SEC2, SEC3, SEC4, SEC5, SEC6, SEC8, SEC9, SEC10, SEC15, SSO1, SSO2, SNC1, SNC2,* and *SCD5* (Novick and Schekman, 1979; Novick *et al.*, 1980; Gerst *et al.*, 1992; Aalto *et al.*, 1993; Protopopov *et al.*, 1993; Nelson *et al.*, 1996). All stages of vesicular transport require members of particular protein families, termed rabs and SNAREs. The rab protein and the soluble *N*-ethyl maleimide-sensitive fusion protein attachment protein receptors (SNAREs) are particular to each stage of transport (Ferro-Novick and Jahn, 1994). In post-Golgi secretion in yeast, the rab family GTPase is Sec4p (Novick and Brennwald, 1993). Sso1p, Sso2p, and Sec9p are target-SNAREs, residing on the target membrane, in this case the plasma membrane (Brennwald *et al.*, 1994). Snc1p and Snc2p are the vesicle-SNAREs, residing on the secretory vesicle (Protopopov *et al.*, 1993). Exocytosis also requires a large protein complex that includes Sec6p, Sec8p, and Sec15p (Bowser *et al.*, 1992; TerBush and Novick, 1995). The function of this complex, which is unique to post-Golgi transport, is not yet known.

The Sec4p GTPase undergoes a cycle of localization between vesicles, plasma membrane and cytosol in parallel with its cycle of nucleotide binding and hydrolysis (Goud *et al.*, 1988). Sec4p is thought to act as a molecular switch, allowing correctly paired v- and t-SNAREs to interact, and thus allowing fusion of exocytic vesicles with the plasma membrane to occur (Brennwald *et al.*, 1994). Genetic studies have shown the Sec4p GTPase to play a central role in regulating the post-Golgi stage of secretion in yeast. *sec4–8*, a temperature-sensitive allele of *SEC4* is synthetically lethal with most of the late-acting *sec* mutants, and an extra copy of the *SEC4* gene is sufficient to suppress many of these mutants (Salminen and Novick, 1987). To further understand the regulation of the exocytic process, we have performed a synthetic lethal screen with *sec4–8*, resulting in isolation of two new *sec3* alleles. The cloning of the *SEC3* gene and phenotypic studies of the new *sec3* alleles are the focus of the present study. The results suggest a role for components of the secretory machinery in bud site selection.

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Techniques

The strains of *S. cerevisiae* used in this study are catalogued in Table 1. Cells were grown in YP medium containing 1% Bacto-yeast extract and 2% Bacto-peptone (Difco Laboratories, Detroit, MI), with either 2% glucose (rich medium, YPD), 0.1% glucose (low glucose medium), 2% galactose (YPG), or 2% raffinose and 0.5% galactose (YPRG). Cells also were grown in minimal medium containing 0.7% yeast nitrogen base without amino acids (Difco), 2% glucose (SD), and supplemented for auxotrophic requirements when necessary as described by Sherman *et al.* (1974). Genetic crosses, sporulation of diploids, and tetrad dissection were performed as described (Sherman *et al.*, 1974). Yeast transformations were performed using the lithium cation method (Ito *et al.*, 1983).

Isolation of New sec3 Alleles Using a Screen for Temperature-sensitive Secretory Mutations That Are Synthetically Lethal with sec4–8

The yeast strain NY876 was constructed by transforming NY774 (*MATα ura3–52 leu2–3,112 sec4–8*) with the plasmid pNB139 (YCp50 *URA3 SEC4*). The cells were mutagenized with ethylmethane sulfonate to give approximately 70% killing (Novick and Schekman, 1979; Novick *et al.*, 1980) and then allowed to recover by growing 28.5 h at 25°C (permissive temperature) in YPD. To enrich for temperature-sensitive secretory mutants, the cells were then shifted to 37°C (restrictive temperature) for 3 h, washed, and resuspended in 1 ml of synthetic minimal medium, layered on top of a 70% Percoll/synthetic minimal medium gradient, centrifuged for 20 min at 20,000 rpm in a Ti50 rotor, and the densest 0.3% of cells collected and plated on SD + leucine. After 4 d of growth at permissive temperature, colonies were replica stamped onto YPD and grown overnight at restrictive temperature. The temperature-sensitive mutants were isolated and screened for the ability to complement *sec4–8*, indicating that there was no mutation in the plasmid copy of *SEC4* that would cause the temperature-sensitive phenotype. Temperature-sensitive colonies were picked and crossed to NY405 (*MAT***a**, *ura3–52 sec4–8*), and the resulting diploids were screened for the ability to grow on rich medium at 37°C. Failure of the diploid cells to grow at the restrictive temperature indicates that the mutant genes are allelic and that the plasmid copy of *SEC4* had acquired a temperature-sensitive mutation. The temperature-sensitive haploid colonies were then stamped onto 5-fluoroorotic acid (5-FOA)-containing plates (Boeke *et al.*, 1987) to screen for the inability of the mutant cells to lose the plasmid, which would suggest synthetic lethality with *sec4–8*. Those mutants that were unable to lose pNB139 would not papillate on 5-FOA plates. The mutants meeting these criteria were crossed to *sec1–1, sec2–41, sec3–2, sec4–8, sec5–24, sec6–4, sec8–9, sec9–4, sec10–2, sec15–1, sec19–1, bet2–1,* and *myo2–66*, and the resultant diploids were analyzed for complementation of the temperature sensitivity. Two mutants did not complement either *sec3–2* or each other and were designated *sec3–4* and *sec3–5*. These two mutants were backcrossed at least three times to wild-type strains to minimize background mutations and to separate the *sec4–8* and the *sec3* mutations. Following backcrossing, these strains were retested for synthetic lethality with *sec4–8* and for noncomplementation of *sec3–2* and each other.

Cloning of SEC3

sec3–4 ura3–52 cells were transformed with a plasmid library of wild-type yeast genomic DNA inserts on the *CEN URA3* vector YCp50 (Rose *et al.*, 1987). Transformants were selected at 25°C on SD and then replica plated to YPD plates, which were incubated at 37°C. Sixteen temperature-resistant colonies were isolated and then tested for plasmid dependency of temperature resistance by growing the transformants on 5-FOA to select for plasmid loss. The Ura$^-$ segregants were

Table 1. Strains used

Strain	Genotype
NY3	*MATa ura3-52,sec1-1*
NY13	*MATa ura3-52*
NY17	*MATa ura3-52 sec6-4*
NY57	*MATa ura3-52 sec9-4*
NY61	*MATa ura3-52 sec10-2*
NY64	*MATa ura3-52 sec15-1*
NY130	*MATa ura3-52 sec2-41*
NY402	*MATa ura3-52, sec5-24*
NY405	*MATa ura3-52 sec4-8*
NY410	*MATa ura3-52 sec 8-9*
NY412	*MATa ura3-52 sec3-2*
NY451	*MATa ura3-52 GAL*$^+$
NY648	*MATa/MATα ura3-52/ura3-52 leu2-3,112/leu2-3,112*
NY774	*MATα ura3-52 leu2-3,112 sec4-8*
NY876	NY774 +pNB139 [YCp50 (*CEN URA3*) *SEC4*]
NY981	*MATa leu2-3,112 his3Δ200*
NY982	*MATα leu2-3,112 his3Δ200*
NY1222	*MATa ura3-52 sec3-2*
NY1225	*MATα leu2-3,112 sec3-2*
NY1241	*MATα ura3 lys2 his3 leu2 ade2 ade3 pfy1-111::LEU2*
NY1280	*MATa psl1/sec3::LEU2 ura3 leu2*
NY1286	*MATa pfy1-111::LEU2 leu2 ade2*
NY1360	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112 HIS4/his4-619 sec1-1/sec1-1 GAL*$^+$*/gal*$^-$
NY1361	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112 HIS4/his4-619 sec2-41/sec2-41 GAL*$^+$*/gal*$^-$
NY1362	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112 HIS4/his4-619 sec3-2/sec3-2 GAL*$^+$*/gal*$^-$
NY1363	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112, HIS4/his4-619 sec4-8/sec4-8 GAL*$^+$*/gal*$^-$
NY1364	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112 HIS4/his4-619 sec5-24/sec5-24 GAL*$^+$*/gal*$^-$
NY1365	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112 HIS4/his4-619 sec6-4/sec6-4 GAL*$^+$*/gal*$^-$
NY1366	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112 HIS4/his4619 sec8-9/sec8-9 GAL*$^+$*/gal*$^-$
NY1367	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112 HIS4/his4-619 sec9-4/sec9-4 GAL*$^+$*/gal*$^-$
NY1368	*MATa/MATα ura3-53/ura3-52 LEU2/leu23,112 HIS4/his4619 sec10-2/sec10-2*
NY1369	*MATa/MATα ura3-53/ura3-52 LEU2/leu2-3,112 HIS4/his4-619 sec15-1/sec15-1*
FY110	*MATa ura3-52,sec3-4*
FY111	*MATα sec3-4 leu2-3,112*
FY112	*MATa sec3-4 leu2-3,112*
FY126	*MATa ura3-52 sec3-5*
FY130	*MATa ura3-52 sec3-5 leu2-3,112*
FY131	*MATα ura3-52 sec3-5 leu2-3,112*
FY136	*MATa/MATα ura3-52/ura3-52 LEU2/leu2-3,112 HIS4/his4-619 sec3-4/sec3-4*
FY137	*MATa/MATα URA3/ura3-52 HIS4/his4-619 sec3-5/sec3-5*
FY139	*MATa ura3-52 leu2-3,112 sec3::GALSEC3-URA3*

then tested for temperature sensitivity. Five transformants demonstrated plasmid-dependent acquisition of temperature resistance. Plasmid DNA from these strains was recovered in the *Escherichia coli* strain DH5α, purified, and then used to retransform *sec3–4* and *sec3–5* cells. All five plasmids complemented both mutations. Restriction mapping demonstrated that three of the five plasmids contained identical inserts. The smallest of the complementing plasmids, called 3YCp50, which contains a 7.5-kb insert, was used for more detailed restriction mapping and subcloning (Figure 1A). All subclones were amplified in *E. coli* DH5α and tested for complementation of *sec3–4* and *sec3–5* mutations at 37°C. This analysis established that the region between the *Xba*I and *Pvu*II sites was within the region necessary for full complementation. This 1.2-kb fragment was ligated into *Xba*I- and *Sma*I-digested pUC119, which was used to transform the *E. coli* strain TG1. SS DNA was sequenced by the Keck Foundation DNA Sequencing Laboratory at Yale University.

To confirm the identity of the cloned gene with the *SEC3* gene, a 6.0-kb complementing restriction fragment, containing the region of the insert from 3YCp50 from the second (more 3′) *Bgl*II site to the *Apa*I site, was inserted into pRS305 (Sikorski and Hieter, 1989), a yeast-integrating plasmid containing *LEU2* as a selectable marker. The plasmid was digested with *Hin*dIII, which cut the plasmid at a unique site within the insert. The digested DNA was used to transform yeast strains NY981 (*MATa leu2–3,112 his3Δ200*) and NY982 (*MATα leu2–3,112 his3Δ200*), so that a copy of the cloned gene tagged with *LEU2* would integrate at the genomic locus of the insert. The transformants, which presumably had the genotype *SEC3::SEC3-LEU2 leu2–3,112 his3Δ200*, were crossed to *sec3–4 leu2–3,112* (FY111 and FY112) and *sec3–5 leu2–3,112* (FY130 and FY131) yeast strains, and the diploids were selected, sporulated, and tetrads dissected and analyzed.

3*Sca*I-*Apa*IpNB419, used in studies on the effects of high-copy *SEC3*, was created by inserting the 4.7-kb *Sca*I-*Apa*I-complementing restriction fragment into pRS426, a yeast 2μ shuttle vector with *URA3* as a selectable marker (Christianson *et al.*, 1992).

Construction of GAL-SEC3 and Turnoff Experiments

A fragment encoding the N-terminal 100 residues of Sec3p was polymerase chain reaction amplified using primers designed to introduce a *Bam*HI site 11 bp upstream and an *Eco*RI site 302 bp downstream of the initiating ATG. This fragment was subcloned

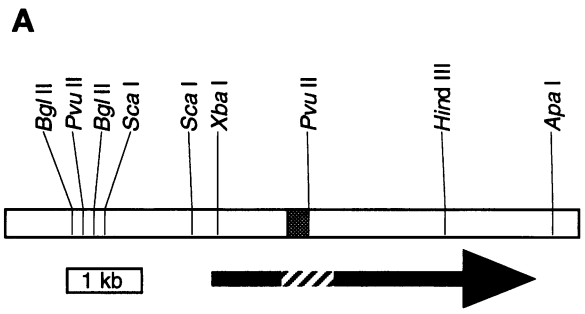

Figure 1. (A) The restriction enzyme map for the *SEC3* locus. The stippled area corresponds to the sequenced region. The Sec3p coding region is shown as an arrow. The coiled coil domain is shown by the striped area. (B) The comparison of the Sec3p sequence to that of the human fibroblast EST, GenBank accession number W42487.

into pNB559, an integrating vector containing the *URA3* gene as a selectable marker, downstream of the *GAL1* promoter. The insert was sequenced to ensure that it contained no mutations. The plasmid was cut within the insert using *Xba*I and used to transform NY648 cells (*MAT***a**/*MATα ura3–5/2 ura3–52 leu2–3,11/leu2–3,112*). Insertion gave one complete copy of *SEC3* under the control of the *GAL1* promoter and one truncated copy. Transformants were selected and sporulated to isolate haploid cells containing *GAL-SEC3* as their sole copy of *SEC3*.

A haploid strain whose *SEC3* gene was under *GAL1* control and an isogenic wild-type strain were grown in YPRG overnight, then harvested and resuspended in either YPG or YPD at 0.05 A_{600nm} units/ml, and grown at either 25°C or 37°C. Growth was monitored by measuring A_{600nm}.

Invertase Secretion

Yeast cultures were grown overnight at 25°C in rich medium (YPD) to early log phase. Approximately 2×10^7 cells were harvested; one-half of the cells was resuspended in 10 mM NaN_3 and stored at 0°C (0 h), the other half was resuspended in YP + 0.1% glucose to derepress synthesis of secreted invertase, incubated at the restrictive temperature of 38°C for 2 h, and then harvested and resuspended in 10 mM NaN_3 at 0°C. Half of the cells in each sample were broken with glass beads by continuous vortexing for 3 min. The broken cells were solubilized in 0.05% Triton X-100. Invertase activity was assayed as described by Goldstein and Lampen (1975). Invertase activity in glass bead lysates = total; invertase activity in unlysed cells = external; external/total × 100 = % secreted.

Thin Section Electron Microscopy

Overnight cultures of wild-type, *sec3–2*, *sec3–4*, and *sec3–5* cells were adjusted to 0.3 A_{600nm} units/ml in YPD, and one-half of each culture was incubated at 25°C and half at 37°C, except for *sec3–2*, which was incubated at 39°C for 2 h. Cells (15 ml) were fixed by adding 50% glutaraldehyde to a final concentration of 1%. The cells were incubated for 5 min on ice and then pelleted in a tabletop centrifuge at 4°C. The cell pellets were resuspended in 1 ml of distilled water and transferred to microfuge tubes. Samples were subsequently fixed with permanganate and processed according to the method of Kaiser and Schekman (1990).

Carboxypeptidase Y (CPY) Pulse-Chase Experiments

Overnight cultures grown to early log phase in minimal medium (SD), supplemented as necessary for auxotrophic requirements, were sedimented in a tabletop centrifuge and resuspended in fresh medium to give approximately 2 A_{600nm} units/ml. Cells were shifted to the experimental temperatures of either 25°C or 37°C for 30 min prior to labeling. For labeling, cells were pulsed for 4 min with 0.15 mCi of ^{35}S-ProMix (Amersham, Arlington Heights, IL) per ml of cells and then chased for 0, 3, 10, and 30 min with 0.5% methionine and cysteine. At each time point, 1 ml of labeled culture was removed to a microfuge tube on ice containing trichloroacetic acid to a final concentration of 5%. Samples were kept on ice for at least 5 min prior to centrifugation for 5 min at 4°C to pellet labeled proteins. Pellets were washed twice with ice-cold acetone and air dried and then resuspended in 75 μl of boiling buffer (10 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, 1% SDS). Glass beads were added to the meniscus of the liquid, and samples were vortexed for 1 min and then boiled for 5 min. Seven hundred microliters of IP buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 0.5% Tween 20) were added to each lysate, which was vortexed for 1 min, then microfuged for 10 min at 4°C. Six hundred fifty microliters of each sample were removed to fresh tubes and diluted with an equal volume of IP buffer. CPY was immunoprecipitated with anti-CPY serum (Valls *et al.*, 1990). Samples were electrophoresed on 8% SDS-polyacrylamide gels.

Calcofluor and Rhodamine-Phalloidin Staining of Cells

Cells were stained with rhodamine-phalloidin to visualize filamentous (F) actin structures (Sigma, St. Louis, MO) and with Calcofluor to visualize chitinous bud scars (Sigma) as follows: Cells were grown at 25°C to early exponential phase. Cells were pelleted at 4°C and resuspended in phosphate-buffered saline (PBS) with 2% glucose, 20 mM EGTA, and 3.7% formaldehyde. Cells were incubated for 1 h at room temperature and then washed twice with PBS. Each sample was then split and the cells processed further either for tetramethylrhodamine isothiocyanate-phalloidin or for Calcofluor staining.

For phalloidin staining, cells were permeabilized for 10 min in 0.1% Triton X-100, then washed twice with PBS, resuspended in 2 μg/ml rhodamine-phalloidin, and incubated for 30 min in the dark at room temperature. The cells were then washed five times with PBS, resuspended in PBS, mounted on slides, and observed on a Zeiss Axiophot microscope using rhodamine filters and the 100× objective.

For Calcofluor staining, cells were resuspended in 0.1 mg/ml Calcofluor and incubated for 5 min in the dark at room temperature. The stained cells were washed three times with water, resuspended in water, briefly sonicated, and mounted on slides. Cells were visualized under UV excitation using a Zeiss Axiophot microscope and the 100× objective.

RESULTS

Screen to Identify New sec Mutations Synthetically Lethal with sec4–8

Synthetic lethality is a genetic interaction where mutants defective in either of two genes are viable, but when the defects are combined, the resultant double mutant is inviable. This strong genetic interaction usu-

Table 2. Growth properties of *sec3* strains

	14°C		25°C		30°C		34°C		37°C	
Allele	YPD	SD	YPD	SD	YPD	SD	YPD	SD	YPD	SD
SEC3	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
sec3-2	+	++	+++	+++	+++	+++	+++	++	−	−
sec3-4	+/−	+	++	+++	+/−	++	−	+/−	−	−
sec3-5	+/−	++	+	++	−	+/−	−	−	−	−
sec3Δ	+/−	+/−	+/−	+/−	−	+/−	−	−	−	−

ally implies that the two gene products are functioning at the same stage of a biological pathway, either in series or in parallel. Synthetic lethality has been previously demonstrated between *sec4–8*, a temperature-sensitive allele of *SEC4*, and mutant alleles of several other genes whose products function in the secretory pathway (Salminen and Novick, 1987). We have used a screen for synthetic lethality with *sec4–8* in combination with density enrichment for *sec* mutants to isolate new mutants that would presumably function at the post-Golgi stage of secretion. Complementation analysis indicated that new alleles of *sec1*, *sec2*, *sec5*, *sec8*, *sec19*, and *bet2* were isolated. Alleles of these genes, with the exception of *sec1*, have been previously shown to be synthetically lethal with *sec4–8* (Salminen and Novick, 1987; Rossi *et al.*, 1991). Most of these mutants have not been studied further.

An important product of this screen was the isolation of two new alleles of *sec3*, *sec3–4*, and *sec3–5*. These new alleles are unable to complement the temperature sensitivity of the *sec3–2* mutant or of each other. Genetic analysis showed that both mutations are linked to the *URA3* locus on chromosome V as well as being centromerically linked, consistent with the assigned map position of *SEC3* (our unpublished results). The synthetic lethality of *sec3–4* and *sec3–5* with *sec4–8* was reconfirmed using backcrossed strains. Both new alleles cause a more severe temperature-sensitive phenotype than *sec3–2*. *sec3–2*, *sec3–4*, and *sec3–5* were tested for growth at 14, 25, 30, 34, and 37°C on both minimal and rich media. The results are summarized in Table 2. *sec3–2* cells are temperature sensitive only at 37°C and grow equally well on minimal or rich medium. *sec3–4* and *sec3–5* cells grow significantly better on minimal than on rich medium and are less temperature-sensitive on minimal medium.

sec3 Mutants Are Blocked for Invertase Secretion

We assayed all three of our temperature-sensitive *sec3* mutants for the ability to secrete the periplasmic form of the enzyme invertase. In the course of these studies, we found that we were unable to use our standard method of spheroplast preparation without spontaneous lysis of the *sec3–4* and *sec3–5* cells. Therefore, we have developed an alternative procedure using glass bead lysates, rather than spheroplast lysates, to measure the internal invertase pool. When assayed using this method at the permissive temperature of 25°C, no defect was seen in secretion of invertase for any of the *sec3* mutants (our unpublished data). We found that all of the *sec3* mutants were blocked for invertase secretion at the restrictive temperature (Figure 2), and that the severity of the secretory block correlated with the severity of the growth defect of each of the mutants (Table 2).

sec3 Mutants Accumulate Endoplasmic Reticulum (ER), Golgi, and Secretory Vesicles

We next used electron microscopy to examine the *sec3* mutant cells for membrane accumulation (Figure 3). We chose to use the permanganate staining method, because in addition to selectively staining cellular membranes, this method does not require removal of the cell wall. Although it has been previously reported that the *sec3–2* mutant accumulates post-Golgi secretory vesicles, the strain used for these studies was later found to contain the *ypt1–2* mutation (Bacon *et al.*,

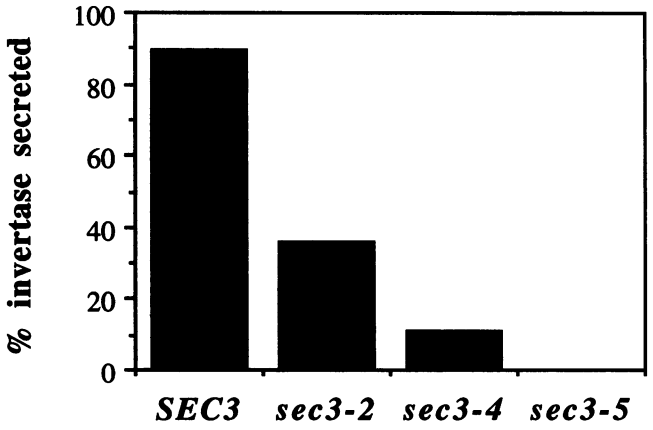

Figure 2. Invertase secretion was measured following a 2-h shift to 38°C. Strains used were *SEC3* = NY13, *sec3–2* = NY1222, *sec3–4* = FY110, *sec3–5* = FY126.

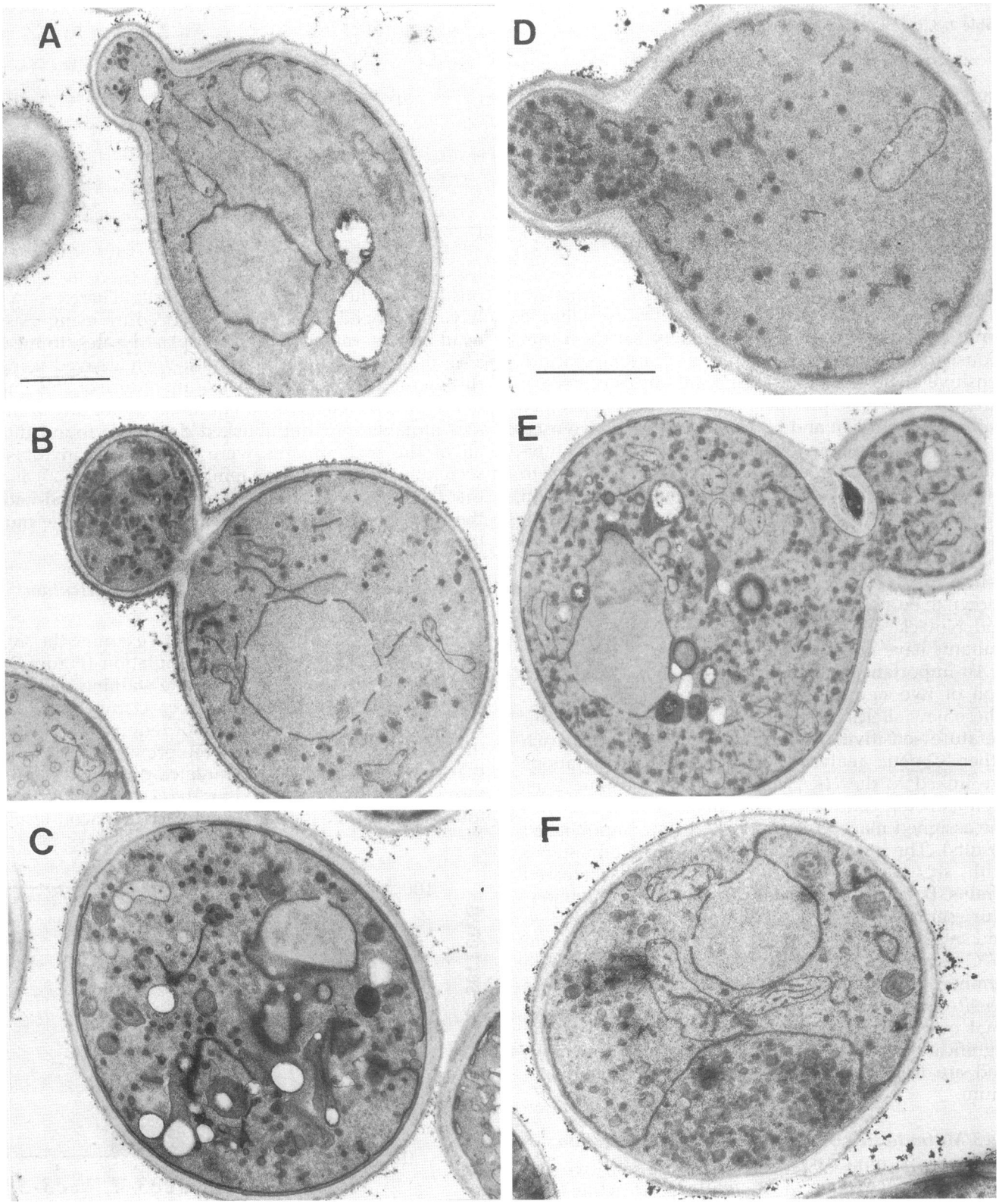

Figure 3. Cells grown at 25°C (B and C), or shifted to 37°C (A, E, and F), or 39°C (D) were fixed and processed for electron microscopy. Strains used were NY13 (*SEC3*, A), NY412 (*sec3–2*, D), FY110 (*sec3–4*, B and E), and FY126 (*sec3–5*, C and F). Bars, 1 μm. Magnification in B, C, and F is the same as in A.

1989). We examined *sec3–2* cells that had been backcrossed away from the *ypt1–2* mutation, and confirmed that although the cells resemble wild-type cells at permissive temperature, at the restrictive temperature they accumulate what appear to be post-Golgi secretory vesicles, primarily in the bud (Figure 3D).

When *sec3–4* cells and *sec3–5* cells were examined, the phenotypes were strikingly different from that of *sec3–2*. At the permissive temperature of 25°C, both mutants displayed an accumulation of Golgi structures and vesicles (Figure 3, B and C). Both mutants also displayed some accumulation of ER membranes; the amount of accumulated ER varied from cell to cell in the sections. At the restrictive temperature, the vesicle accumulation was more pronounced (Figure 3, E and F). These pleiotropic phenotypes suggest that these mutations affect multiple stages of the secretory pathway. The *sec3–4* and *sec3–5* cells also frequently displayed thick necks and improperly formed septa at the mother bud-neck. An example of this is seen in Figure 3E, where the septum forming between the mother cell and the bud does not completely span the neck, and electron-dense material appears to be trapped between the layers of septum. This may reflect improperly directed secretion at the time of cytokinesis.

An alternative hypothesis for the presence of early secretory pathway structures in these slow-growing strains is that the secretory pathway is constitutively backed up and that Sec3p has no direct effect on early secretory stages. To address this question, *sec4–8* and *sec9–4* cells were grown at the semirestrictive temperature of 28.5°C for 16 to 20 h prior to fixation and processing. At this temperature, these strains grow at rates similar to those of *sec3–4* and *sec3–5* strains. If the membrane accumulation was solely due to a constitutive impairment of the late secretory pathway, these strains would be expected to accumulate ER and Golgi structures. The only structures that accumulated in either *sec4–8* or *sec9–4* cells were post-Golgi secretory vesicles (our unpublished data), implying that the aberrant organelle structures seen in *sec3* strains are the result of impaired function of Sec3p specifically and not of post-Golgi secretion generally.

sec3 Mutants Display Partial Blocks in Transport of CPY to the Vacuole

sec3 mutants were next examined for defects in the transport of the vacuolar protease CPY to determine whether there was a transport defect at the earlier stages of the secretory pathway (Figure 4). CPY is modified in the ER and in the Golgi en route to its arrival in the vacuole where it is proteolytically cleaved to a 61 kDa (M) active form. The ER and Golgi forms of CPY, respectively, 67 kDa (P1) and 69 kDa (P2), accumulate in mutants with a secretory block at

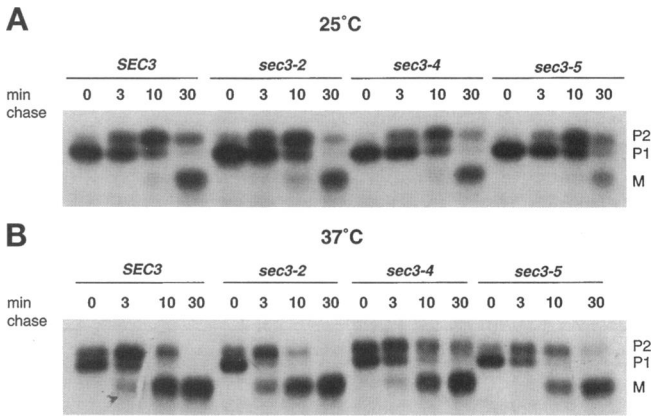

Figure 4. Cells were preshifted to 37°C for 30 min prior to labeling. Cells were pulsed with ^{35}S-ProMix for 4 min, then chased with unlabeled cysteine and methionine. At time points, aliquots were removed and CPY was immunoprecipitated. Samples were electrophoresed on 8% SDS-polyacrylamide gels. P1, 67-kDa ER form of CPY; P2, 69-kDa Golgi form of CPY; M, 61-kDa mature vacuolar form of CPY. Strains used were: *SEC3* = NY13, *sec3–2* = NY1222, *sec3–4* = FY110, *sec3–5* = FY126.

these stages (Stevens *et al.*, 1982). Pulse-chase studies on lysates from *sec3* mutant cells showed that *sec3–2* cells were able to transport CPY to the vacuole with wild-type kinetics at both permissive and restrictive temperatures (Figure 4). At permissive temperature, *sec3–4* cells also transported CPY to the vacuole with transit times similar to those of wild type (Figure 4A). *sec3–5* cells displayed a delay in the conversion of CPY from the Golgi to the vacuolar form at 25°C. At 30 min of chase, in *sec3–5* cells, CPY was found in ER, Golgi, and vacuolar forms, whereas the other strains no longer had any of the ER form present. At the restrictive temperature, processing from the Golgi form to the vacuolar form was slowed in both *sec3–4* and *sec3–5*, although some mature CPY is produced, indicating that the block in transport is not tight (Figure 4B). In the wild-type and *sec3–2* cells, ER and Golgi forms of CPY were present at 0 min of chase. By 3 min of chase, mature CPY began to appear, although the Golgi form predominated. At 10 min of chase, most of the CPY was mature, although some Golgi form remained. At 30 min of chase, all CPY was mature. In *sec3–4* cells, ER and Golgi forms of CPY were present at 0 min of chase and at 3 min of chase. At 10 min of chase, mature CPY appeared, although ER and Golgi forms were still present. After 30 min of chase, although there was an increase in the amount of mature CPY, the Golgi form was still present. In *sec3–5* cells, there was very little of the Golgi form of CPY present at 0 min of chase; the ER form predominated. At 3 min of chase, ER and Golgi forms of CPY were present. Mature CPY first appeared at 10 min of chase although ER and Golgi forms were still present. Even after 30 min of chase, the Golgi form of CPY was still present

Table 3. Synthetic lethality of *sec3* alleles with profilin

	4 Viable spores			3 Viable spores		2 Viable spores		1 Viable spore
Cross	4−/0+	3−/1+	2−/2+	2−/1+	0−/3+	2−/0+	0−/2+	1−/0+
NY1225 (*sec3-2*) × NY1286 (*pfy1-111*)[a]	3	5	2	1	0	1	0	0
NY1286 (*pfy1-111*) × FY111 (*sec3-4*)[b]	3	0	0	8	0	0	0	1
NY1286 (*pfy1-111*) × FY223 (*sec3-5*)[b]	2	0	0	7	1	0	2	0

[a]Double mutants are ±25°C, −30°C.
[b]Synthetically lethal.

in *sec3–5* cells. This suggests that transport of CPY from the ER to the Golgi, and from the Golgi to the vacuole, is slowed or partially blocked in *sec3–4* and *sec3–5* cells at the restrictive temperature.

Cloning of SEC3

The restrictive growth properties of the new *sec3–4* allele were useful for cloning of the *SEC3* gene by complementation of its temperature sensitivity. Five plasmids, containing three different yeast genomic inserts that were able to fully complement *sec3–4* and *sec3–5* at 37°C, were recovered from a yeast genomic library. The smallest of the inserts was 7.5 kb and was subcloned to a 6.0-kb insert that retained the ability to fully complement both mutations (Figure 1). This 6.0-kb insert was shown to contain the *SEC3* gene by crossing a strain where the insert tagged with *LEU2* was integrated at its genomic locus (presumed genotype *SEC3::SEC3-LEU2*), with *sec3–4* and *sec3–5* cells that had the *leu2–3,112* auxotrophy. Thirty-nine tetrads were dissected and analyzed for temperature sensitivity and requirement for leucine. Leu^+ and Ts^+ phenotypes segregated 2:2, and all Leu^+ spores were also Ts^+ (parental ditypes), demonstrating that the cloned sequence is *SEC3*.

SEC3 Is Identical to PSL1

We sequenced a 1.2-kb *Xba*I-*Pvu*II restriction fragment cloned into pUC119 because our subcloning and complementation results had shown that a deletion of this region resulted in partial complementation of the temperature sensitivity of *sec3–4* and *sec3–5*, indicating that these sites were either within the *SEC3* coding sequence or in regulatory sequences. The sequence of this fragment is contained within that of a gene cloned and sequenced by Haarer *et al.* (1996) as *profilin synthetic lethal 1* (*PSL1*; Figure 1A, GenBank accession number L22204). The predicted restriction map for *PSL1* was in close agreement with the *SEC3* restriction map. All three *sec3* strains were unable to complement a temperature-sensitive strain in which *PSL1* had been disrupted. Furthermore, in a cross between *sec3–2* and *psl1Δ* strains, all four spores in each tetrad were temperature sensitive, indicating tight linkage of the genes. These results demonstrate that *SEC3* and *PSL1* are the same gene.

The *SEC3* sequence predicts a 1336-amino acid protein of 154 kDa containing a coiled coil of 145 amino acids (Figure 1A). The Sec3 protein is hydrophilic, with many charged residues. We have found an expressed sequence tag (EST) derived from human fibroblast cells (GenBank accession number W42487) which shows 59% identity to a 22-amino acid region near the C-terminus of Sec3p from codons 1090–1111 (Figure 1B). This EST may represent a portion of a mammalian Sec3p-encoding gene.

The *SEC3* gene is not essential. Yeast cells whose sole copy of the *SEC3* gene was under the control of the inducible *GAL1* promoter were viable, although severely growth impaired and temperature sensitive when grown under conditions where transcription should not occur (Figure 5). These results are consistent with those of Haarer *et al.* (1996), who obtained the same phenotype by deleting a portion of the *SEC3* gene.

A strain containing the mutant allele of profilin, *pfy1–111*, used by Haarer *et al.* (1993, 1996) in their genetic screen, was crossed to our *sec3* strains to see whether this genetic interaction is a general property of *sec3* mutants or allele specific. All three *sec3* alleles genetically interact with this profilin mutation; for two of the alleles, *sec3–4* and *sec3–5*, the interaction is lethal (Table 3). The profilin mutant is altered in a central basic region thought to be important for binding of phosphatidylinositol bisphosphate as well as for interaction with actin (Haarer *et al.*, 1993). We also examined the *pfy1–111* mutant by thin section electron microscopy to see whether this profilin mutation conferred a secretory defect. Although we saw a few secretory vesicles, there was no striking membrane accumulation in this strain after 1 h of growth at the restrictive temperature, indicating that any secretion defect would probably be quite mild (our unpublished data).

Table 4. Effects of high-copy *SEC3*

Suppression	Growth inhibition	No effect
sec3-2[a]	*sec10-2*[b]	*sec1-1*
sec3-4[a]	*sec15-1* (very slight)[b]	*sec2-41*
sec3-5[a]	*sec8-9* (very slight)[b]	*sec4-8*
sec5-24[b]	*ypt1-3*[a]	*sec6-4*
pfy1-111[b]	*ypt1*$^{I121, V161}$[b]	*sec7-1*
		sec9-4
		sec12-4
		sec13-1
		sec14-1
		sec16-2
		sec17-1
		sec18-1
		sec19-1
		sec20-1
		sec21-1
		sec22-3
		sec23-1
		act1-2
		act1-3

Mutants containing the high-copy *SEC3* plasmid were scored for growth phenotype in comparison to cells containing the plasmid without *SEC3*.

[a]Phenotype was seen at 37°C.

[b]Phenotype was seen at 34°C.

Effects of High-Copy SEC3

By causing cells to produce an excess of a wild-type gene product in various mutant backgrounds, other genetic interactions that can reflect functional interactions may be observed. One such interaction is termed dosage suppression, where overproduction of one gene product suppresses the mutant phenotype. Another interaction is termed synthetic dosage toxicity, where the presence of excess gene product exacerbates the mutant phenotype, causing growth inhibition under conditions where the mutant would otherwise be viable (Kroll *et al.*, 1996).

Both types of interactions were observed when a high-copy *SEC3* plasmid was transformed into secretory and cytoskeletal mutants (Table 4). High-copy *SEC3* was able to complement all of the *sec3* mutants. Dosage suppression was observed with *sec5–24* and *pfy1–111*. Strong negative effects on growth were observed with *sec10–2* and two alleles of *ypt1*, whereas slight negative effects were observed with *sec8–9* and *sec15–1*. No effects were observed in wild-type cells, actin mutants, or other *sec* mutants.

Actin Is Polarized in sec3 Mutants and in Cells Depleted of Sec3p

The genetic interactions of *sec3* alleles with a profilin mutation, *pfy1–111*, and the previously described role of actin in secretion (Novick and Botstein, 1985), raised the possibility that actin may be abnormally distributed in *sec3* strains. Actin was observed in wild-type and *sec3* strains by staining with rhodamine-conjugated phalloidin, which binds to F-actin and prevents its depolymerization (Figure 6 and Table 5). Since two of the *sec3* mutants, *sec3–4* and *sec3–5*, are severely growth impaired even at the permissive temperature, we observed polymerized actin structures only at the permissive temperature. Wild-type and *sec3–2* cells had normal morphology and actin polarization; actin cables were seen and actin patches were found in the bud (Figure 6, A and B). The *sec3–4* (Figure 6C) and *sec3–5* (Figure 6D) strains, which have abnormal morphology even at permissive temperature, had partially depolarized actin patches, which were seen in the mother cell and in the bud. The actin patches still appeared to be largely concentrated in the bud. Actin cables were present in both of these strains.

To see whether these effects are due to the *sec3* mutations specifically or whether they reflect the loss of Sec3p function, we looked at actin structure in cells whose sole copy of *SEC3* is under the control of the inducible *GAL1* promoter under conditions where the gene would and would not be expressed (Figure 6, G–K). Cells grown at 25°C in YPRG, expressing the *SEC3* gene, had normal actin morphology (Figure 6G). Cells growing at 25°C were also observed at different times after shift into medium with glucose as the carbon source, where the *GAL1* promoter would be repressed and Sec3p would not be produced. These cells grown in YPD had a slow doubling time after about 17 h of growth in YPD. After 14 h (Figure 6H), 17 h (Figure 6I), and 20 h (Figure 6J) of growth in YPD, actin morphology was essentially normal, with little depolarization of actin patches and actin cables present, although cell shape became increasingly abnormal. Following 30 h of growth in YPD (Figure 6K), actin appeared to be somewhat abnormal, with patches in mother cells and few cables. Many cells were enlarged and round. Even under these conditions, the actin patches tended to be concentrated in the bud. These results, that only severe long-term loss of Sec3p function is reflected in F-actin structural changes, imply that Sec3p does not directly affect actin structure. The genetic interactions of *SEC3* with *PFY1* are unlikely to be the result of Sec3p having a similar role to profilin in regulating actin polymerization and depolymerization. Actin morphology was also examined in the other late-acting *sec* mutants. All showed the normal polarized distribution of actin (our unpublished results).

Calcofluor Staining Reveals Abnormal Morphology and Diploid-specific Bud Site Selection Defects in sec3–4, sec3–5, sec4–8, *and* sec9–4 *Cells*

Calcofluor staining of chitin in bud scars revealed a diploid-specific defect in bud site selection in *sec3–101/sec3–101* mutants; cells budded randomly rather than

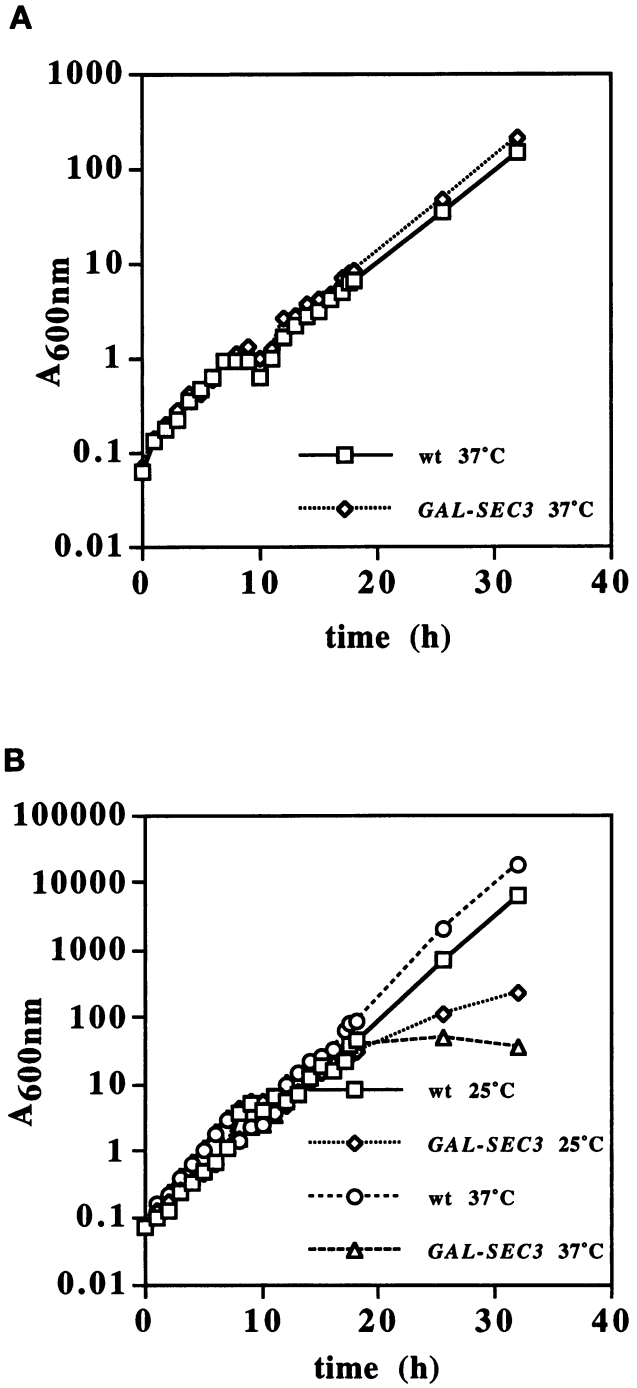

Figure 5. Cells were grown overnight to early log phase in YPRG and then diluted into either YPG (A) or YPD (B) at the indicated temperatures. wt, NY451; *GAL-SEC3*, FY139.

in the usual bipolar pattern (Haarer *et al.*, 1996). We have examined budding patterns in haploid and homozygous diploid *sec3* mutants (Figure 7 and Table 5). Haploid *sec3–2* (Figure 7B), *sec3–4* (Figure 7C), and *sec3–5* (Figure 7D) cells all budded in the expected axial pattern, although the Calcofluor staining revealed enlarged, brightly staining necks and abnormal chitin deposits in the *sec3–4* and *sec3–5* strains. The abnormal septa seen by electron microscopic examination of *sec3–4* and *sec3–5* cells may be identical to these abnormal chitin deposits. The homozygous diploids all bud randomly (Figure 7, O–Q). Additionally, the *sec3–4/sec3–4* (Figure 7P) and *sec3–5/sec3–5* (Figure 7Q) strains showed enlarged necks and abnormal chitin deposits.

Bud site selection was also examined in haploid cells whose sole copy of *SEC3* was under the control of the inducible *GAL1* promoter. These cells had the normal axial budding pattern and normal morphology when grown in medium containing raffinose and galactose as carbon sources (Figure 7G). Cells depleted of Sec3p by growth in medium with glucose as the sole carbon source had a phenotype similar to that seen with the haploid *sec3–4* and *sec3–5* mutants; budding was axial, but enlarged necks and abnormal chitin deposits were seen (Figure 7, H–K).

We then examined the other nine post-Golgi *sec* mutants for budding pattern abnormalities. The *sec4–8* (Figure 7R) and *sec9–4* (Figure 7S) mutations caused random budding patterns in diploid cells homozygous for these alleles. Enlarged necks and abnormal chitin deposits were also seen with these strains. *sec1–1*, *sec2–41*, *sec5–24*, *sec6–4*, *sec8–9*, *sec10–2*, and *sec15–1* had no striking effects on either budding pattern in haploids and diploids or on cell morphology (our unpublished results).

DISCUSSION

Sec4p, the rab family member involved in post-Golgi secretion in yeast, occupies a central role in exocytosis. New alleles of many, although not all, of the genes previously shown to be synthetically lethal with *sec4–8* were isolated in the screen used here for isolation of temperature-sensitive secretory mutants synthetically lethal with *sec4–8*. The new alleles of *sec1* isolated in this screen are the first indication of synthetic lethal interactions between *sec1* and *sec4*. The principle benefit of this screen was the isolation of two new *sec3* alleles, which proved especially useful for cloning the *SEC3* gene.

The result of this cloning and sequencing was the discovery, made independently by Haarer *et al.* (1996), that *SEC3* is identical to *PSL1*. The *SEC3* gene has thus been identified in two screens for temperature-sensitive secretory mutations and in a screen for mutations synthetically lethal with profilin. The identity of *SEC3* and *PSL1* provides new evidence for the connection between the actin cytoskeleton and the secretory pathway first documented by Novick and Botstein (1985) in their studies of temperature-sensitive actin mutants. Among the defects of the actin mutants were the ac-

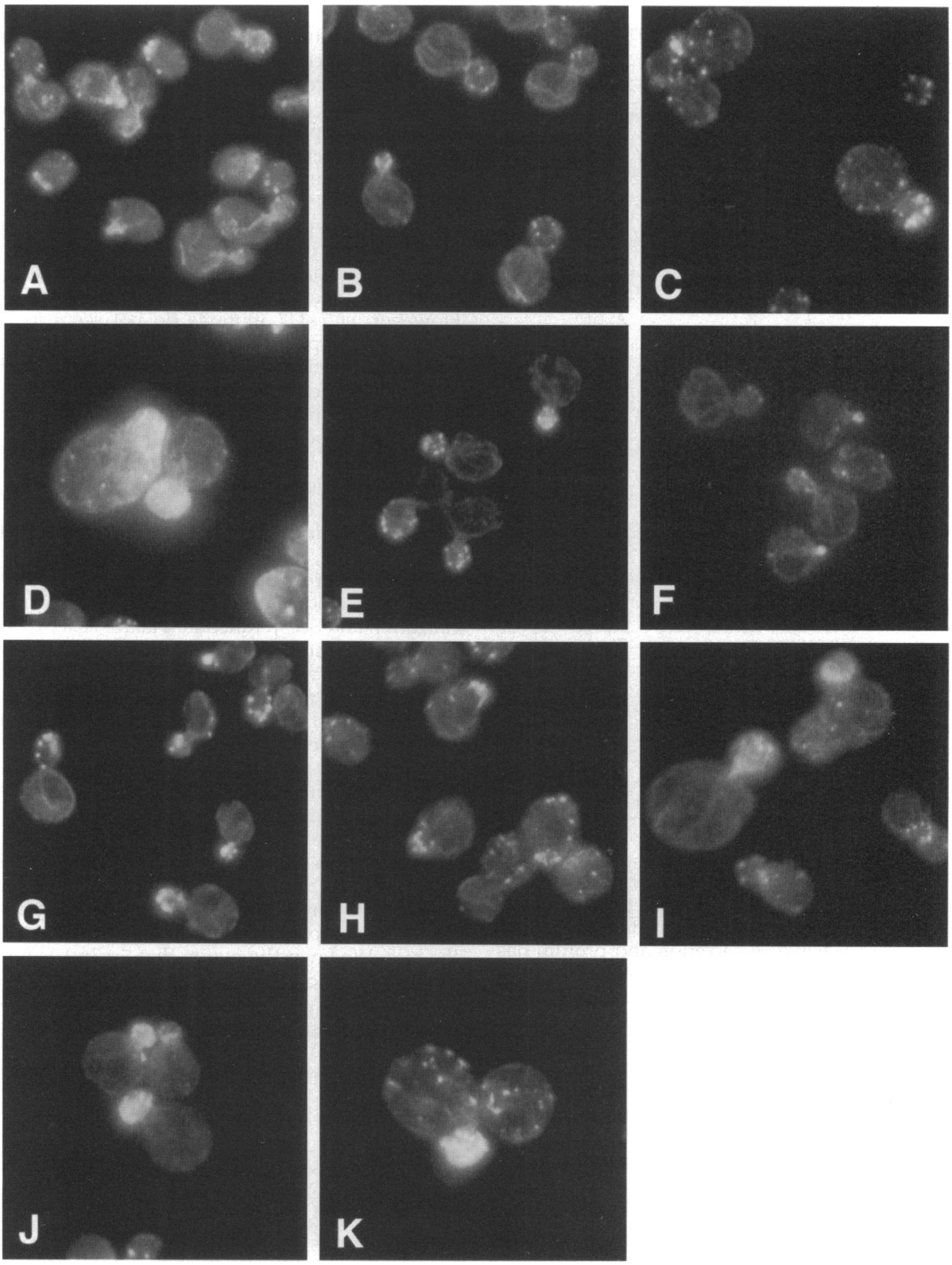

Figure 6. Cells grown at 25°C were stained with tetramethylrhodamine isothiocyanate-phalloidin. Unless otherwise specified, cultures were grown in YPD. A, NY13, *SEC3*; B, NY1222, *sec3–2*; C, FY110, *sec3–4*; D, FY126, *sec3–5*; E and F, NY451, *SEC3*; E is in YPRG. Cells shown in G–K are FY139 = *GAL-SEC3* cells grown in YPRG (G) or shifted into YPD for 14 h (H), 17 h (I), 20 h (J), and 30 h (K). Bar, 5 μm.

Table 5. Summary of actin and budding pattern results for *sec* mutants

Genotype	Actin	Budding pattern
SEC	+	Axial
sec3-2	+	Axial
sec3-4	Depolarized[a]	Axial[b]
sec3-5	Depolarized[a]	Axial[b]
GALSEC3		
YPRG	+	Axial
YPD, 14 h	+	Axial[b]
YPD, 17 h	+	Axial[b]
YPD, 20 h	+	Axial[b]
YPD, 30 h	Depolarized[a]	Axial[b]
sec1-1	+	Axial
sec2-41	+	Axial
sec4-8	+	Axial[b]
sec5-24	+	Axial
sec6-4	+	Axial
sec8-9	+	Axial
sec9-4	+	Axial[b]
sec10-2	+	Axial
sec15-1	+	Axial
SEC/SEC	ND	Bipolar
sec3-2/sec3-2	ND	Random
sec3-4/sec3-4	ND	Random[b]
sec3-5/sec3-5	ND	Random[b]
sec1-1/sec1-1	ND	Bipolar
sec2-41/sec2-41	ND	Bipolar
sec4-8/sec4-8	ND	Random[b]
sec5-24/sec5-24	ND	Bipolar
sec6-4/sec6-4	ND	Bipolar
sec8-9/sec8-9	ND	Bipolar
sec9-4/sec9-4	ND	Random[b]
sec10-2/sec10-2	ND	Bipolar
sec15-1/sec15-1	ND	Bipolar

[a]These strains had actin cables, but patches were somewhat depolarized and found in mother and daughter cells.

[b]These strains had enlarged necks and abnormal chitin deposits.

cumulation of post-Golgi secretory vesicles and a partial block in the release of the secreted form of invertase following a shift to the restrictive temperature.

The relationship between secretion and profilin has not yet been defined, although there are several lines of evidence indicating such a connection. The isolation of the gene encoding profilin, *PFY1*, and the gene for one of the post-Golgi v-SNAREs, *SNC1*, in the same genetic screen was the first indication that profilin and secretion may be related (Vojtek *et al.*, 1991; Gerst *et al.*, 1992). Both *PFY1* and *SNC1* are suppressors of loss of C-terminal function of the adenylate cyclase-associated protein (CAP). The mechanism of suppression of the loss of C-terminal CAP function is unclear for both *PFY1* and *SNC1*. Mutant forms of CAP are not reported to affect secretory function (Gerst *et al.*, 1992). CAP is found associated with the actin cytoskeleton, and its C-terminal domain binds to actin (Freeman *et al.*, 1996; Zelicof *et al.*, 1996). CAP also contains an SH3 domain which can bind to the actin-binding protein Abp1p (Freeman *et al.*, 1996). There is a significant amount of additional genetic evidence indicating interaction between profilin and exocytosis. All known *sec3* alleles have negative synthetic lethal interactions with *pfy1–111*, and we have found that high-copy *SEC3* can suppress the *pfy1–111* mutation. *pfy1–111* also has synthetic negative interactions with the late-acting *sec* mutants *sec2–41*, *sec4–8*, *sec8–9*, and *sec9–4* (Haarer *et al.*, 1996).

The phenotype of profilin mutants is similar in many respects to that of actin or *cdc24* mutants: chitin is delocalized and cells are enlarged, indicating a defect in the polarity of secretion (Haarer *et al.*, 1990). However, *pfy1–111* mutants do not seem to have a significant *sec* phenotype. Electron microscopic studies on this profilin mutant show the accumulation of only a few secretory vesicles 1 h after shift to the restrictive temperature, indicating that any secretory defect would be fairly mild.

Haarer *et al.* (1996), in their studies of the *sec3–101* mutant, observed a relatively normal pattern of localization of F-actin as visualized with rhodamine-labeled phalloidin at permissive temperature, but suggest that the disappearance of this fluorescence following a prolonged incubation at the restrictive temperature indicates a connection between actin localization and Sec3p function (Haarer *et al.*, 1996). The *sec3–2* mutant has F-actin structures indistinguishable from those in wild-type cells at the permissive temperature, with actin patches in the bud and actin cables. Using *sec3* alleles that cause more severe growth defects and aberrant morphology even at permissive temperature (*sec3–4* and *sec3–5*), we see significant abnormalities in actin localization at permissive temperature. Depletion of Sec3p from cells does not seem to perturb the actin cytoskeleton immediately, although growth under conditions of chronic Sec3p depletion causes partial depolarization of actin patches. Even under conditions of chronic loss of Sec3p function, the actin patches are still largely concentrated in the bud. We favor the notion that Sec3p has no direct effect on actin localization since only the most extreme and chronic impairments of Sec3p function affect F-actin structures. Therefore, the genetic interactions of *SEC3* with *PFY1* do not reflect a role for Sec3p in regulation of actin structure. Other post-Golgi *sec* mu-

Figure 7 (facing page). Cells grown at 25°C were stained with Calcofluor. Unless otherwise specified, cells were grown in YPD. A, NY13, *SEC3*; B, NY1222, *sec3–2*; C, FY110, *sec3–4*; D, FY126, *sec3–5*; E and F, NY451, *SEC3*; E is in YPRG. Cells shown in G–K are FY139 = *GAL-SEC3* cells grown in YPRG (G) or shifted into YPD for 14 h (H), 17 h (I), 20 h (J), and 30 h (K). L, NY405, *sec4–8*; M, NY57, *sec9–4*; N, NY648, *SEC3/SEC3*; O, NY1362, *sec3–2/sec3–2*; p, 136, *sec3–4/sec3–4*; Q, 136, *sec3–5/sec3–5*; R, NY1363, *sec4–8/sec4–8*; S, NY1367, *sec9–4/sec9–4*. Bar, 5 μm.

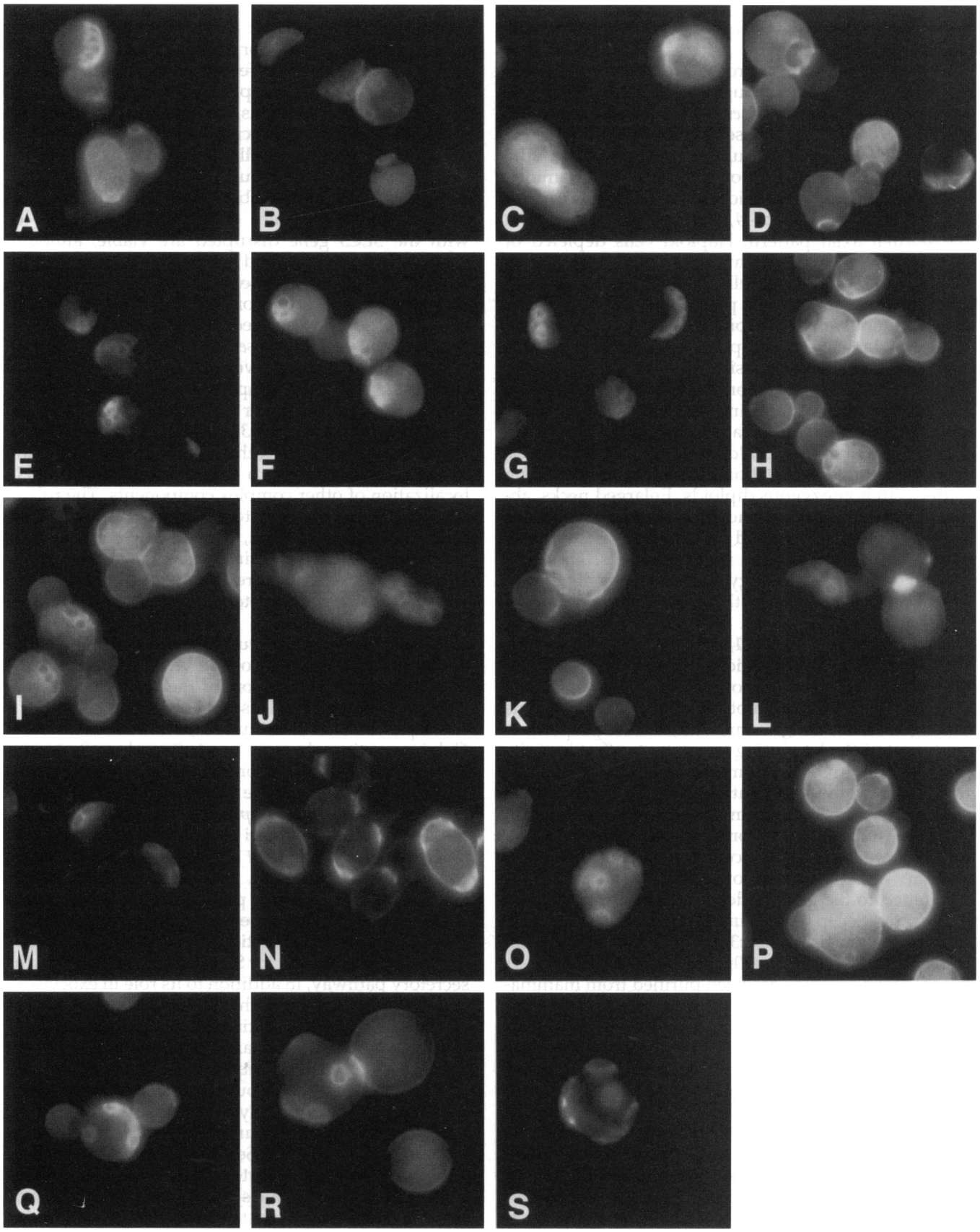

Figure 7.

tants also have normal polarized actin structures at the permissive temperature.

Bud site selection is a process intimately connected with cell polarity and thought to be mediated by the actin cytoskeleton. Haarer *et al.* (1996) have found that the *sec3–101* mutation causes a diploid-specific defect in bud site selection, resulting in random budding instead of the normal bipolar pattern. We examined budding patterns in haploid and homozygous diploid *sec3* mutants. *sec3–2*, *sec3–4*, and *sec3–5* cells all bud in the normal axial pattern. Haploid cells depleted of Sec3p also have the normal axial budding pattern characteristic of haploid cells. Diploid *sec3* mutants all have the random budding phenotype. The *sec3–4* and *sec3–5* mutants, both haploids and diploids, and haploid cells depleted of Sec3p also have enlarged necks and abnormal chitin deposits. Thus, Sec3p appears to be specifically required for maintenance of the diploid-specific bipolar budding pattern.

We examined all of the late-acting *sec* mutants, haploids and diploids, for budding pattern abnormalities. *sec4–8* and *sec9–4* mutations cause random budding patterns in homozygous diploids. Enlarged necks, abnormal chitin deposits, and misshapen cells are also seen in haploid and diploid cells with these mutations, similar to the defects seen in *sec3* mutants. These defects in cellular morphology might reflect a disruption of the normal polarity of the secretory pathway and suggest a need for Sec3p, Sec4p, and Sec9p in neck formation. *sec1–1*, *sec2–41*, *sec5–24*, *sec6–4*, *sec8–9*, *sec10–2*, and *sec15–1* mutations have no obvious effects on budding patterns or morphology. Thus, defects in bud site selection are not unique to *sec3* mutants among the late-acting *sec* mutants, but only a particular subset of the late-acting *sec* mutants affect bud site selection patterns. Furthermore, those *SEC* gene products that affect budding patterns appear to play a role in bud site selection only in diploids. This may reflect the need in diploid cells for reorientation of the secretory pathway to a nonadjacent bud site for the bipolar pattern, whereas in haploid cells the pathway has reoriented to the axial budding site at cytokinesis.

Recently, Sec3p was found to be a component of a complex comprising Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo70p (TerBush *et al.*, 1996). A homologous complex has been purified from mammalian brain; however, short peptide sequences of the components do not identify any of them as a clear Sec3p homologue (Ting *et al.*, 1995; Hsu *et al.*, 1996). It is not yet known whether Sec3p is only found as a component of this complex, or whether it may also function independently of some or all of the complex components. The effects of high-copy *SEC3* on the growth of *sec5*, *sec10*, and *sec15* mutants may reflect physical interactions of complex components. Sec8p has been localized to the tip of the growing bud (TerBush and Novick, 1995), the site of polarized secretion, and it has been suggested that this complex functions as a "targeting patch" for secretory vesicles (Drubin and Nelson, 1996). A defect in localization of such a targeting patch might explain the morphological defects of the *sec3* mutants, by depolarizing bud tip secretion and perhaps secretion to the neck between mother and daughter cells at cytokinesis. Although this complex may be required for vesicle docking or fusion, Sec3p cannot be absolutely required for either of these processes, since cells depleted of Sec3p or with the *SEC3* gene disrupted are viable, although temperature-sensitive and severely growth-impaired, while secretion is an essential process. Sec3p could, however, facilitate one or both of these processes. These functions are implied by the temperature-sensitive secretory defects of *sec3* mutants such as the accumulation of secretory vesicles in the bud in *sec3–2* cells at the restrictive temperature. Sec3p may help to specify the correct site for exocytosis, consistent with the polarity defects of *sec3* mutants. Sec3p is the only member of this complex that affects bud site selection in diploids, suggesting a possible role for Sec3p in localization of other complex components. The role of actin in secretion may be to polarize delivery of secretory vesicles into the bud, where Sec3p could participate in defining the docking/fusion site.

The new *sec3* mutants are atypical of late *sec* mutants with respect to the secretory block. Although *sec3–2* cells display a block in post-Golgi secretion only, *sec3–4* and *sec3–5* cells accumulate ER and Golgi membranes in addition to exocytic vesicles and are partially defective in transport of the vacuolar protease CPY to the vacuole. This cannot be explained as a consequence of a constitutive partial block in post-Golgi secretion, because *sec4–8* and *sec9–4* cells blocked in growth to a comparable extent do not display this phenotype. The temperature sensitivity of two different alleles of *ypt1*, the rab family member involved in ER to Golgi and intra-Golgi transport (Bacon *et al.*, 1989; Jedd *et al.*, 1995), is exacerbated by overproduction of Sec3p. The effects of overproduction of other *SEC* gene products in a *ypt1* mutant background have not been studied; therefore, the specificity of this interaction is not clear. These data suggest a possible role for Sec3p in earlier stages of the secretory pathway, in addition to its role in exocytosis. It is possible, however, that these effects are not the direct result of Sec3p acting at the ER or Golgi, but may result from the mutant Sec3p titrating out some limiting factor that is required for both Sec4p and Ypt1p function. This would imply that *sec3–4* and *sec3–5* are at least partially dominant, which does not appear to be the case (our unpublished results). The polarity defects of the Sec3p mutant proteins may have ramifications on cytoplasmic organization that would affect other stages of the secretory pathway beyond the effects on post-Golgi secretion.

Sec3p appears to have a dual role as a *SEC* gene product shown to play a role in establishment of cell polarity in addition to its role in the secretory pathway. The development of the bud appears to be a more complex process than previously thought, with components of the secretory apparatus influencing the site and development of cell polarity, rather than simply responding to polarization cues determined by cytoskeletal elements. A possible role of Sec3p as a spatial landmark for the site of exocytosis will need to be considered in future studies of Sec3p's function.

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