Molecular Biology of the Cell
Vol. 9, 1725–1739, July 1998

Dominant Negative Alleles of *SEC10* Reveal Distinct Domains Involved in Secretion and Morphogenesis in Yeast

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Submitted November 24, 1997; Accepted April 17, 1998
Monitoring Editor: Randy W. Schekman

The accurate targeting of secretory vesicles to distinct sites on the plasma membrane is necessary to achieve polarized growth and to establish specialized domains at the surface of eukaryotic cells. Members of a protein complex required for exocytosis, the exocyst, have been localized to regions of active secretion in the budding yeast *Saccharomyces cerevisiae* where they may function to specify sites on the plasma membrane for vesicle docking and fusion. In this study we have addressed the function of one member of the exocyst complex, Sec10p. We have identified two functional domains of Sec10p that act in a dominant-negative manner to inhibit cell growth upon overexpression. Phenotypic and biochemical analysis of the dominant-negative mutants points to a bifunctional role for Sec10p. One domain, consisting of the amino-terminal two-thirds of Sec10p directly interacts with Sec15p, another exocyst component. Overexpression of this domain displaces the full-length Sec10 from the exocyst complex, resulting in a block in exocytosis and an accumulation of secretory vesicles. The carboxy-terminal domain of Sec10p does not interact with other members of the exocyst complex and expression of this domain does not cause a secretory defect. Rather, this mutant results in the formation of elongated cells, suggesting that the second domain of Sec10p is required for morphogenesis, perhaps regulating the reorientation of the secretory pathway from the tip of the emerging daughter cell toward the mother–daughter connection during cell cycle progression.

INTRODUCTION

The polarized transport of secretory vesicles to distinct domains of the plasma membrane helps to establish the directionality of cell growth in eukaryotic cell types as diverse as yeast, epithelial cells, and developing neurons. The budding yeast *Saccharomyces cerevisiae* has proven to be a particularly useful system for the genetic identification of proteins required for vesicular transport (Novick *et al.*, 1980). The terminal stage of the yeast-secretory pathway relies on 10 *SEC* gene products (Sec1p, Sec2p, Sec3p, Sec4p, Sec5p, Sec6p, Sec8p, Sec9p, Sec10p, and Sec15p) (Novick *et al.*, 1981), as well as the Snc (Gerst *et al.*, 1992; Protopopov *et al.*, 1993) and Sso proteins (Aalto *et al.*, 1993). Mammalian homologues have been identified for most of these yeast proteins (Bennett and Scheller, 1994; Ferro-Novick and Jahn, 1994; Rothman, 1994). This evolutionary conservation may reflect the importance of these components of the secretory machinery for all eukaryotic cells. The challenge now is to establish the physical and functional relationships among these proteins and to define their mechanism of action in vesicle targeting, docking, and fusion.

Research into the mechanisms of vesicle targeting and fusion has focused most intensely on two groups of proteins: a set of proteins collectively called SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors), and a family of low molecular weight GTP-binding proteins, known as the Rab proteins. Specific SNAREs are required for each

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stage of vesicular transport (Ferro-Novick and Jahn, 1994; Rothman, 1994; Rothman and Wieland, 1996). The current hypothesis suggests that an interaction between SNAREs on the vesicle (v-SNAREs) and cognate SNAREs on the target membrane (t-SNAREs) ensures close spatial proximity between the two membranes for vesicle fusion (Sollner *et al.*, 1993a,b). In post-Golgi transport in yeast, the v-SNARE is encoded by *SNC1/2* and the t-SNAREs by *SSO1/2* and *SEC9* (Aalto *et al.*, 1991, 1993; Gerst *et al.*, 1992; Protopopov *et al.*, 1993; Brennwald *et al.*, 1994).

The family of Rab GTPases, whose founding member is the yeast Sec4 protein (Salminen and Novick, 1987), act as nucleotide-dependent molecular switches to control membrane traffic (Bourne *et al.*, 1990; Novick and Brennwald, 1993; Zerial and Stenmark, 1993). However, the molecular mechanisms underlying the action of Rabs remain unknown to date. Rab proteins may act to promote SNARE complex formation by activating individual SNAREs (Dascher *et al.*, 1991; Novick and Brennwald, 1993; Lian *et al.*, 1994; Sogaard *et al.*, 1994). This idea is mainly based on genetic studies in yeast. At the ER to Golgi stage, cells defective in the Rab homologue Ypt1 fail to form the relevant SNARE complex (Lian *et al.*, 1994; Sogaard *et al.*, 1994), and the overexpression of SNARE components can bypass the lethality of a deletion of *YPT1*. Furthermore, at the post-Golgi stage of transport, overexpression of the t-SNARE Sec9p can bypass the growth defect of a cold-sensitive mutation of Sec4p (Brennwald *et al.*, 1994). Although these findings suggest a role for Rab proteins in SNARE complex formation, it is unclear whether the SNARE activation is direct or indirect.

Rab proteins may not only function in the activation of SNARE proteins, but may also act on the cytoskeleton to polarize vesicular transport. Activated forms of Rab8 have recently been found to cause changes in the morphology of cultured fibroblasts resulting in the outgrowth of cellular processes (Peranen *et al.*, 1996). These morphological changes were correlated with rearrangements of the actin cytoskeleton and microtubules, both of which relocalize to the newly formed processes. Sec4p is the protein most homologous to Rab8 in budding yeast (Huber *et al.*, 1993). The nucleotide state of Sec4p is critical for its role in polarized vesicle transport. If Sec2p, the guanyl nucleotide exchange factor for Sec4p, is mutated thereby decreasing the amount of GTP-bound Sec4p, vesicles accumulate randomly in the mother cell instead of concentrating at the tip of the daughter cell (Walch-Solimena *et al.*, 1997).

Of the remaining proteins required for post-Golgi transport in yeast, six (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and a previously uncharacterized protein termed Exo70p) form a multisubunit complex termed the exocyst (TerBush and Novick, 1995; TerBush *et al.*, 1996). A similar, high-molecular weight complex has recently been purified from brain (Hsu *et al.*, 1996), and the mammalian homologues of Sec6p, Sec8p, and Sec10p have been cloned (Ting *et al.*, 1995; Guo *et al.*, 1997; Hazuka *et al.*, 1997). This evolutionary conservation of the members of the exocyst may reflect their importance for the flow of vesicles from the Golgi to the plasma membrane in all eukaryotic cells (TerBush *et al.*, 1996). An important clue to the function of the exocyst stems from the concentration of some of its members at sites of active secretion at the bud tip or at the mother–daughter connection in yeast (TerBush and Novick, 1995; Mondesert *et al.*, 1997; Finger *et al.*, 1998). It has, therefore, been hypothesized that the exocyst acts as a "target patch" for the delivery of vesicles to sites of active surface growth (Drubin and Nelson, 1996).

Genetic data in yeast suggest a close interplay between individual members of the exocyst and the Rab homologue Sec4p (Salminen and Novick, 1987). Temperature-sensitive alleles of most members of the exocyst (*sec3–2, sec5–24, sec8–9, sec10–2* and *sec15–1*) are synthetically lethal with the temperature-sensitive *sec4–8* allele (Salminen and Novick, 1987). Furthermore, simply doubling the amounts of Sec4p within a cell can suppress the growth defects of *sec15–1* and *sec8–9* and, to a lesser extent, of *sec5–24*, and *sec10–2*. *SEC4* and *SEC15* show particularly strong genetic interactions with each other (Salminen and Novick, 1987).

In this study we have identified two functional domains of Sec10p. One domain interacts with Sec15p to generate an active exocyst complex while the second domain is involved in morphogenesis, perhaps mediating the reorientation of exocytosis from the bud tip to the neck region during the progression of the cell cycle.

MATERIALS AND METHODS

Yeast Genetic Techniques

Table 1 lists the yeast strains used in this study. Cultures were grown in rich medium (YP) containing 1% Bacto yeast extract and 2% Bacto peptone (Difco Laboratories, Detroit, MI) or in synthetic medium containing 0.7% yeast nitrogen base without amino acids (Difco). Synthetic medium was supplemented with nutrients if necessary as described (Sherman *et al.*, 1974). Glucose was generally used as a carbon source (2% final concentration) except in experiments requiring expression from the *GAL1* promotor. In this case, cells were first grown to early log phase ($A_{600} \sim 0.2$) in YP containing glycerol (2% final concentration) as a nonfermentable carbon source, and then shifted to 2% galactose and grown further for 10 h.

Yeast transformations were performed with the lithium-acetate method (Ito *et al.*, 1983), and transformants were selected on synthetic medium supplemented with nutrients as required at 25°C.

Generation of Sec10 Domains for Expression in Yeast

Sec10 fragments were generated by PCR and subcloned into integrating vectors containing a *GAL1* promotor and *ADH* terminator

with *URA3* (pNB 530) or *LEU2* (pNB 529) as auxotrophic markers. The resulting plasmids were linearized with *Afl*II (for pNB 529-derived plasmids) or with *Stu*I (for pNB 530-derived plasmids) to allow for homologous recombination.

SDS-PAGE and Western Blotting

SDS-PAGE was carried out according to Laemmli (1970), and proteins were transferred onto nitrocellulose (Schleicher & Schuell, Keene, NH). Nonspecific binding sites were blocked with 3% milk in PBS for 1 h. Proteins were then probed for 1 h at room temperature with anti-Sec10 antibody at 1:1000 dilution. The filters were then washed and probed with secondary antibody (HRP-conjugated goat-anti mouse or rabbit IgG) at 1:5000 for 20 min at room temperature in PBMT (PBS containing 3% milk and 0.2% Tween 20). After five washes in PBMT, blots were developed by ECL (Amersham, Arlington Heights, IL).

Immunofluorescence

Yeast were grown at 25°C to an A_{600} of approximately 0.2 in YP containing 2% glycerol. Overexpression of proteins from the *GAL1* promotor was induced by addition of galactose (2% final concentration) directly to the medium. After a further 10 h of growth, 5 A_{600} units of cells were fixed for 4 h at room temperature by addition of an equal volume of fixative solution (8% formaldehyde in 2× PBS) to the medium.

For cell wall removal, fixed cells were washed twice in KPi/sorbitol (0.1 M potassium phosphate, pH 7.4, and 1.2 M sorbitol), resuspended in 0.5 ml KPi/sorbitol containing 0.5% 2-mercaptoethanol and 80 μg/ml zymolyase, and incubated for 30 min at 37°C. Cells were then washed once in cold PBS/BSA (PBS containing 1 mg/ml BSA) and resuspended in 100 μl PBS/BSA. A portion (25 μl) of this cell suspension was applied to eight-well slides (model 10086; Carlson Scientific, Peotone, IL) coated with polylysine (1 mg/ml; MW, 400,000). Cells were then permeabilized with 0.5% SDS in PBS/BSA for 5 min at room temperature and washed 10 times with PBS/BSA before addition of the primary antibodies. Primary antibody incubation was for 1 h at room temperature with the following antibody dilutions into PBS/BSA: affinity-purified anti-Sec10p and affinity-purified anti-Sec15p at 1:100; and monoclonal or polyclonal antiSec4p, anti-Myo2p (gift from S. Reck-Peterson), and monoclonal antiactin (clone C4; ICN, Costa Mesa, CA) at 1:1000 dilution. Before addition of the secondary antibodies, wells were washed 10 times in PBS/BSA. Secondary antibody incubation was for 30 min at room temperature with dichlorotriazinyl-amino fluorescein-conjugated donkey anti-rabbit or Texas Red-conjugated goat anti-mouse antibodies (Jackson Immunoresearch, West Grove, PA) at 1:250 dilutions into PBS/BSA. Wells were washed as above, mounted in antifade solution (90% glycerol, 1 mg/ml *p*-phenylenediamine), and sealed with nail varnish. Cells were observed with a Zeiss Axiophot (Carl Zeiss, Thornwood, NY) using a 100× objective. For double-labeling experiments, cells were incubated sequentially with primary and respective secondary antibody solutions to the first antigen followed by incubations with primary and secondary antibodies to the second antigen.

TRITC-Concanavalin A (TRITC-Con A) Staining

For cell wall labeling, 5 A_{600} units of cells grown as before in YP glycerol/galactose were pelleted, resuspended in 100 μl YP, and incubated with 10 μg/ml TRITC-Con A for 5 min at room temperature in the dark. Cells were then washed three times to remove unbound TRITC-Con A and incubated further in YP-galactose. Wild-type cells and cells overexpressing Sec10CT were grown for 1 h after TRITC-Con A labeling before fixing and mounting on slides. Observation of the cells was with a 100× objective and a Zeiss axiophot.

Electron Microscopy

Cells were grown in YP-glycerol, and overexpression of proteins was induced by addition of galactose to the medium as described for the immunofluorescence. Preparation of cells for electron microscopy was as previously described (Salminen and Novick, 1987). In brief, 10 A_{600} units of cells were fixed in 2% glutaraldehyde and washed, and the cell wall was removed by incubation in KPi/sorbitol containing 80 μg/ml zymolyase at 37°C. The extent of cell wall removal was monitored by light microscopy. Cells were then stained with osmium tetroxide and uranyl acetate, dehydrated, and embedded in low-viscosity epoxy resin (Spurr; Polysciences, Warrington, PA). Blocks were sectioned and poststained with uranyl acetate and lead citrate.

Immunoprecipitations from [^{35}S]-Methionine/Cysteine–labeled Cells

Yeast were grown to early log phase in YP-glycerol, and then shifted to synthetic media with 2% galactose for 10 h. For metabolic labeling, 5 A_{600} units of cells per immunoprecipitation were incubated with 10 μl of label mix (14.3 μCi/μl of [^{35}S]-methionine/cysteine; Amersham, Arlington Heights, IL) for 4 h at 25°C. Spheroplasts were prepared by incubation in KPi/sorbitol containing 80 μg/ml zymolyase and 0.5% 2-mercaptoethanol at 37°C for 20 min. For cell lysis, cells were washed with KPi/sorbitol and resuspended in 1 ml of cold IP buffer [20 mM piperazine-N,N′-bis-(ethanesulfonic acid), pH 6.8, 100 mM NaCl, 1 mM EDTA, 0.5% Tween 20, 2 μM PMSF, 10 μM antipain, 30 μM chymostatin, 1 μM pepstatin, 1 μg/ml aprotinin, and 30 μM leupeptin]. The lysates were centrifuged at 14,000 × *g* for 10 min at 4°C and precleared with 4 mg/ml protein A Sepharose for 1 h at 4 C. Immunoprecipitations were performed for 2 h at 4°C with anti-Sec10 affinity-purified antibodies at 1:100 dilution or anti-myc 9E10 ascites at 1:500 dilution. Antibodies were precipitated by further incubation for 1 h at 4°C with 4 mg/ml protein A Sepharose. Beads were then washed five times in IP buffer, resuspended in 80 μl SDS sample buffer, and boiled, and released proteins were loaded onto SDS-polyacrylamide gels. Gels were fixed, incubated in autofluor (National Diagnostics, Atlanta, GA), dried, and exposed to film (Kodak X-Omat AR, Rochester, NY).

In Vitro Transcription/Translation

Constructs for the in vitro transcription/translation were generated by PCR and subcloned into mammalian expression vectors containing a CMV promotor. Preparation of [^{35}S]-cysteine and -methionine–labeled proteins was carried out with an in vitro transcription/translation kit according to the manufacturer's instructions (Promega, Madison, WI). For binding experiments, the proteins to be tested for binding were cotranscribed/translated in vitro. After the transcription/translation reaction, 3 μl of the reaction mixture were diluted 100-fold into IP buffer (see above), and immunoprecipitations were performed as described above. In the end, beads were resuspended in 25 μl SDS sample buffer and boiled, and released proteins were separated by SDS-PAGE. Gels were fixed and dried, and radioactive bands were visualized by autoradiography.

RESULTS

Construction of Dominant-Negative Fragments of Sec10p

The yeast Sec10 protein and its homologues in *Caenorhabditis elegans* and mammals share sequence identity on the order of 20–25%. Major regions of homology are contained in three blocks of approximately 150 amino acids each, present at the N terminus, the mid-

dle region, and the C terminus of Sec10p as indicated in Figure 1A. The amino-terminal block contains a domain (amino acids 77–98) predicted to form a coiled-coil structure. The carboxy-terminal block is somewhat more hydrophobic than the rest of the protein, as predicted by the algorithm of Kyte and Doolittle (1982) (Figure 1A).

Given the conservation of amino acid residues in the N terminus and the C terminus together with the hydrophobicity of the C terminus, we investigated whether these regions of the protein might constitute distinct functional domains of Sec10p. Based on the hydropathy plot and the conserved regions, we constructed three fragments of Sec10p, termed Sec10NT, Sec10ΔC, and Sec10CT. Sec10NT comprised a 30-kDa fragment that contained the predicted coiled-coil region and the first region of homology. Sec10ΔC comprised the first two regions of homology, while Sec10CT comprised the more hydrophobic, third region of the protein (Figure 1A). These fragments were overexpressed in yeast from the galactose-inducible *GAL1* promotor. The rational behind the approach is that Sec10p may normally interact with several partners through its different domains. Overexpression of an isolated domain of Sec10p would compete against the endogenous Sec10p for the binding to one of its interactive partners. Because such a protein would not be fully functional, it would competitively inhibit the function of the endogenous full-length Sec10p.

While overexpression of Sec10p itself is not toxic to the cells, we found that cells overexpressing either Sec10ΔC or Sec10CT had a growth defect (Figure 1B). Quantitative immunoblot analysis indicated that Sec10ΔC was expressed between 200- to 300-fold over the normal Sec10p level. The growth inhibition suggested that both Sec10p fragments were able to inhibit the function of the endogenous protein. The extent of the growth rate inhibition by Sec10ΔC was ~70%, while Sec10CT overexpression gave ~45% growth rate inhibition compared with wild type. Sec10NT, although overexpressed, did not cause a growth defect (Figure 1B). The growth inhibition observed upon overexpression of the entire hydrophilic (Sec10ΔC) or hydrophobic (Sec10CT) regions of the Sec10p suggests that this protein has at least two distinct protein–protein interaction domains. The N-terminal one-third of the protein, which covers the predicted coiled-coil region, may not constitute a fully functional binding domain.

Phenotypic Characterization of the Sec10 Dominant-Negative Mutants

When we examined the cells overexpressing Sec10ΔC or Sec10CT by light microscopy, we noticed almost diametrically opposite phenotypic effects on cell morphology (Figure 2, A–C). Cells that had been grown in YP-glycerol and had been induced for overexpression of the Sec10 dominant-negative mutants for 10 h were processed for indirect immunofluorescence and double labeled with anti-Sec4p and anti-actin antibodies. At this time point, maximal expression of the Sec10 fragments was reached, and the phenotypic changes were quite evident. Cells overexpressing Sec10ΔC were frequently enlarged, and most cells were unbudded (92% unbudded, compared with 39% for wild type) or had only small buds (Figure 2B). The actin cytoskeleton was depolarized, with cortical actin patches present throughout mother and daughter cells, and actin cables were no longer detectable (Figure 2H). Sec4p staining, marking the location of secretory vesicles, was still localized to the bud site (Figure 2E). Cells were usually mononucleate. At this level, the phenotype of these cells is similar to that of a post-Golgi blocked *sec* mutant, such as the temperature-sensitive mutant *sec10–2*.

Cells overexpressing Sec10CT showed a distinctly different phenotype typified by elongated cells (Figure 2C). These cells were 70% longer than the average small budded wild-type cell and 30% longer than the

Table 1. Strains used in this study

Strain	Genotype
NY 1060	Mat a/α *ura3-52/ura3-52 leu2-3,112/leu2-3,112*
NY 604	Mat α *ura3-52 leu2-3,112*
NY 1573	Mat α *ura3-52::(URA3, Gal-SEC10ΔC)*
NY 1574	Mat α *ura3-52::(URA3, Gal-SEC10CT)*
NY 1377	Mat α *ura3-52 leu2-3,112::(LEU2, SEC8-3x-c-myc) sec8-9*
NY 1570	Mat a/α *ura3-52::(URA3, Gal-SEC10ΔC)/ura3-52 leu2-3,112::(LEU2, SEC8-3x-c-myc)/leu2-3,112*
NY 1571	Mat a/α *ura3-52::(URA3, Gal-SEC10CT)/ura3-52 leu2-3,112::(LEU2, SEC8-3x-c-myc)/leu2-3,112*
NY 1572	Mat a/α *ura3-52/ura3-52 leu2-3,112::(LEU2, SEC8-3x-c-myc)/leu2-3,112*
NY 1562	Mat a/α *ura3-52::(URA3, Gal-SEC15)/ura3-52 leu2-3,112/leu2-3,112*
NY 1565	Mat a/α *ura3-52::(URA3, Gal-SEC15)/ura3-52 leu2-3,112::(LEU2, Gal-SEC10ΔC)/leu2-3,112*
NY 1566	Mat a/α *ura3-52::(URA3, Gal-SEC15)/ura3-52 leu2-3,112::(LEU2, Gal-SEC10CT)/leu2-3,112*
NY 1563	Mat a/α *ura3-52/ura3-52 leu2-3,112::(LEU2, Gal-SEC10ΔC)/leu2-3,112*
NY 1564	Mat a/α *ura3-52 leu2-3,112::(LEU2, Gal-SEC10CT)/leu2-3,112*

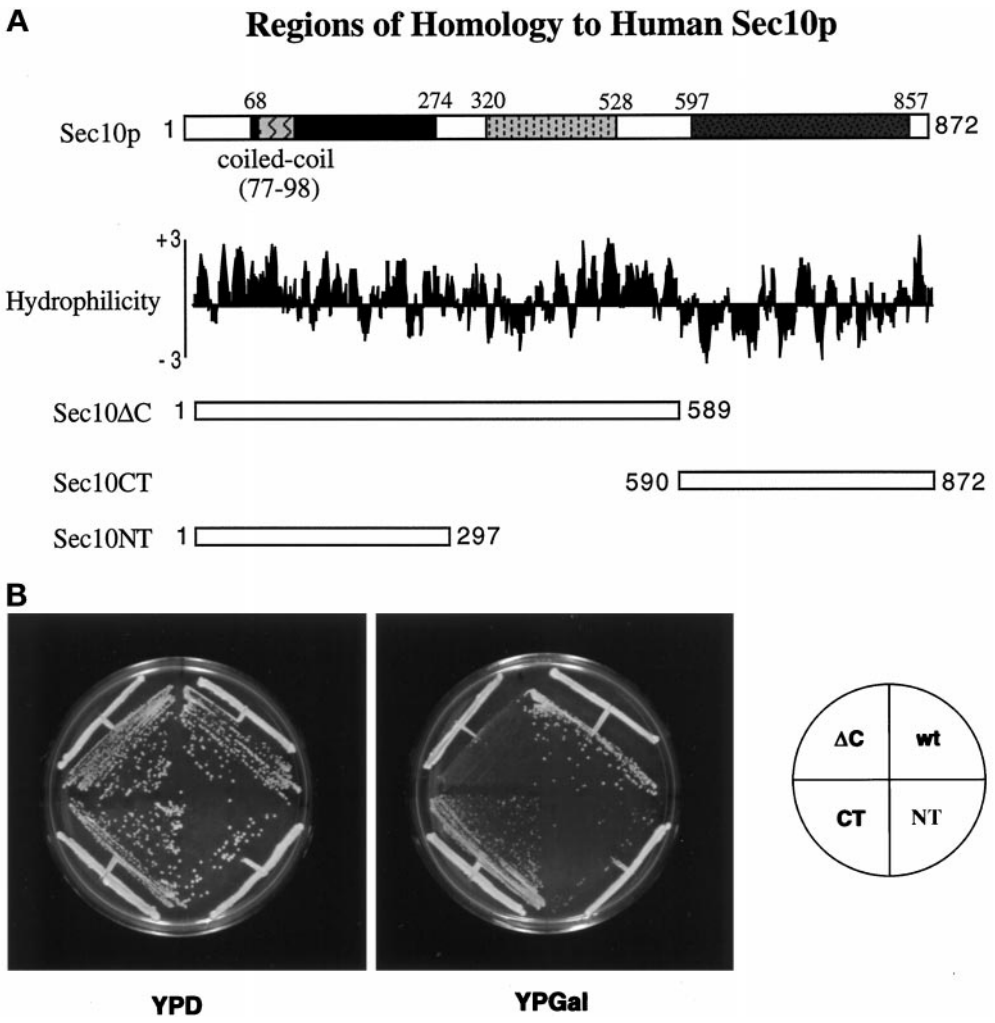

Figure 1. Overexpression of Sec10 deletion mutants inhibits cell growth. (A) Hydropathy analysis and schematic representation of deletion mutants of Sec10p. The upper panel shows the regions in Sec10p that are homologous to *C. elegans* and human sequences and the region predicted to form a coiled-coil structure. The middle panel shows the hydropathy profile of Sec10p generated by Protean software (DNA star) based on the algorithm of Kyte and Doolittle (1982). Hydrophilic regions are represented by positive values and hydrophobic regions by negative values. The lower panels depict the deletion mutants of Sec10p. Numbers next to the cartoons indicate amino acids. (B) Inhibition of cell growth by overexpression of the Sec10p domains, Sec10ΔC and Sec10CT. Wild-type yeast or yeast expressing the Sec10 mutants (Sec10ΔC [ΔC], Sec10 CT [CT], and Sec10NT [NT]) from the *GAL1* promoter were grown at 25°C on rich medium containing glucose (YPD), a repressing carbon source, or galactose (YPGal) for the induction of the mutants. The order of strains is depicted schematically on the right.

average large budded wild-type cell. Actin was predominantly present at the tip of the bud, but also extended to the neck region (Figure 2I). Interestingly, Sec4p staining was frequently (39% of the cells) visible simultaneously at the tip of the bud and at the neck between mother and daughter cells (Figure 2F). To investigate whether secretion in these cells occurred simultaneously at both bud tip and neck, we performed cell wall labeling with TRITC-Con A and examined by fluorescence microscopy where newly synthesized cell wall material was deposited after a growth period in the absence of TRITC-Con A. The incorporation of newly synthesized cell wall material, as indicated by the absence of TRITC-Con A fluorescence, was only detected at the bud tip (our unpublished observations), suggesting that growth preferentially occurred at this site. The method used, however, may not have been sensitive enough to detect smaller amounts of secretion occurring at the neck.

Accumulation of Vesicles in Sec10ΔC but Not in Sec10CT-overexpressing Cells

An important question from the above data was whether the growth defects of the dominant negative Sec10p mutants were due to blocks on the exocytic pathway. Such a block would result in the accumulation of secretory vesicles. To address this, we performed electron microscopy on the dominant-negative Sec10p mutants and wild-type cells. In cells overexpressing Sec10ΔC, vesicles accumulated (81 ± 31 vesicles/cell section, compared with 4 ± 2 for wild-type) (Figure 3, C and D). In small budded cells (Figure 3C), the vesicles were primarily concentrated in the bud, but were also distributed randomly in the mother cell. In large budded cells, vesicles accumulated approximately equally in daughter and mother cell. However, vesicles in the bud appeared more tightly concentrated in groups than they were in the mother cell (Figure 3D). In summary, the data demonstrated that

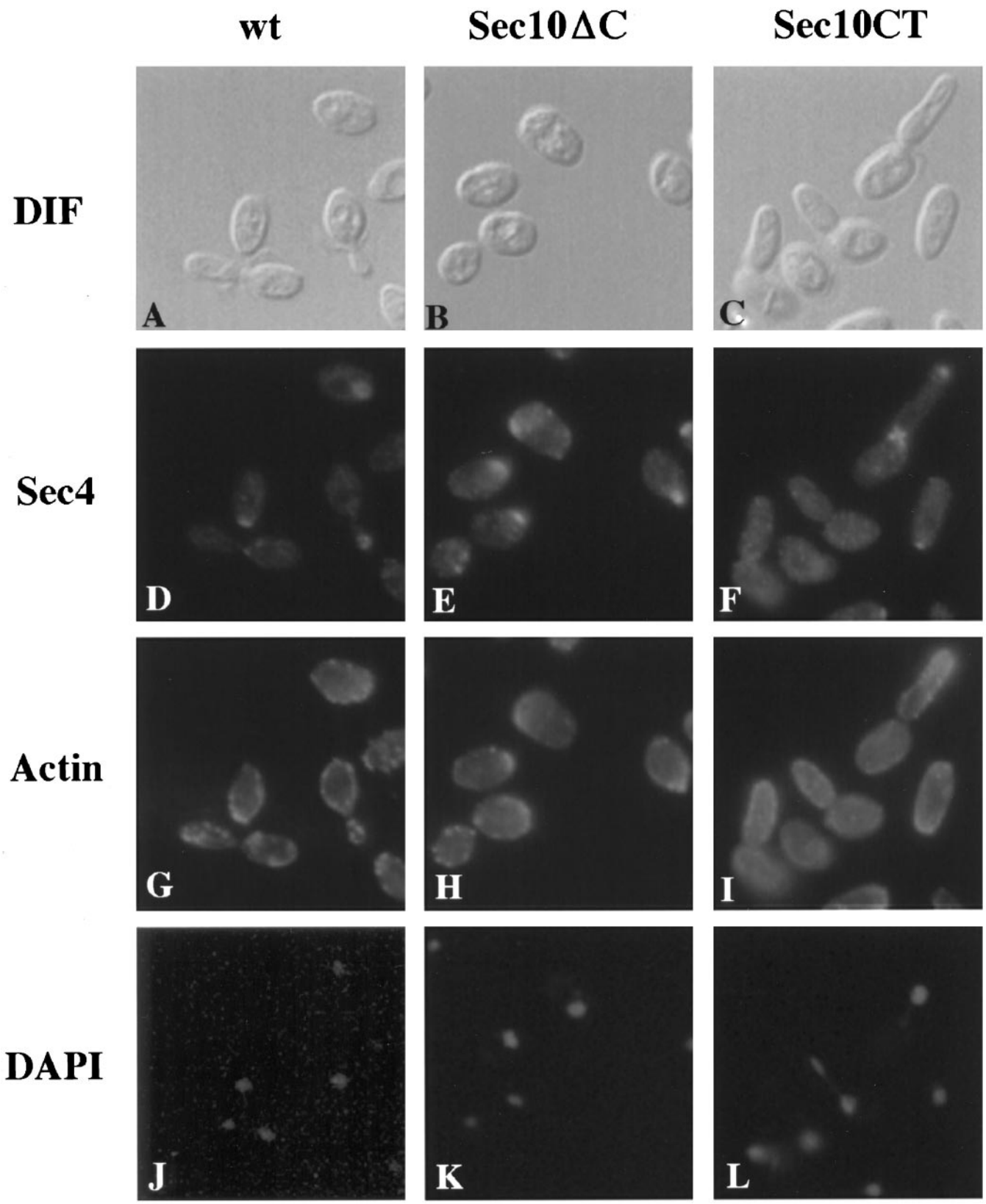

Figure 2.

Table 2. Genetic interactions between mutants

sec mutant	Overexpression of Sec10ΔC	Overexpression of Sec10CT
sec1-1	+	−
sec2-41	+	+
sec3-2	−	+
sec4-8	+	−*
sec5-24	−	+
sec6-4	+/−	−
sec8-9	+	−
sec10-2	−	+
sec15-1	−	−

+, Viable cells; −, synthetic lethality; +/−, synthetic sickness.

overexpression of Sec10ΔC resulted in a block of the exocytic pathway.

In contrast, cells overexpressing the C-terminal region of Sec10p, Sec10CT, were largely devoid of vesicles (5 ± 2 vesicles/cell section) (Figure 3B). Since these cells did not accumulate any other forms of intracellular membranes, we assume that the slow growth of these cells was not due to a defect in vesicular traffic. Rather, the elongated shape of these cells suggested that exocytosis continued, but that the switch from bud tip growth to isotropic growth and subsequent cytokinesis was delayed.

Sec10ΔC and Sec10CT Are Synthetically Lethal with Different Subsets of Exocytic Mutants

As an initial clue to the identification of interactive partners of the Sec10 fragments, we searched for genetic interactions between the dominant-negative Sec10 mutants and mutants in other genes of the post-Golgi pathway (Table 2). Toward this aim, the Sec10p domains were expressed in temperature-sensitive mutants of members of the exocyst (*sec3–2*, *sec5–24*, *sec6–4*, *sec8–9*, *sec10–2*, *sec15–1*), of a t-SNARE *(sec9–4*), of a potential SNARE-associated protein (*sec1–1*), of the Rab (*sec4–8*), and of its nucleotide exchange protein (*sec2–41*). The two dominant negative mutants of Sec10p displayed distinct patterns of genetic interactions. Expression of Sec10ΔC resulted in cell death at 25°C exclusively with mutants of the exocyst, namely *sec3–2*, *sec5–24*, *sec6–4*, *sec10–2*, and *sec15–1*. On the other hand, overexpression of Sec10CT was synthetically lethal with a mutant of the t-SNARE, *sec9–4*, a mutant of the potentially SNARE-interacting protein Sec1p, as well as with the exocyst mutants, *sec6–4*, *sec8–9*, and *sec15–1.* Somewhat weaker synthetic effects were found with *sec4–8*. In conclusion, Sec10ΔC genetically interacts exclusively with members of the exocyst; in contrast, Sec10CT interacts with components of the SNARE apparatus and a subset of the exocyst proteins.

Figure 2 (facing page). Phenotypic analysis of cells expressing the Sec10p dominant-negative mutants. Wild-type yeast (A, D, G, and J) or yeast expressing the dominant-negative Sec10p mutants Sec10ΔC (B, E, H, and K), and Sec10CT (C, F, I, and L) after a 10-h induction in galactose were fixed and processed for immunofluorescence. Double labeling was performed with polyclonal anti-Sec4p antibodies (D–F) followed by monoclonal anti-actin antibodies (G–I), and DAPI staining (J–L). The morphology of the cells in the same field was observed under phase-contrast with differential interference (DIF; A–C).

Sec10ΔC Physically Interacts with Sec15p

Given the different effects of the Sec10p fragments on cell morphology and their distinct patterns of genetic interactions, we examined whether the Sec10p fragments would bind to different partners. We have investigated the interactions of Sec10ΔC and Sec10CT with other members of the exocyst by using in vitro synthesized [^{35}S]-methionine/cysteine–labeled proteins. The proteins to be tested for interaction were cotranslated, and the reaction mixture was subjected to immunoprecipitation using a c-*myc* tag, which had been added to the protein sequence of Sec10ΔC and Sec10CT. To test a direct interaction between the two different Sec10p domains themselves, c-*myc*-tagged Sec10ΔC and untagged Sec10CT were used. The C-terminal fragment of Sec10p did not bind to any other member of the exocyst tested in vitro, nor did it bind to the Sec10ΔC fragment (our unpublished observations). However, we found an in vitro interaction between the Sec10ΔC fragment and Sec15p (Figure 4). Binding of Sec10ΔC to Sec15p was almost stoichiometric.

Effect of the Dominant-Negative Sec10p Domains on the Composition of the Exocyst Complex

The composition of the exocyst complex is altered in temperature-sensitive mutants of some of its members (*sec3–2*, *sec5–24*, *sec15–1*, and *sec10–2*) (TerBush *et al.*, 1995). Since different mutants cause the loss of different subunits from the complex, important clues can be deduced about the structural interactions within the complex. We have, therefore, examined the effects of the dominant-negative Sec10p mutants on the composition of the exocyst complex. Strains carrying a c-myc_3–tagged *SEC8* allele, as well as expressing Sec10ΔC or Sec10CT under the control of the inducible *GAL1* promotor, were induced for overexpression of the Sec10p fragments and then metabolically labeled with [^{35}S]-cysteine and -methionine. Sec8p and associated proteins were retrieved from lysates by precipitation with anti–c-*myc* antibody, and labeled proteins were visualized by autoradioagraphy (Figure 5A). While overexpression of Sec10ΔC displaced full-length Sec10p from the complex, no other subunits

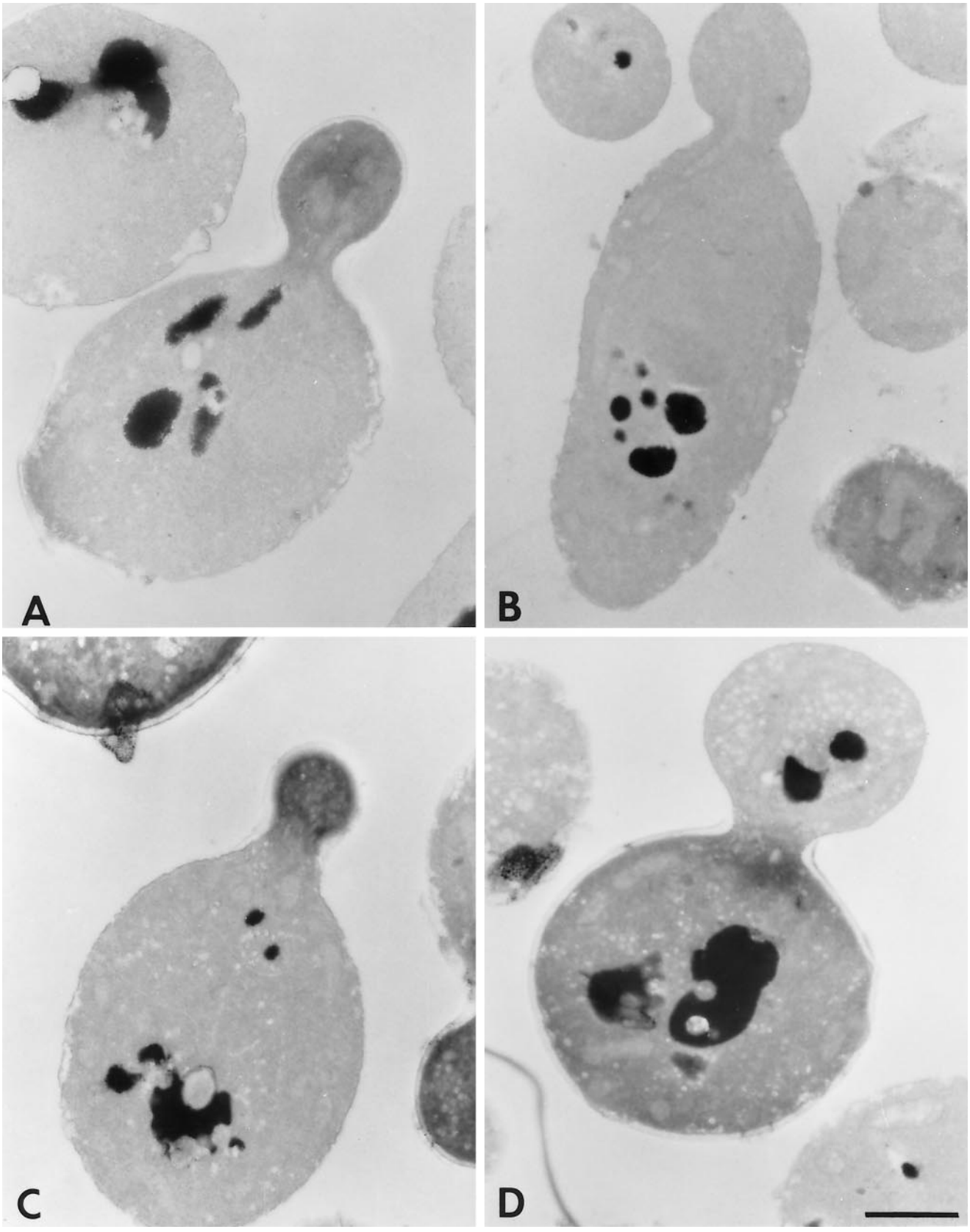

Figure 3.

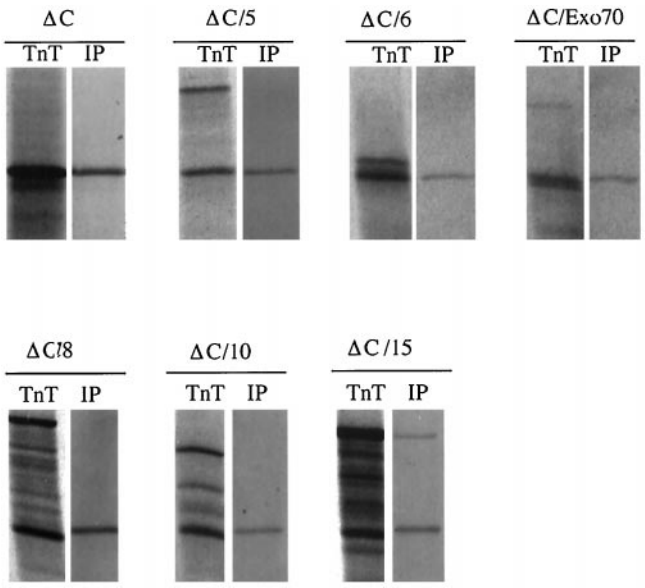

Figure 4. Sec10ΔC interacts with Sec15p in vitro. c-*myc*–tagged Sec10ΔC and members of the exocyst were cotranslated in vitro and immunoprecipitated with anti–c-*myc* antibodies. Two microliters of the translation reaction mixtures were loaded onto the gel (TnT) as a reference for the proteins present in the immunoprecipitation (IP).

were displaced, although a slight reduction in the level of Sec6p and Sec15p was noted. The composition was not altered when the Sec10CT was overexpressed (Figure 5A). As a marker for the position of Sec10p in the immunoprecipitates, an immunoprecipitate from a strain overexpressing full-length Sec10p was used. When Sec10p and Sec15p were cotranscribed and translated in vitro, Sec15p could be coprecipitated with Sec10p (Figure 5B). Addition of GST-Sec10ΔC, but not GST alone, was able to displace Sec15p from full-length Sec10p.

Based on these results, we suggest that, in Sec10ΔC-overexpressing cells, the growth defect and the accumulation of vesicles are due to the loss of endogenous Sec10p from the exocyst complex, possibly by competition for binding to Sec15p. On the other hand, Sec10CT does not appear to exert its effect on cell function by interacting with the other components of the exocyst complex. The presence of a complete exocyst complex is in agreement with the lack of vesicle accumulation seen in cells overexpressing Sec10CT. Furthermore it is in agreement with the failure of Sec10CT to bind in vitro to other components of the exocyst.

Figure 3 (facing page). Electron microscopic analysis of cells expressing the Sec10p-dominant mutants. Wild-type and mutant yeast were grown in galactose for induction of Sec10ΔC and Sec10CT and then processed for electron microscopy as described in MATERIALS AND METHODS. (A) Wild-type yeast. (B) Yeast expressing Sec10CT. (C) Small-budded cell expressing Sec10ΔC. (D) Large-budded cell expressing Sec10ΔC. Scale bar, 1 μm.

The Effects of Co-overexpression of Sec15p and Fragments of Sec10p

Given the binding of Sec10ΔC to Sec15p, we investigated the functional interaction of Sec10p and Sec15p in vivo. The overexpression of Sec15p alone results in growth inhibition and the accumulation of vesicles (Salminen and Novick, 1989). Many of the vesicles accumulate in the form of a tight cluster, which is distinct from the pattern of vesicle accumulation seen in temperature-sensitive post-Golgi *sec* mutants such as *sec1–1* or *sec6–4*. When we compared the growth of yeast coexpressing Sec10ΔC and Sec15p with the growth of strains expressing each protein alone, we noticed a synergistic negative effect on growth rate (our unpublished observations). Cells co-overexpressing Sec10CT and Sec15p, however, had the same approximate growth rate of cells overexpressing Sec10CT alone (our unpublished observations). However, when examined by light microscopy, these cells no longer appeared elongated like the cells overexpressing Sec10CT alone, implying that co-overexpression of Sec15p modifies the Sec10CT phenotype (Figure 6).

Sec15p and Sec4p Colocalize upon Sec15p Overexpression

When Sec15p is overexpressed, it can be localized by immunofluorescence as a patch in the vicinity of the bud tip or sometimes in the neck region of small budded cells, whereas the normal level of Sec15p in wild-type cells is not detectable (Salminen and Novick, 1989). In cells overexpressing Sec15p, Sec4p is localized in regions similar to Sec15p. We investigated the potential colocalization of Sec15p and Sec4p in cells overexpressing Sec15p (Figure 6). Upon overexpression of Sec15p, both Sec4p and Sec15p were detectable in a bright spot that colocalized in the majority of the cells (64% colocalization). Some cells, however, could not be stained by Sec15p antibody, although a distinct patch of Sec4p was visible (Figure 6, C and D). Yet, whenever Sec15p and Sec4p are detected within the same cell, they colocalize. These patches of Sec4p and Sec15p most likely correspond to the vesicle clusters seen by electron microscopy in Sec15p-overexpressing cells (Salminen and Novick, 1989).

We next used the formation of the Sec4p/Sec15p patch as an assay by which to further examine the potential function of the Sec10p domains. In particular, we were interested to determine whether the overexpression of either of the Sec10p domains would cause a change in the formation of the Sec15p patch.

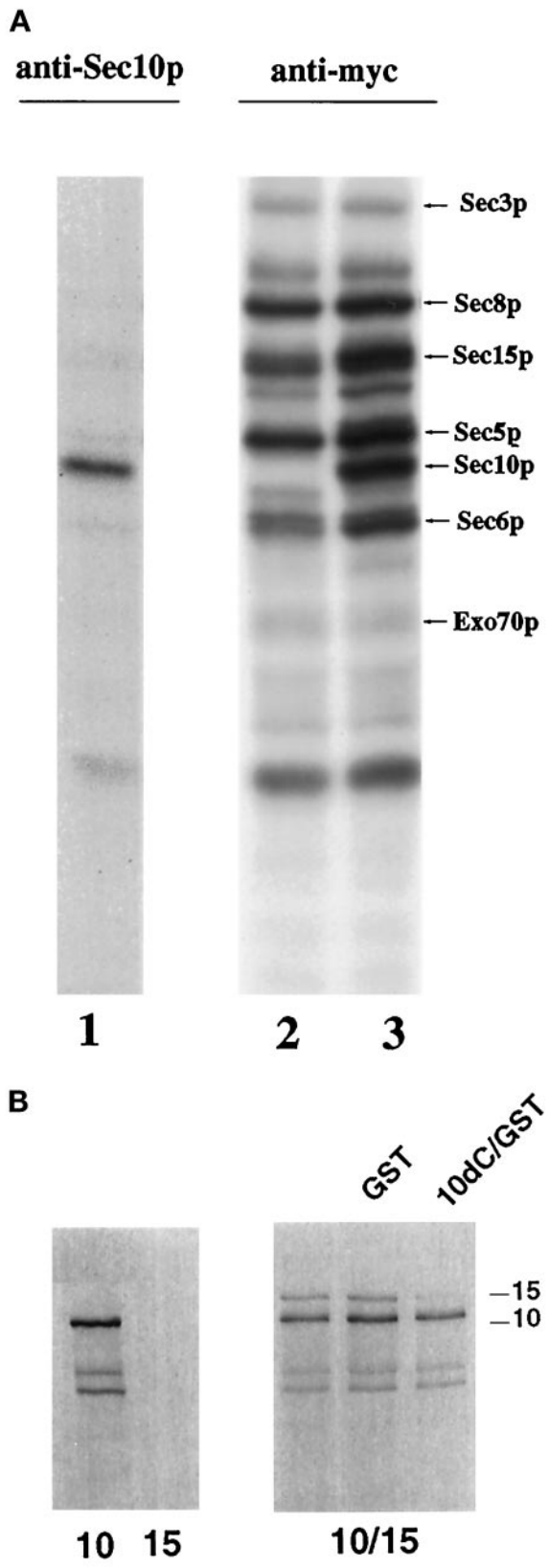

Figure 5.

Prior studies demonstrated that formation of the Sec15 patch requires the function of the GTPase Sec4p and its exchange protein Sec2p (Salminen and Novick, 1989). In cells co-overexpressing Sec15p and the Sec10CT, we noticed that Sec15p colocalized with Sec4p in 64% of cells (Figure 6, G and H), and the morphology of these cells was similar to that of wild-type cells. Thus, while the coexpression of Sec10CT and Sec15p prevents the formation of elongated cells, typical of Sec10CT expression, it does not prevent the formation of the Sec15p/Sec4p patch, typical of Sec15p overexpression.

In contrast, when Sec15p and Sec10ΔC were co-overexpressed, the patch of Sec15p staining was no longer visible (Figure 6F). Given that Sec10ΔC binds to Sec15p, we speculated that the Sec10ΔC may be recruited to the site of the Sec15p patch and that the lack of Sec15p fluorescence in the Sec10ΔC/Sec15p co-overexpressing cells was due to the masking of the epitope by Sec10ΔC. In this case we would expect to see colocalization of Sec4p and Sec10ΔC by immunofluorescence. As shown in Figure 7, double labeling with monoclonal Sec4p antibody and polyclonal Sec10p antibody demonstrated colocalization in distinct patches in the cell (Figure 7, E and F). As in the case of the Sec15p staining, not all the cells showed staining with both Sec4p and Sec10p antibodies (79% colocalization). It seems most likely that Sec10ΔC is recruited to the site of Sec15p localization and thus masks the epitope for the Sec15p antibody. Since the antibody used recognizes both endogenous Sec10p, as well as the Sec10ΔC fragment, it was important to verify that the patches observed with this antibody upon Sec10ΔC expression correspond to concentrations of Sec10ΔC and not the full-length protein. As shown in Figure 7C, the immunofluorescence is more intense, when Sec10ΔC was overexpressed compared with wild-type cells, where only the endogenous Sec10p was recognized (Figure 7A). This suggests that the patch stained with Sec10p antibody in the Sec15p/Sec10ΔC co-overexpressors predominantly reflects the presence of Sec10ΔC, although we cannot rule out that

Figure 5 (cont). (A) Effect of Sec10Δ and Sec10CT on the composition of the exocyst complex. Yeast containing c-myc$_3$–tagged Sec8p and expressing Sec10ΔC (lane 2) or Sec10CT (lane 3) after 10 h growth in galactose were labeled with [^{35}S]-cysteine and -methionine. The exocyst complex was precipitated using the c-myc$_3$ tag present on Sec8p as described in MATERIALS AND METHODS. Individual proteins in the exocyst were identified according to an immunoprecipitate from cells containing only c-*myc*$_3$–tagged Sec8p. The position of Sec10p was further identified by immunoprecipitation from yeast overexpressing Sec10p (lane 1). (B) Sec10ΔC displaces Sec10p from Sec15p. c-*myc*–tagged Sec10p and Sec15p were translated either separately (left panel) or were cotranslated (right panel) in vitro. After addition of either GST, GST-Sec10ΔC (200 μg/ml final concentration), or no addition, the c-*myc*–tagged Sec10p was immunoprecipitated with anti–c-*myc* antibodies.

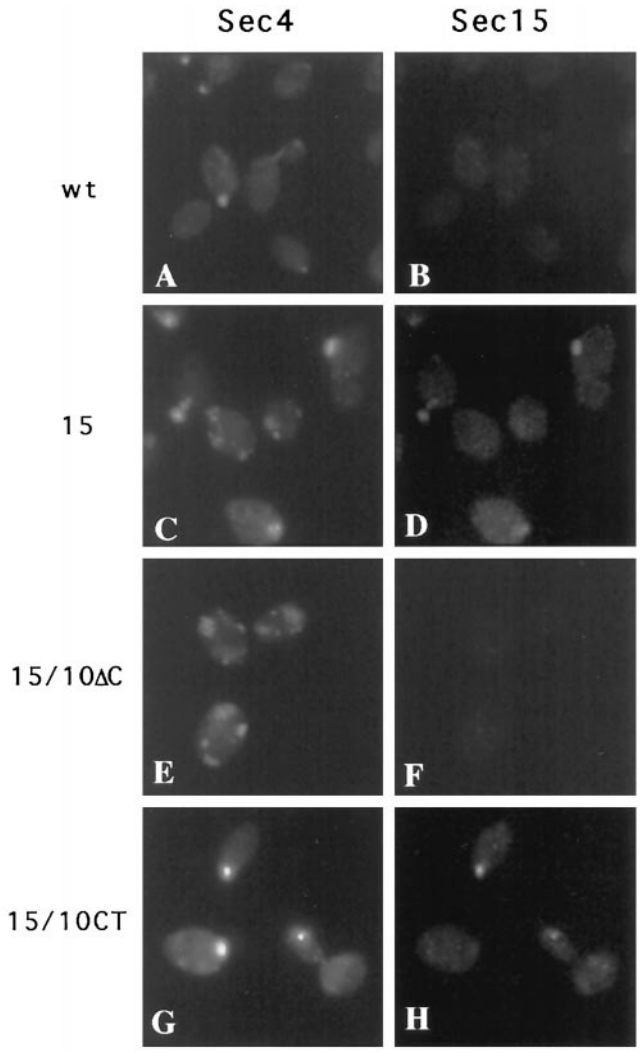

Figure 6. Colocalization of Sec15p and Sec4p upon Sec15p overexpression. Wild-type yeast (A and B) or yeast overexpressing Sec15p (C and D) or cooverexpressing Sec15p with Sec10ΔC (E and F) or with Sec10CT (G and H) were grown in galactose for 10 h to induce overexpression of the proteins. Cells were then processed for immunofluorescence as described in MATERIALS AND METHODS. Double labeling was performed with monoclonal anti-Sec4p antibodies (A, C, E, and G) and polyclonal anti-Sec15p antibodies (B, D, F, and H).

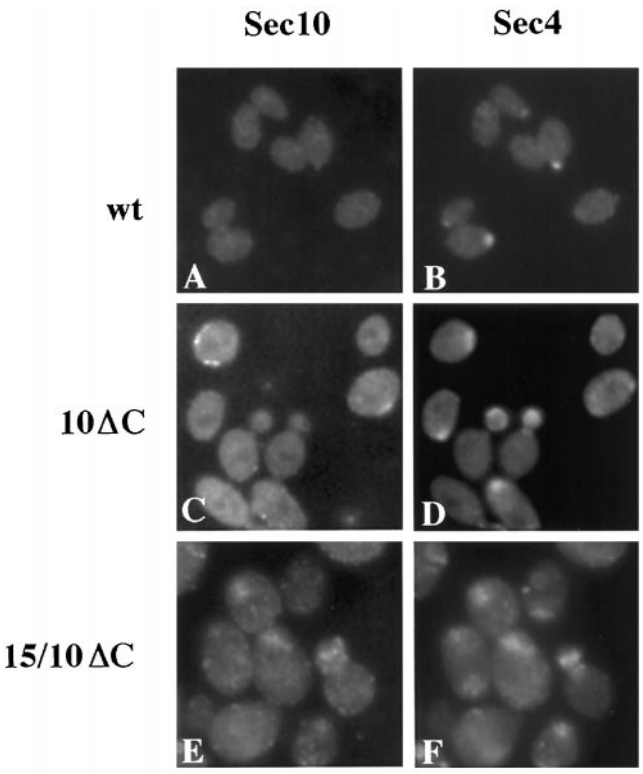

Figure 7. Sec10ΔC is recruited to the Sec4p patch upon Sec15p co-overexpression. Yeast were grown for 10 h in galactose to induce the overexpression of Sec10ΔC and Sec15p. Immunofluorescence was performed on the fixed cells with double labeling using anti-Sec10p (A, C, and E) and anti-Sec4p antibodies (B, D, and F). (A and B) Wild-type cells. (C and D) Cells overexpressing Sec10ΔC alone. (E and F) Cells co-overexpressing Sec15p and Sec10ΔC.

endogenous Sec10p was also recruited to the site of Sec4p colocalization. Since we did not observe a concentration of Sec10p staining in cells overexpressing Sec15p alone (our unpublished observations), it seems that most of Sec10p is not recruited into a patch, in this situation. Also, as shown in Figure 7, C and D, overexpression of Sec10ΔC alone did not result in the colocalization of Sec4p and Sec10ΔC. Sec10ΔC was distributed throughout the cells. Thus, Sec15p has the ability to colocalize with Sec4p in a patch when overexpressed, and Sec10ΔC can become incorporated into these patches through its interaction with Sec15p.

To extend this line of investigation, we used electron microscopy to examine the ultrastructure of yeast co-overexpressing Sec15p with either of the Sec10 fragments to assay for the formation of vesicle clusters (Figure 8). Cells co-overexpressing Sec10ΔC and Sec15p (Figure 8B) contained a comparable number of vesicles (170 ± 23 vesicles/cell section) as cells overexpressing Sec15p alone (160 ± 123 vesicles/cell section) (Figure 8A). Frequently, cells accumulated additional membranes such as Golgi and endoplasmic reticulum. The severe constitutive block in post-Golgi transport may lead to the accumulation of membrane at earlier stages of the secretory pathway. Vesicles in these cells often appeared in clusters similar to those in Sec15p-overexpressing cells (Figure 8B). The binding of Sec10ΔC to Sec15p did not appear to interfere with the ability of Sec15p to cluster vesicles.

We also analyzed the extent of vesicle accumulation in cells co-overexpressing Sec10CT and Sec15p. However, somewhat fewer vesicles were present (52 ± 17

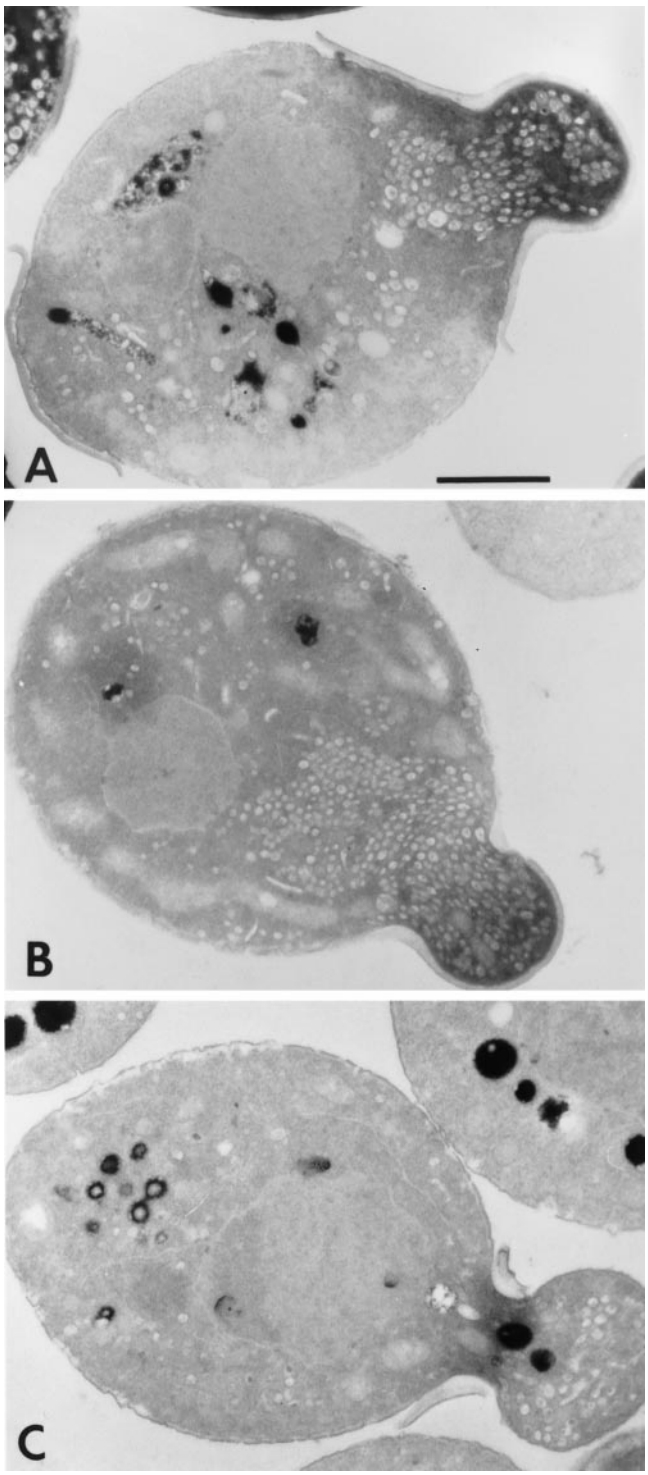

Figure 8. Electron microscopic analysis of cells co-overexpressing Sec15p with Sec10ΔC or Sec10CT. Yeast were grown for 10 h in galactose to induce the overexpression of Sec10ΔC, Sec10CT, and Sec15p. Cells were then processed for electron microscopy as described in MATERIALS AND METHODS. (A) Cell overexpressing Sec15p alone. (B) Cell co-overexpressing Sec15p and Sec10ΔC. (C) Cell co-overexpressing Sec15p with Sec10CT. Scale bar, 1 μm.

vesicles/cell section) than when Sec15p alone was overexpressed (160 ± 123 vesicles/cell section) (Figure 8C). While a minor fraction of cells did show a vesicle cluster (our unpublished observations), such clusters were generally less prominent (as in Figure 8C). In contrast, overexpression of Sec10CT alone caused an elongated cell shape without any accumulation of vesicles (5 ± 2 vesicles/cell section). The phenotypic interaction of Sec15p and Sec10CT may not result from a direct interaction of Sec10CT and Sec15p, but from the counterbalancing of two opposing effects on the cell. Overexpression of Sec15p may, by inhibiting the secretory pathway, prevent the formation of elongated cells, while overexpression of Sec10CT may, by slowing the growth rate of the cells, limit the accumulation of vesicles.

Myo2p Colocalizes with Sec4p

Both actin and the class V unconventional myosin, Myo2p, have been implicated in the targeting of vesicles. To explore the possibility of the presence of components of the cytoskeleton in the vesicle cluster formed in response to Sec15p overexpression, we performed double labeling with Sec4p and actin or Sec4p and Myo2p antibodies. The localization of Sec4p was taken as a reference point for the formation of the vesicle cluster. Similar to the situation in wild-type cells, actin and Sec4p, although generally present in similar regions of the cell, never colocalized exactly (our unpublished observations). Some overlap of localization was observed, particularly in large budded cells overexpressing Sec15p. In these cells, Sec15p was frequently found in the neck region between mother and daughter cells as was actin. Yet even in these situations, the colocalization was still only partial. The additional overexpression of Sec10ΔC or Sec10CT did not further alter the pattern of actin localization.

The class V unconventional myosin, Myo2p, has been localized to the sites of exocytosis in yeast (Lillie and Brown, 1994). Furthermore, the temperature-sensitive mutant, *myo2–66*, leads to an accumulation of vesicles, clearly showing a connection between this myosin and exocytosis (Govindan *et al.*, 1995). In wild-type cells, Myo2p and Sec4p did colocalize in the bud tip and at the neck between mother and daughter cells (Figure 9, A and B). The additional punctate cytoplasmic staining of Myo2p, which had also been previously reported (Lillie and Brown, 1994), did not colocalize with Sec4p. In cells overexpressing Sec15p, Myo2p remained, in many cases, colocalized with Sec4p staining in patches (Figure 9, C and D). Sec15p, therefore, seemed to be able to also mislocalize Myo2p together with Sec4p. However, not all cells displayed Myo2p staining, and when multiple spots of Sec4p were present in a single cell, only the ones with the highest intensity of Sec4p staining would costain for

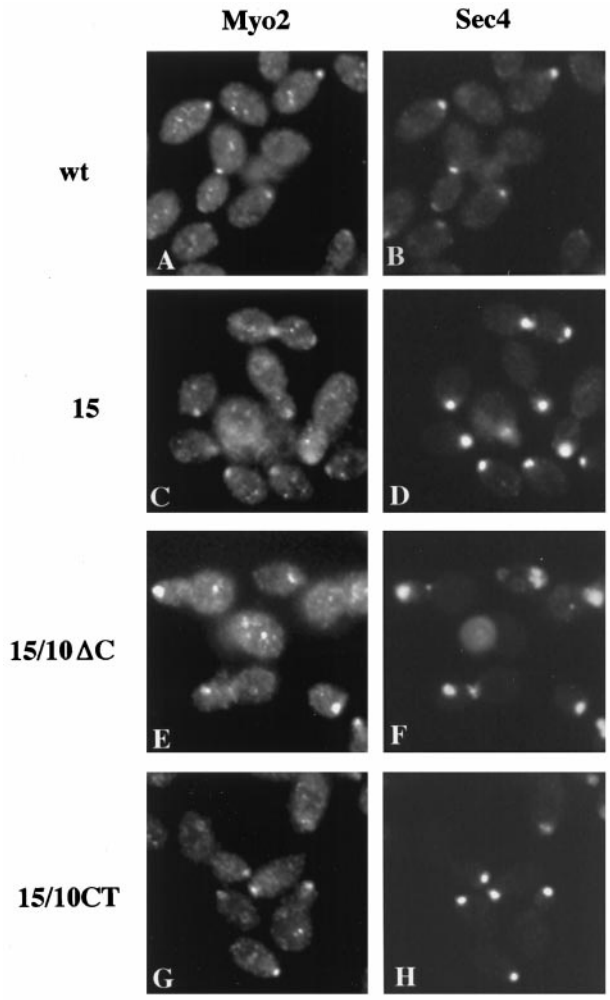

Figure 9. Myo2p colocalizes with Sec4p. Yeast were grown for 10 h in galactose to induce the overexpression of Sec10ΔC, Sec10CT, and Sec15p. Cells were then processed for immunofluorescence and costaining performed with anti-Myo2p (A, C, E, and G) and anti-Sec4p antibodies (B, D, F, and H). (A and B) Wild-type. (C and D) Cells overexpressing Sec15p. (E and F) Cells co-overexpressing Sec15p with Sec10ΔC. (G and H) Cells co-overexpressing Sec15p with Sec10CT.

Myo2p. The additional overexpression of Sec10p fragments did not alter Sec4p and Myo2p colocalization (Figure 9, E–H). By extrapolation, we conclude that Myo2p also colocalizes with Sec15p in this patch.

DISCUSSION

All of the components of the yeast exocyst complex have recently been identified (TerBush *et al.*, 1996), and a similar protein complex has been purified from brain (Hsu *et al.*, 1996). A number of lines of evidence indicate that the exocyst functions to specify sites on the plasma membrane for the docking and fusion of secretory vesicles. However, the specific functions of the individual subunits have remained unknown. In this study we have generated dominant-negative mutants of Sec10p to examine the potential functions of this exocyst component and have used these dominant-negative Sec10p constructs to investigate the relationship of Sec10p to the exocyst complex.

The Sec10p dominant-negative constructs were based on analysis of the hydrophilicity of the amino acid sequence as well as the regions of homology between Sec10p and its homologues in *C. elegans* and mammals. Overexpression of either the amino-terminal two-thirds (Sec10ΔC) or the carboxy-terminal region (Sec10CT) resulted in the inhibition of cell growth. The effects of Sec10ΔC and Sec10CT overexpression on cell morphology, however, were strikingly distinct (see Table 3 for summary). Expression of Sec10ΔC causes an accumulation of post-Golgi vesicles indicating a block in exocytosis. In these cells, the endogenous Sec10p is missing from the exocyst complex, but all other subunits are present. The high concentration of Sec10ΔC may competitively inhibit the binding of full-length Sec10p to the complex. Sec10ΔC is apparently lost from the complex during the extensive washing necessary for immunopurification. We suggest, therefore, that the incorporation of Sec10p into the exocyst is essential for normal exocytosis and cell growth. In cells overexpressing Sec10ΔC, the incomplete composition of the exocyst complex may be the cause for the block in exocytosis and the accumulation of vesicles.

We have identified Sec15p as an interactive partner of Sec10ΔC. Prior studies have shown that overexpression of Sec15p leads to growth inhibition, the forma-

Table 3. Effects of Sec10ΔC and Sec10CT overexpression

Characteristic	Sec10ΔC	Sec10CT
Morphology	Cells enlarged, frequently unbudded	Hyperpolarized cells
Actin	Delocalized	Localized
Vesicle accumulation	Yes	No
Synthetic lethality	With members of Exocyst	With t-SNARE, Sec1p, and some Exocyst members
Interaction	With Sec15p	?
Sec15 co-overexpression	Recruited to Sec15p patch	Co-suppression of hyperpolarized phenotype

tion of a patch of Sec15p, and the accumulation of a cluster of secretory vesicles. Sec15p appears to respond to activated Sec4p, since mutations in either Sec4p or in its nucleotide exchange protein, Sec2p, block patch formation (Salminen and Novick, 1989), and we have shown here that the patch of Sec15p colocalizes with Sec4p, a marker for secretory vesicles. To help establish the relationship of Sec10p to Sec4p and Sec15p, we explored the effects of co-overexpression of Sec15p and Sec10p fragments. We have shown that Sec10ΔC is recruited to the Sec4p vesicle patch formed upon overexpression of Sec15p. These data suggest that Sec15p has an innate ability to cluster vesicles that is not interrupted by the binding of Sec10ΔC to Sec15p. We have further shown that Myo2p is found together with Sec4p and Sec15p in this patch structure. Recently, the brain homologue of Myo2p, myosin V, was reported to reside on synaptic vesicles (Prekeris and Terrian, 1997). The potential interaction of Myo2p with vesicles is consistent with its colocalization with Sec4p and Sec15p in the patch.

While overexpression of Sec10CT did cause growth inhibition, it did not result in a block in exocytosis as revealed by the absence of vesicle accumulation (see Table 3 for summary). Furthermore, Sec10CT was not observed to physically interact with any component of the exocyst, and expression of Sec10CT did not alter the subunit composition of the complex. However, overexpression of Sec10CT did result in an elongated cell phenotype. In wild-type cells, secretion during cell cycle progression is initially restricted to a region at the tip of the bud, becomes isotropic as the bud becomes larger, and then finally relocates to the neck region for the formation of the septum between mother and daughter cell. The reorientation of vesicular traffic from the tip of the bud may be disturbed or delayed by Sec10CT overexpression. We suggest, therefore, that Sec10CT may interact with a protein that mediates the reorientation of secretion during cell cycle progression. By binding to such a protein, Sec10CT may block the interaction with full-length Sec10p and thereby block the reorientation of the secretory machinery from the bud tip, resulting in the formation of elongated cells. Sec10p appears to play an important role functioning at the interface of the secretory pathway with the morphogenetic machinery.

ACKNOWLEDGMENTS

We would like to thank Fern Finger and Christiane Walch-Solimena for invaluable help with immunofluorescence and electron microscopy, Linda Cichione for thin sectioning, and Laurie Daniels and Kohji Takei for helpful assistance with electron microscopy. Furthermore, we are grateful to Samara Reck-Peterson for the generous gift of anti-Myo2 antibody. We also thank Pietro DeCamilli for helpful discussions and Chavelar Carr and Fern Finger for critical reading of the manuscript. D.R. was supported by a fellowship from the Deutsche Forschungsgemeinschaft and W.G. was supported by Brown-Cox and National Institutes of Health fellowships. This work was supported by grant GM-35370 from the National Institutes of Health (to P.N.).

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