Yeast *SEC16* Gene Encodes a Multidomain Vesicle Coat Protein that Interacts with Sec23p

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Abstract. Temperature-sensitive mutations in the *SEC16* gene of *Saccharomyces cerevisiae* block budding of transport vesicles from the ER. *SEC16* was cloned by complementation of the *sec16-1* mutation and encodes a 240-kD protein located in the insoluble, particulate component of cell lysates. Sec16p is released from this particulate fraction by high salt, but not by nonionic detergents or urea. Some Sec16p is localized to the ER by immunofluorescence microscopy. Membrane-associated Sec16p is incorporated into transport vesicles derived from the ER that are formed in an in vitro vesicle budding reaction. Sec16p binds to Sec23p, a COPII vesicle coat protein, as shown by the two-hybrid interaction assay and affinity studies in cell extracts. These findings indicate that Sec16p associates with Sec23p as part of the transport vesicle coat structure.

Genetic analysis of *SEC16* identifies three functionally distinguishable domains. One domain is defined by the five temperature-sensitive mutations clustered in the middle of *SEC16*. Each of these mutations can be complemented by the central domain of *SEC16* expressed alone. The stoichiometry of Sec16p is critical for secretory function since overexpression of Sec16p causes a lethal secretion defect. This lethal function maps to the NH_2-terminus of the protein, defining a second functional domain. A separate function for the COOH-terminal domain of Sec16p is shown by its ability to bind Sec23p. Together, these results suggest that Sec16p engages in multiple protein–protein interactions both on the ER membrane and as part of the coat of a completed vesicle.

TRANSPORT vesicles mediate the movement of protein cargo between the organelles of the secretory pathway (Palade, 1975). The yeast *Saccharomyces cerevisiae* has been useful for identifying genes required for the proper function of these transport vesicles. Through the use of genetic screens and purification of proteins active in cell-free transport assays, ~20 yeast gene products have been identified that act in vesicle transport between the ER and Golgi apparatus (for review see Pryer et al., 1992). Some of these gene products appear to be evolutionarily conserved since mammalian homologues have been isolated that have been shown to complement *SEC* gene function or to be located in the transitional region between the ER and Golgi apparatus in mammalian cells (Wilson et al., 1989; Orci et al., 1991; Griff et al., 1992; Hosobuchi et al., 1992; Kuge et al., 1994; Shaywitz et al., 1995).

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; DAPI, 4′,6-diamidino-2-phenylindole; GST, glutathione-*S*-transferase; SC, synthetic complete medium; ts, temperature sensitive; YPD, yeast extract/peptone/dextrose medium.

Four genes, *SEC12*, *SEC13*, *SEC16*, and *SEC23*, are recognized to be required for the process of vesicle formation from the ER by morphological assays (Kaiser and Schekman, 1990). *SEC16*, the least well characterized member of this group of genes, was identified in a screen for conditional mutants that block secretory protein transport to the cell surface (Novick et al., 1980). At the nonpermissive temperature, *sec16* mutations cause rapid accumulation of secretory protein precursors in core-glycosylated ER forms showing that Sec16p is needed for protein transport to the Golgi apparatus (Novick et al., 1981; Stevens et al., 1982). *SEC16* was implicated in the process of vesicle budding since mutants do not form 50-nm transport vesicles observed by thin section electron microscopy (Kaiser and Schekman, 1990). The other genes with a similar phenotype, *SEC12*, *SEC13*, and *SEC23*, have been shown to be necessary for vesicle budding in cell-free extracts (Rexach and Schekman, 1991; Hicke et al., 1992; Pryer et al., 1993). *SEC13* and *SEC23* encode components of the COPII protein coat of ER vesicles formed in vitro (Barlowe et al., 1994).

Additional evidence for the involvement of *SEC16* in vesicle budding comes from the genetic interactions between *SEC16* and genes required for vesicle formation. *sec16-1* or *sec16-2* alleles are lethal at 25°C when combined with mutations in *SEC12*, *SEC13*, or *SEC23*, but have no

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pronounced effect on mutations in *SEC* genes that act later in the secretory pathway (Kaiser and Schekman, 1990). These stage-specific genetic interactions show that at 25°C, SEC*16* alleles impair vesicle formation at the ER. Further, *sec16* mutations are partially suppressed by overexpression of *SAR1*, a small GTP-binding protein that is required for ER to Golgi transport (Nakano and Muramatsu, 1989). The functions of *SAR1*, *SEC12*, and *SEC23* constitute a GTP hydrolysis cycle coupled to vesicle formation. Sec23p stimulates GTPase activity of Sar1p (Yoshihisa et al., 1993), and Sec12p stimulates exchange of GTP for GDP by Sar1p (Barlowe and Schekman, 1993). The genetic interactions outlined above suggest that the function of *SEC16* may also relate to this GTPase cycle. Sec16p could generate a local signal that acts on the Sar1p GTPase cycle; alternatively Sec16p could be acted upon by a signal generated by Sar1p.

In this paper, we describe the isolation of *SEC16* and the characterization of its product. Sec16p was found to be both on the ER and on vesicles that had budded from the ER. Moreover, the COOH-terminal domain of Sec16p is shown to bind to Sec23p, indicating that Sec16p is part of the vesicle coat structure and may serve as a platform for incorporation of cytosolic proteins into the vesicle coat. A genetic dissection of *SEC16* identifies at least three functionally distinguishable domains of the protein. Analysis of Sec16p and its association with other vesicle components will likely uncover many of the subunit interactions that are important for transport vesicle assembly and integrity.

Materials and Methods

Strains, Media, and Recombinant DNA Techniques

Table I lists the *S. cerevisiae* strains and Table II describes the plasmids used in this study. Standard genetic manipulations and yeast transformations were performed as described (Kaiser et al., 1994). Unless otherwise noted, cultures were grown in synthetic complete (SC) medium with the indicated carbon source and without the supplements appropriate for selection. DNA manipulations were performed using standard techniques (Sambrook et al., 1989). PCR was performed using Taq polymerase according to the manufacturer's specifications (Perkin-Elmer Cetus, Norwalk, CT).

Cloning and Sequencing of SEC16 and SEC16 Mutations

SEC16 was isolated from a library of *S. cerevisiae* genomic sequences in YCp50 (Rose et al., 1987). Insert sequences from a plasmid that complemented *sec16-1* were subcloned into the centromere, *URA3* vector pRS316 (Sikorski and Hieter, 1989). The smallest complementing subclone contained a 7.2-kb BamHI–SphI genomic fragment. Deletion derivatives were produced by digestion of linear plasmid DNA with exonuclease III and S1 (Henikoff, 1987). Using nested deletions from both ends of the cloned DNA as the templates, the gene was sequenced following the protocol for the Sequenase kit (United States Biochemical Corp., Cleveland, OH). Gaps in the sequence were filled using synthetic oligonucleotide primers that matched the sequence of the first strand.

To test for linkage between the cloned sequences and the *SEC16* locus, the integrating plasmid YIp352 (Hill et al., 1986) with a SphI–StuI fragment containing ~ the COOH-terminal two-thirds of *SEC16* was directed to integrate at the homologous chromosomal locus by cleaving the plasmid in the insert sequences with PstI before transformation of CKY8. Two transformants were crossed to CKY51 and tetrads showed complete linkage of the plasmid sequences to *sec16-2*.

Because the NH_2-terminal domain of *SEC16* is not essential, we per-

Table I. Saccharomyces cerevisiae Strains

Strain	Genotype	Source
CKY8	*MATα ura3-52 leu2-3, 112*	Kaiser Lab Collection
CKY10	*MAT***a** *ura3-52 leu2-3, 112*	Kaiser Lab Collection
CKY19	*MAT***a** */MATα ura3-52/ura3-52 leu2-Δ1/leu2-Δ1 his3-Δ200/his3-Δ200 ade2-101/ade2-101 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63*	Kaiser Lab Collection
CKY50	*MATα sec16-2 ura3-52 his4-619*	Kaiser Lab Collection
CKY51	*MAT***a** *sec16-2 ura3-52*	Kaiser Lab Collection
CKY52	*MAT***a** *sec16-1 ura3-52 leu2-3, 112*	Kaiser Lab Collection
CKY93	*MATα ura3-52 leu2-3, 112 pep4::URA3*	Kaiser Lab Collection
CKY96	*MATα ura3-52 leu2-3, 112 his4-619* Gal^+	Kaiser Lab Collection
CKY107	*MAT***a** *sec16-3 ura3-52 leu2-3, 112*	Kaiser Lab Collection
CKY200	*MAT***a** *sec16-2 ura3-52 leu2-3, 112 his4-619* Gal^+	Kaiser Lab Collection
CKY230	*MAT***a** *sec16-4 sec13-1 ura3-52 leu2-3, 112 ade2 ade3* (pKR4)	K. Roberg and C. A. Kaiser, unpublished data
CKY231	*MAT***a** *sec16-5 sec13-1 ura3-52 leu2-3, 112 ade2 ade3* (pKR4)	K. Roberg and C. A. Kaiser, unpublished data
CKY232	CKY96 (pPE4)	This study
CKY233	*MAT***a**/*MATα SEC16/sec16-Δ1::TRP1 ura3-52/ura3-52 leu2-Δ1/leu2-Δ1 his3-Δ200/his3-Δ200 ade2-101/ade2-101 lys2-801/lys2-801 trp1-Δ63/trp1-Δ63*	This study
CKY237	*MAT***a** *sec16-Δ1::TRP1 ura3-52 leu2 trp1-Δ63* Gal^+ (pPE4)	This study
CKY238	CKY10 (pPE8)	This study
CKY239	CKY10 (pPE26)	This study
CKY240	CKY96 (pRS315)	This study
CKY241	*MAT***a**/*MATα ura3-52/ura3-52 leu2-3, 112/leu2-3, 112 HIS4/his4-619* Gal^+ (pPE4)	This study
CKY247	CKY237 (pCD43)	This study
CKY282	*MAT***a** *ura3-52 leu2-3, 112 his4-619* Gal^+	This study
CKY283	CKY93 (pPE26)	This study
EGY40	*MATα ura3-52 leu2 his3 trp1*	Golemis and Brent, 1992

Table II. Plasmids

Plasmid	Description	Source
pRS304	integrating vector marked with *TRP1*	Sikorski and Hieter, 1989
pRS315	centromere vector marked with *LEU2*	Sikorski and Hieter, 1989
pRS316	centromere vector marked with *URA3*	Sikorski and Hieter, 1989
YCp50	centromere vector marked with *URA3*	Rose et al., 1987
YEp352	2μ vector marked with *URA3*	Hill et al., 1986
pCD43	*GAL1/GAL10* promoter in pRS316	Shaywitz et al., 1995
pPE4	*GAL1* promoted *SEC16* in pRS315	This study
pPE8	*SEC16* in pRS315	This study
pPE26	*SEC16-HA* in pRS315	This study
pPE38	*GAL1* promoted *SEC16*(565-2194) in pRS315	This study
pPE46	*GAL1* promoted *SEC16*(1017-2194) in pRS315	This study
pPE113	*SEC16* disruption in pRS304	This study
pPE129	*SEC16*(565-2194) in YEp352	This study
pPE130	*SEC16*(565-2164) in YEp352	This study
pPE131	*SEC16*(565-1475) in YEp352	This study
pPE132	*SEC16*(565-1171) in YEp352	This study
pPE133	*SEC16*(565-1027) in YEp352	This study
pCK1615	*SEC16*(565-2194) in YEp352	This study
pKR4	*SEC13* and *ADE3* in pRS315	K. Roberg, and C. A. Kaiser, unpublished data
pPE12	*SUC2* fusion vector marked with *URA3*	This study
pPE14	*GAL1* promoted *SEC16*(1-103)-*SUC2* in pRS316	This study
pPE27	*GAL1* promoted *SEC16*(1-661)-*SUC2* in pRS316	This study
pPE29	*GAL1* promoted *SEC16*(1-1092)-*SUC2* in pRS316	This study
pPE30	*GAL1* promoted *SEC16*(1-1967)-*SUC2* in pRS316	This study
pPE36	*GAL1* promoted *SEC16*(1-499)-*SUC2* in pRS316	This study
pPE37	*GAL1* promoted *SEC16*(1-824)-*SUC2* in pRS316	This study
pPE53	*GAL1* promoted *SEC16*(565-1235)-*SUC2* in pRS316	This study
pEG202	lexA DNA binding domain in a 2μ, *HIS3*-marked vector	Gyuris et al., 1993
pJG4-5	acidic activation domain in a 2μ, *TRP1*-marked vector	Gyuris et al., 1993
pSH18-34	*lacZ* reporter gene in a 2μ, *URA3*-marked vector	Gyuris et al., 1993
pPE58	*SEC16*(1645-2194) in pEG202	This study
pPE59	*SEC16*(1-824) in pEG202	This study
pPE74	*SEC16*(447-1737) in pEG202	This study
pPE81	*SEC23* in pJG4-5	This study
pPE86	*GAL1*-promoted *SEC16C-HA*(1638-2194) in pRS315	This study
pPE119	*GAL1*-promoted *GST-SEC23* fusion in pRS316	This study
pRD56	*GAL1*-promoted *GST* in pRS316	R. Deshaies

The numbers in parentheses indicate the amino acid numbers of the preceding gene's product.

formed several tests to establish that the putative initiation ATG preceded by in-frame stop codons was in fact the start of translation. First, a frame-shift mutation was generated by a 2-bp deletion at a SacII site at the codon for amino acid 103. A clone with this mutation did not complement a *sec16-2* mutation, indicating that the SacII site was within the *SEC16* coding sequence. Second, a fusion to the *GAL1* promoter was constructed to initiate translation at the putative initiation ATG (pPE4). This plasmid was shown to express functional *SEC16* by complementation of a chromosomal deletion of *SEC16*. To show that *SEC16* expressed from the *GAL1* promoter initiated at the wild-type initiation codon, both wild-type *SEC16* and *SEC16* expressed from the *GAL1* promoter were fused at amino acid 103 to the *SUC2* gene. The size of fusion proteins expressed from the wild-type promoter and the *GAL1* promoter were identical as determined by SDS-PAGE and Western blotting with anti-invertase antibodies (not shown).

Five temperature-sensitive *sec16* mutations were mapped by marker rescue recombination with plasmid-borne *SEC16* sequences (Falco et al., 1983). A *sec16* mutation to be mapped was transformed with each of a nested set of deletion plasmids. To stimulate mitotic recombination between the plasmid sequences and chromosomal *SEC16*, transformant cultures were exposed briefly to light from germicidal lamps such that ~50% of the cells survived. Temperature-resistant recombinants, scored after plating at 37°C, arose only if the deletion did not remove the site of the mutation.

Once located by deletion mapping, each of the mutant alleles was cloned by gap repair of plasmid pCK1615 containing a gap within the *SEC16* sequences produced by cutting with XbaI and BstXI before transformation (Rothstein, 1991). The base changes responsible for the mutations were obtained by sequencing the appropriate deletion interval using synthetic oligonucleotide primers.

SEC16 Deletion

A chromosomal deletion *sec16-Δ1::TRP1* that replaced all but the first 103 amino acids of *SEC16* coding sequence with *TRP1* was made by the method of γ transformation (Sikorski and Hieter, 1989). The disruption plasmid, pPE113, contained both a 0.4-kb KpnI–StuI fragment from the 3′ noncoding sequence and a BamHI–SacII fragment containing 5′ sequences and the first 103 codons inserted into pRS304 (*TRP1*). A *trp1* diploid, CKY19, was transformed with linearized pPE113 to yield CKY233 with a heterozygous chromosomal disruption of *SEC16* that extended from amino acid 103 to 30 basepairs past the end of the gene. Integration was confirmed by Southern blot analysis (ECL kit; Amersham Corp., Arlington Heights, IL).

Construction of GAL Promoter Fusions

SEC16 was fused to the *GAL1* promoter in pCD43 (Shaywitz et al., 1995) using the primer 5′-GCGGATCCAAGA**ATG**ACACCTGAAGCCAAG-3′ and PCR to create a junction between the BamHI site (underlined) adjacent to the *GAL1* promoter and the beginning of the *SEC16* coding sequence (bold). The plasmid expressing full-length *SEC16* from the *GAL1* promoter, pPE4, contained the complete coding sequence of *SEC16* extending to the StuI site in the 3′ noncoding sequence inserted into the

BamHI–SmaI sites of pRS315. An NH_2-terminally truncated *GAL1* promoter fusion, pPE38, that contained amino acids 565–2194 of Sec16p was made using the primer 5′-GCGGATCCAACC**ATG**CGTCAAGAG-CAAGTTC-3′ to create the junction between an ATG codon at position 565 and the BamHI site adjacent to the *GAL1* promoter. A third *GAL1* promoter fusion, pPE46, that contained amino acids 1017–2194 of Sec16p was made using the primer 5′-TTGGATCC**ATG**ATTTCATCAAG-CATTGTAC-3′ to create the junction between an ATG codon at position 1017 and the BamHI site adjacent to the *GAL1* promoter.

Construction of Invertase Fusions

A plasmid pPE12 was created to fuse portions of *SEC16* to the cytoplasmic form of invertase, encoded by *SUC2*. The plasmid pPE12 has the XhoI–SacII polylinker of pBluescript (Stratagene Inc., La Jolla, CA) fused to *SUC2* by use of the following oligonucleotide primer: 5′-TC-CCCGCGGCATCA**ATG**ACAAACGAAAC-3′. The SacII site is underlined and the ATG for the internal form of invertase is in bold face. All of the *SEC16–SUC2* fusions were expressed from the *GAL1* promoter and Table II lists the amino acids of *SEC16* contained in each fusion.

Epitope Tagging of SEC16

The epitope-tagged *SEC16-HA* was constructed as follows: a 2-kb PstI–NotI fragment of pPE4 was subcloned into pBluescript-SK+ (Stratagene Inc.). Oligonucleotide site–directed mutagenesis was used to insert a NotI site between amino acids 1892 and 1893 of *SEC16* (Kunkel et al., 1987). The oligonucleotide sequence was 5′-CATCGCCTGCTATATATG-CAGGCGGCCGCAGAACTCACCAAGCACATGC-3′. This plasmid was partially digested with NotI, and a 100-bp NotI–NotI fragment from pGTEPI (Tyers et al., 1993), containing three tandem copies of the hemagglutinin epitope recognized by the 12CA5 mAb (BAbCO, Richmond, CA), was inserted (Kolodziej and Young, 1991). The epitope-tagged fragment of *SEC16* was inserted into the full-length *SEC16* plasmid, pPE8 to generate full-length *SEC16-HA*, pPE26.

Carboxypeptidase Y (CPY) Immunoprecipitation

To analyze cells overexpressing Sec16p, a strain carrying plasmid pPE4 expressing *SEC16* from the *GAL1* promoter (CKY232) was grown at 30°C to exponential phase in minimal medium containing 2% raffinose and 3% glycerol. Expression from the *GAL1* promoter was induced in medium containing 2% galactose for 10 h. To analyze cells with decreased levels of Sec16p, a strain with a chromosomal deletion of *SEC16* carrying pPE4 (CKY247) was grown at 30°C to exponential phase in SC medium containing 1% glucose and 1% galactose. Sec16p expression was shut off by growth in medium containing 2% glucose for 15 h. The *sec16-2* strain (CKY50) was grown at 25°C, then 37°C for 1 h. Cultures were labeled for 10 min at a concentration of $\sim 1 \times 10^8$ cells/ml using 150 μCi/ml of ^{35}S-radiolabeled cysteine and methionine (Expre $^{35}S^{35}S$, DuPont-NEN, Boston, MA). After 10 min, the chase was initiated by addition of cysteine and methionine to a final concentration of 30 and 40 μg/ml, respectively. An aliquot of 2×10^7 cells was removed at time points and lysed using NaOH as described below for Western blots. The resulting extract was resuspended in 50 μl sample buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 0.1 M DTT, 10% glycerol, and 0.01% bromophenol blue) and heated at 95°C for 5 min. CPY isolated from extracts by immunoprecipitation was analyzed by gel electrophoresis and autoradiography (Laemmli, 1970; Rothblatt and Schekman, 1989).

Electron Microscopy

CKY50 carrying *sec16-2* was grown at 25°C in yeast extract/peptone/dextrose medium (YPD) to exponential phase ($\sim 1 \times 10^7$ cells/ml) and shifted to 37°C for 1 h. To prepare cells depleted of Sec16p, a strain with a chromosomal deletion of *SEC16* carrying a plasmid with *SEC16* expressed from the *GAL1* promoter (CKY237) was grown at 30°C to exponential phase in medium containing 1% glucose and 1% galactose, diluted into medium containing 2% glucose, and grown for 16 h. Cells were prepared for electron microscopy by fixation with glutaraldehyde and $KMnO_4$ (Kaiser and Schekman, 1990). Fixed, dehydrated cells were embedded in Spurr's resin and were sectioned to a thickness of ~70 nm. Sections were stained with a 1:5 dilution of Reynold's lead citrate for 2.5 min (Reynolds, 1963) to enhance membrane profiles and were viewed in an electron microscope (1200CX; JEOL USA, Analytical Instruments Division, Cranford, NJ) at 80 kV.

Sec16p Antiserum

Sec16p antiserum was elicited against a hybrid protein composed of a segment of Sec16p fused to *Staphylococcal* protein A. A 1.4-kb PvuII–PstI fragment encoding 460 amino acids from the central region of *SEC16* was inserted into protein A fusion vector pRIT31 (Nilsson and Abrahmsen, 1990). Fusion protein was prepared from *Escherichia coli* extracts and antibody to this protein elicited as previously described (Griff et al., 1992).

The serum was affinity purified using a β-galactosidase-Sec16p hybrid protein constructed in the vector pEX1 by fusing to *lacZ* the same PvuII–PstI fragment of Sec16p used in the protein A fusion (Stanley and Luzio, 1984). The hybrid protein was isolated and used for affinity purification of the antibody as described (Griff et al., 1992).

Western Blotting

Yeast cultures were grown to exponential phase ($\sim 1 \times 10^7$ cells/ml) in YPD or SC medium containing either 2% glucose or, for Sec16p overexpression, 2% raffinose and 3% glycerol followed by growth in 2% galactose for 4.5 h. Cell extracts were prepared by suspending $\sim 6 \times 10^7$ cells in 100 μl medium and adding 17 μl 1.85 M NaOH, 1 M β-mercaptoethanol for 10 min at 4°C (Yaffe and Schatz, 1984). Proteins were precipitated with TCA, washed with acetone, dried, and resuspended in 0.1 ml sample buffer by heating to 100°C. Protein extracts were assayed for total protein using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Protein samples were separated by electrophoresis on a gel of 6% polyacrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS without a stacking layer. Electrophoretic transfer of proteins to nitrocellulose was performed in the presence of 0.1% SDS (Harlow and Lane, 1988). Proteins were detected using a 1:500 dilution of 12CA5 mouse mAb (BAbCO) and a 1:10,000 dilution of anti–mouse IgG, peroxidase coupled whole antibody from sheep (Amersham Corp.), or a 1:500 dilution of affinity-purified anti-Sec16p rabbit polyclonal antibody and a 1:10,000 dilution of anti–rabbit IgG, peroxidase-linked whole antibody from donkey (Amersham Corp.). Western blots were developed using chemiluminescence (ECL kit; Amersham Corp.).

Cell Fractionation

To analyze the subcellular distribution of Sec16p, a wild-type strain (CKY10) was grown at 30°C in YPD to exponential phase. 2×10^9 cells were suspended in 50 ml 0.1 M Tris sulfate, pH 9.4, 28 mM β-mercaptoethanol for 10 min at 25°C, and then spheroplasted for 1 h at 30°C using 3,700 U lyticase in 10 ml spheroplasting buffer (2% yeast extract, 1% peptone, 10 mM Tris-HCl, pH 8.0, 0.7 M sorbitol). Metabolic activity was regenerated by aeration of spheroplasts in YPD with 0.7 M sorbitol for 1 h at 30°C. Cells were washed in 0.7 M sorbitol, 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and gently lysed in 0.5 ml cell lysis buffer (20 mM MES, pH 6.5, 0.1 M NaCl, 5 mM $MgCl_2$, and protease inhibitor cocktail) using 0.3 g of acid-washed glass beads. The inhibitor cocktail consisted of 1 mM PMSF, 10 μg/ml E-64, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 2 μg/ml aprotinin, and 0.5 U/ml α_2-macroglobulin (all Boehringer Mannheim Biochemicals, Indianapolis, IN) (Jones, 1991). Cell lysis was complete as judged by light microscopy. After lysis, the cell extract was subjected to a series of centrifugation steps using an ultracentrifuge rotor (TLA100; Beckman Instruments, Palo Alto, CA): 500 *g* for 20 min; 10,000 *g* for 30 min; and 150,000 *g* for 60 min, all at 4°C.

Extraction of Sec16p from the particulate fraction was tested by treating 0.1 ml cell extract with 1% Triton X-100, 0.1 M sodium carbonate (pH 11.5), 2.5 M urea or 0.5 M NaCl. Samples were incubated at 4°C for 1 h and separated into soluble and particulate fractions by centrifugation at 150,000 *g* for 1 h at 4°C. In both experiments, samples representing equal amounts of cell extract were solubilized in sample buffer and analyzed by Western blotting as described above.

Indirect Immunofluorescence

The intracellular location of Sec16p was examined by indirect immunofluorescence performed essentially as described (Pringle et al., 1991). A diploid yeast strain expressing *SEC16* from the *GAL1* promoter (CKY241) was grown at 30°C to early exponential growth phase (10^6–10^7 cells/ml) in SC medium containing 2% raffinose and 3% glycerol. To facilitate localization, Sec16p was overexpressed by transferring the cells to medium containing 2% galactose for 2 h before fixation.

Cells were fixed for 1 h with 3.7% formaldehyde in the medium. A 10-ml culture was collected by centrifugation and then spheroplasted with

100 U lyticase in 0.1 M potassium phosphate, pH 7.5, 28 mM β-mercaptoethanol for 30 min at 30°C. Antibody incubations were performed on coverslips in a humid chamber at 25°C for 1 h. Sec16p was detected using a 1:100 dilution of affinity-purified Sec16p antibody and a 1:500 dilution of anti-rabbit Ig-FITC antibody (Boehringer Mannheim Biochemicals). Samples mounted in medium containing 4,6-diamidino-2-phenylindole (DAPI) and *p*-phenylenediamine were photographed with an axioscope (Carl Zeiss, Inc., Thornwood, NY) using hypersensitized Technical Pan Film 2415 (Lumicon, Livermore, CA) at ASA400 and developed using D-19 (Eastman Kodak Co., Rochester, NY) for 4 min at 23°C (Schulze and Kirschner, 1986).

In Vitro Vesicle Synthesis and Purification

Membranes and cytosol used in the vesicle synthesis reaction were prepared as previously described with the exception that donor membranes were collected at 12,000 *g* (Wuestehube and Schekman, 1992). Membranes from CKY283 were prepared from spheroplasts that were lysed gently with glass beads in the presence of protease inhibitors. Cytosol was prepared from CKY93 without added guanine nucleotide. Guanine nucleotides were later added as indicated to budding reactions at a final concentration of 0.1 mM. A standard vesicle synthesis reaction of 1 ml contained 200 μg membranes, 2.4 mg cytosol, 1 mM GDP-mannose, 0.1 mM guanine nucleotide, and an ATP regeneration system in reaction buffer (20 mM Hepes-KOH, pH 6.8, 150 mM KOAc, 5 mM MgOAc, 250 mM sorbitol) with protease inhibitors (Wuestehube and Schekman, 1992). The reaction with apyrase added contained 10 U/ml apyrase in the place of the ATP regeneration system. Reactions were incubated at 20°C for 2 h unless otherwise noted. Donor membranes were removed by centrifugation of Eppendorf tubes at 17,000 rpm (12,000 *g*) for 10 min at 4°C in a rotor (TLA 100.3; Beckman Instruments, Inc., Palo Alto, CA). Vesicles were collected from the supernatant by centrifugation at 60,000 rpm for 30 min at 4°C in a TLA 100.3. Vesicle pellets were solubilized in sample buffer and proteins were analyzed by Western blotting using either the 12CA5 mAb, a 1:1,000 dilution of Sec22p polyclonal antibody (gift of Dr. Charles Barlowe, Dartmouth Medical School, Hanover, NH), or a 1:1,000 dilution of Sec61p polyclonal antibody (gift of Dr. Randy Schekman, University of California, Berkeley, Berkeley, CA). Protein detected by Western blotting was quantitated by densitometry using an Ultroscan (2202; LKB Instruments, Inc., Gaithersburg, MD). The protease inhibitor cocktail used for these experiments contained 1 mM PMSF, 10 μg/ml E-64, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 2 μg/ml aprotinin, 50 μg/ml antipain, 1 mg/ml Pefabloc, 0.1 mg/ml phosphoramidon, 40 μg/ml bestatin, and 0.25 U/ml α_2-macroglobulin (all Boehringer Mannheim Biochemicals).

Vesicles formed in vitro from donor membranes prepared at 32,000 *g* were fractionated by gel filtration on a 14-ml (18 cm) Sephacryl S-1000 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in reaction buffer as described (Barlowe et al., 1994). A 0.7-ml sample of the 32,000 *g* supernatant from a 1.0-ml reaction was applied to the column, eluted with reaction buffer, and 0.75-ml fractions were collected. Vesicles in each fraction were concentrated by centrifugation at 60,000 rpm for 30 min at 4°C in a TLA 100.3 rotor. Proteins solubilized in sample buffer were analyzed by Western blotting. Column fractions were also assayed for total protein using the DC protein assay kit.

Two-Hybrid Protein–Protein Interaction Assay

Potential interactions between Sec16p and Sec23p were investigated using the two-hybrid protein interaction assay and the plasmids pEG202, pJG4-5, and pSH18-34 (Gyuris et al., 1993). Plasmids derived from pEG202 contained the lexA DNA-binding domain fused to fragments of Sec16p: amino acids 1645–2194 (pPE58), 1–824 (pPE59), and 447–1737 (pPE74). The full-length coding sequence of *SEC23* was fused to an acidic transcriptional activator in the plasmid pJG4-5 to create pPE81.

Combinations of control or fusion protein plasmids together with a reporter plasmid, pSH18-34, were transformed into the strain EGY40 (Golemis and Brent, 1992). Positive interactions were scored as blue colonies on SC medium (pH 7.0) containing 2% galactose and 40 mg/l X-gal. β-galactosidase activity was assayed as described (Kaiser et al., 1994). Strains were grown to exponential phase in SC medium containing 2% raffinose and 3% glycerol. 10 h before the assay galactose was added to a final concentration of 2% to induce production of the acidic activator protein. Activity was normalized to total protein determined by the Bradford assay (Bio-Rad Laboratories).

Affinity Isolation of Sec16p and Sec23p

The full-length coding sequence of *SEC23* was fused to the glutathione-*S*-transferase (GST) gene (Smith and Johnson, 1988) expressed from the *GAL1* promoter to create pPE119, a derivative of pRD56 (the kind gift of Dr. Ray Deshaies, California Institute of Technology, Pasadena, CA). To produce a soluble and detectable COOH-terminal domain of *SEC16*, we used plasmid pPE86 that expressed the COOH-terminus of Sec16p-HA (amino acids 1638–2194), from the *GAL1* promoter. Binding was tested in extracts from CKY282 transformed with pPE119 and pPE86.

Protein extracts were prepared from cells grown to exponential phase in SC medium, then for 4 h in SC medium containing 2% galactose. 10^8 cells were lysed using glass beads in extraction buffer (20 mM Hepes-KOH, pH 6.8, 80 mM KOAc, 5 mM MgOAc, 0.02% Triton X-100) with protease inhibitor cocktail. The extract was diluted to 1 ml with extraction buffer and the lysate was cleared by centrifugation at 13,000 *g* for 2 min. Glutathione Sepharose 4B beads (Pharmacia Fine Chemicals) were added and samples were incubated for 1 h at 25°C. Beads were washed three times in 1 ml extraction buffer and once in buffer without Triton X-100. Proteins were solubilized in sample buffer and resolved by SDS-PAGE on a 7% gel. Lysate samples were prepared by adding 5× sample buffer to an aliquot of cleared lysate. Proteins were transferred to nitrocellulose and Western blots were developed as described above.

Results

Isolation and Sequence of SEC16

A genomic library in the centromere vector YCp50 (Rose et al., 1987) was screened for clones that complemented *sec16-1* at 38°C. A complementing 7.2-kb BamHI–SphI fragment was isolated. This segment was shown to contain the authentic *SEC16* locus by directing integration of a plasmid carrying this fragment to the homologous chromosomal site, and showing that the integrated plasmid sequences were tightly linked to *sec16-2* by segregational analysis.

The 7.2-kb complementing fragment was sequenced and an open reading frame encoding 2,194 amino acids was found. The predicted amino acid sequence is shown in Fig. 1 *a* and the nucleotide sequence is available from Genbank. *SEC16* did not appear to be closely related to other genes in the database, but two features of the sequence are noteworthy. Overall, the sequence was hydrophilic with no obvious signal sequence or transmembrane domains, suggesting a cytoplasmic protein. In addition, the sequence contained three regions with a high density of prolyl and glycyl residues and with few charged or hydrophobic residues; amino acids 581–1050 (18% P and G), amino acids 1501–1800 (15% P and G), and amino acids 2076–2175 (32% P and G). The other regions of the protein had an unusually high density of both basic and acidic residues. The domains rich in residues that disrupt secondary structure could be extended linkers connecting charge-rich domains.

Temperature-sensitive Alleles

Five recessive temperature-sensitive mutations in *SEC16* have been isolated: *sec16-1* and *sec16-2* were found in the original screen for *sec* mutations (Novick et al., 1980), *sec16-3* was isolated in a more recent screen for secretion defective mutants (Wuestehube, L., and R. Schekman, personal communication) and *sec16-4* and *sec16-5* were isolated as mutations synthetically lethal with *sec13-1* (Roberg, K., and C. A. Kaiser, unpublished results). Each mutation was mapped by in vivo recombination tests with

a

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   1 MTPEAKKRKN QKKKLKQKQK KAAEKAASHS EEPLELPEST INSSFNDDSV   50
  51 NRTESDIASK SDVPPVSSST NISPANETQL EIPDTQELHH KLLNDSDQHD  100
 101 ITADSNDLPD NSIVEHDSVI TQTKPAMSQE YEETAAHLSS RNPSLDVVAG  150
 151 ELHNNNEHTQ KIAVSAVEED SFNEEEGENH DSIIISSLND ATPSQYNHFL  200
 201 PSDGNLLSPE LSSGDTPTHN VPLGTKDNEI NDDEYCNDKE ISLNANNVLP  250
 251 DELSKEEDER LKLETHVSTE EKKQDIADQE TAENLFTSST EPSENKIRNS  300
 301 GDDTSMLFQD DESDQKVPWE EDVKKDFHNE NTNNTQESAP NTDDRDKGYE  350
 351 GNEALKKSES CTAADERSYS EETSEDIFHG HDKQVVEGQN DFTGKNIENE  400
 401 SQKLMGEGNH KLPLSAEADI IEPGKDIQDQ AEDLFTQSSG DLGEVLPWES  450
 451 TDKNADVTSK SQEKHEDLFA ASGNDEKLPW EVSDGEVSSG KTENSMQTST  500
 501 EKIAEQKFSF LENDDDLLDD DSFLASSEEE DTVPNTDNTT NLTSKPVEEK  550
 551 KASRYKPIFE EEAGMRQEQV HFTNTTGIVT PQQFHGLTKT GLGTPNQQVS  600
 601 VPNIVSPKPP VVKDNRSNFK INEEKKKSDA YDFPLEIISE SSKKGHAKPV  650
 651 AVPTQRFGSG NSFSSLDKPI PQSRKGSNNS NRPPVIPLGT QEPRSSRTNS  700
 701 AISQSPVNYA FPNPYKIQQL QQAPIQSGMP LPNTNIPPPA LKVETTVSAP  750
 751 PIRARGVSNA SVGSSASFGA RHATQYGLNN GVPPVSPYGQ ATINLPTANK  800
 801 YAPVSPTVQQ KQYPSVVQNL GASAVNTPNF VKTHRGHTSS ISSYTPNQNE  850
 851 HASRYAPNYQ QSYQVPYTSQ PVGPVAGNSS YQSQTRSSYA VPMMPQAQTS  900
 901 ASIQPHANIQ PPTGILPLAP LRPLDPLQAA TNLQPRASNI TAANSLPLAN  950
 951 LPLAENILPE IITHRATSSV APPRQENNPI KIDNEALLRR QFPIFHWSAA 1000
1001 NKVVYAVPPI PDQSQYMISS SIVQEIKVTP IDQIIKPNDM LKSFPGPLGS 1050
1051 AKLKKKDLTK WMETTIKSIS ENESSTDMTI WQLLEMKLND KVNWKNISKL 1100
1101 LYNSDELLMY LSQPFPNGDM IPNAYRLDIN CQMRVLAFLQ TGNHDEALRL 1150
1151 ALSKRDYAIA LLVGSLMGKD RWSEVIQKYL YEGFTAGPND QKELAHFLLL 1200
1201 IFQVFVGNSK MAIKSFYTNN ETSQWASENW KSIVAAVLIN IPENNEDPLL 1250
1251 IPPVVLEFLI EFGIFLTKKG LTAAASTLFI IGNVPLSNEP VMADSDVIFE 1300
1301 SIGNMNTFES ILWDEIYEYI FSYDPKFKGF SSILPQKIYH ASLLQEQGLN 1350
1351 SLGTKYTDYL SSSVRKLPKK DILTINLTRE LSEVASRLSE SNTGWLAKPK 1400
1401 LSSVWGQLDK SFNKYIGGDD IDALNKKNDK KKVFDGFTPG SSANSSTVDL 1450
1451 TQTFTPFQAQ VTSQSYVDTT ALLHNAHNVP SHSVLHSKPS NVSKGLVEAN 1500
1501 LPYTHRIGDS LQGSPQRIHN TQFAAAEPQM ASLRRVRTDQ HTNEKALKSQ 1550
1551 QILEKKSTAY TPQFGQNHSV PMEKSNSNVP SLFADFPAPP KLGTVPSNYV 1600
1601 SSPDLVRRES IISTGSEFLP PPKIGVPTKA NSSQGSLMYS PSVEALPIDP 1650
1651 VVPQVHETGY NDFGNKHSQK SMPEDESHTS HDNSNADQNT LKDSADVTDE 1700
1701 TMDIEGPGFN DVKNLLPMEP NHQPTSTVNP IQTISDDIQP ILQTNVEVRG 1750
1751 TDASKMENSL PSIENERSSE EQPENISKSA SSAYLPSTGG LSLENRPLTQ 1800
1801 DENSISETVQ STYLPAGSIS MEAKPISQVQ DVPRNVNNKA SKLVEQHMAP 1850
1851 PKPKSTDATK MNYSPYVPQS TAASADGDES TILKTSPAIY ARTHQAHASN 1900
1901 PSQYFPLVNQ ANETASFELS ESTSQAQSNG NVASENRFSP IKKAEVVEKD 1950
1951 TFQPTIRKAS TNQYRAFKPL ESDADKYNDV IEDESDDDNM STDEAKNRKE 2000
2001 EKKNVNMKKE TKPSNKDIDD KSNGWFGWLK KDTGDKKVYK AKLGHKNTLY 2050
2051 YDEKLKRWVN KDATEEEKQK IIESSAPPPP PIVKRKDGGP KTKPRSGPIN 2100
2101 NSLPPVHATS VIPNNPITGE PLPIKTSPSP TGPNPNNSPS PSSPISRISG 2150
2151 VNLTSKKANG LDDLLSLAGG PKPASTRRKK KTARGYVNVM DNIQ       2194
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a nested set of *SEC16* deletions (Falco et al., 1983). The mutations were recovered onto plasmids by gap repair, and the appropriate region of the plasmid clone was sequenced. The five mutations were located near the middle of the gene; *sec16-1* changed Trp 1230 to Arg, *sec16-2* and *sec16-5* were identical and changed Leu 1088 to Pro, *sec16-3* changed Leu 1083 to Pro, and *sec16-4* changed Leu 1058 to Ser (see Fig. 1 *b*).

A series of *SEC16* deletions was constructed on a high copy vector to find the regions of *SEC16* necessary for complementation of the *sec16* alleles. The NH_2-terminus was not required for complementation since a truncation of *SEC16* that removed the first 565 codons (pPE129) complemented all *sec16* alleles (Fig. 2). Complementation depended on the truncated allele being expressed from a 2μ vector and transcription presumably was initiated within vector sequences. Extensive COOH-terminal deletions partially complemented in the sense that *sec16* mutant strains carrying the truncated allele on a plasmid grew slowly at 36°C, whereas strains carrying the vector only did not grow at all at this temperature. pPE131 (amino acids 565–1475) partially complemented both *sec16-1* and *sec16-2*, and pPE132 (amino acids 565–1171), which had lost the site of the *sec16-1* mutation, partially complemented *sec16-2*, but did not complement *sec16-1* (Fig. 2). This intragenic complementation behavior shows that the central domain of *SEC16* can, by itself, provide some function lost in the *sec16* mutants, and therefore ascribes a discrete function to the central portion of the protein.

NH_2-terminal Domain of SEC16 Is Not Essential

A null allele of *SEC16* was constructed by replacing the sequences from amino acid 103 to beyond the end of the coding sequence with the *TRP1* gene. This allele, *sec16-Δ1::TRP1*, behaved as a recessive lethal since a heterozygous diploid segregated as two viable and two dead ascospores on sporulation and none of the viable spore clones carried the *TRP1* marker.

The ability of truncated *SEC16* to complement temperature-sensitive alleles suggested that parts of *SEC16* may not be essential. We tested the plasmid-borne truncations of *SEC16* for the ability to complement *sec16-Δ1::TRP1* by segregational analysis in diploids heterozygous for *sec16-Δ1::TRP1* (Fig. 2). The NH_2-terminus of Sec16p (amino acids 1–564) was not essential since viable segregants car-

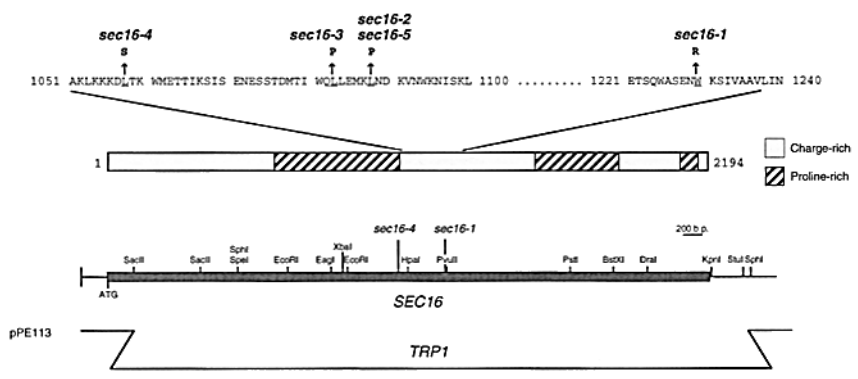

Figure 1. (*a*) The predicted amino acid sequence of *SEC16*. The *SEC16* DNA sequence data are available from GenBank/EMBL/DDBJ under accession number U23819. (*b*) Domain structure of Sec16p and locations of the ts mutations. Hatched regions designate portions of the protein that are rich in proline residues. (*c*) Restriction map of the *SEC16* gene and the *sec16-Δ1::TRP1* allele.

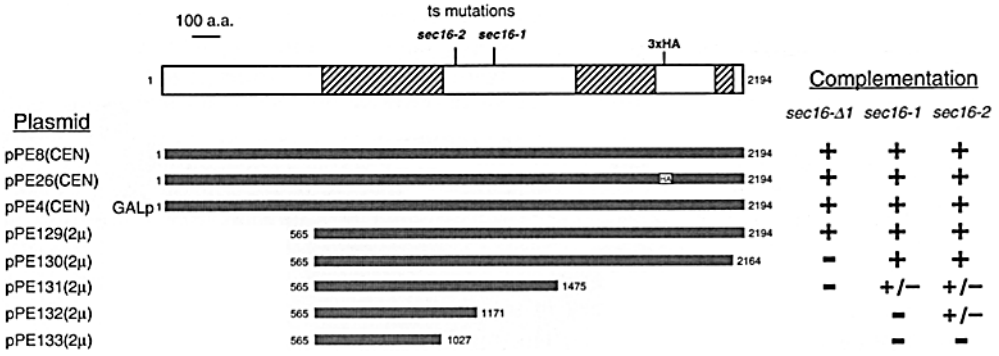

Figure 2. Complementing activity of *SEC16* truncations. Complementation of *sec16-2* (CKY200) and *sec16-1* (CKY52) by plasmids bearing different truncations was tested by growth at 36°C. Complementation of *sec16-Δ1::TRP1* at 30°C was tested by segregational analysis of diploids heterozygous for the *SEC16* null allele (CKY233). Complementation of the *sec16-Δ1::TRP1* allele by pPE4 was assayed by plasmid shuffle on medium containing 1% glucose and 1% galactose and 5-fluoro-orotic acid (Boeke et al., 1984). Plasmids pPE8, pPE26, and pPE4 are low copy, centromere plasmids. Plasmids pPE129, pPE130, pPE131, pPE132, and pPE133 are high copy, 2μ plasmids. Shaded bars and amino acid numbers indicate the portion of *SEC16* contained on the plasmid.

rying pPE129 covering the chromosomal deletion could be isolated. pPE130, which has the COOH-terminal 30 amino acids deleted, was also tested. No segregants carrying pPE130 covering the chromosomal deletion were found in 20 tetrads dissected showing that the COOH-terminus of Sec16p was essential.

Depletion of Sec16p

To test whether loss of Sec16p resulted in a phenotype similar to that of the temperature-sensitive (ts) mutations, we examined cells depleted of Sec16p. To do this a gene fusion was made that placed the expression of *SEC16* under control of the *GAL1* promoter. Full expression of *SEC16* from the *GAL1* promoter on 2% galactose is toxic to cells (see below). However, growth on medium containing 1% glucose and 1% galactose gave modest expression of Sec16p that was not toxic. A strain with *GAL1* regulated *SEC16* covering *sec16-Δ1::TRP1* on the chromosome (CKY247) allowed *SEC16* expression to be shut off by growth in glucose. ER to Golgi transport was measured by following the maturation of the vacuolar protease CPY. In a pulse–chase experiment, the core-glycosylated p1 form of the CPY proenzyme in the ER is converted to the p2 form by further glycosylation in the Golgi apparatus and finally is proteolytically processed in the vacuole to give the mature (*m*) form (Fig. 3, lanes *1–3*) (Stevens et al., 1982). When CKY247 grown in medium containing 1% glucose and 1% galactose was transferred to 2% glucose medium, the cells stopped growing within 15 h. The cessation of growth was accompanied by a complete block in the conversion of CPY from the p1 to p2 form (Fig. 3, lanes *4–6*). This defect in ER to Golgi transport is comparable to that in a *sec16-2* mutant at the restrictive temperature (Fig. 3, lanes *13–15*).

The phenotype that results from depletion of Sec16p was examined more closely by electron microscopy. CKY247 was grown in glucose medium for 16 h to develop a secretory block due to depletion of Sec16p. These cells were fixed and stained with permanganate to visualize membranes. The mutant cells accumulated excess ER membranes to a similar degree as that of *sec16-2* at the restrictive temperature (Fig. 4, *b* and *c*). Importantly, there was no accumulation of 40–50 nm vesicles as observed in mutants defective in vesicle fusion such as *sec17* and *sec18* (Kaiser and Schekman, 1990). Thus, like the ts alleles of *SEC16*, depletion of Sec16p appears to block vesicle budding.

Sec16p Is Lethal When Overexpressed

We found that a high copy, 2μ plasmid carrying *SEC16* transformed yeast at a very low frequency, suggesting that overexpression of Sec16p was toxic to cells. The effect of *SEC16* overexpression was examined systematically using the inducible *GAL1* promoter. A wild-type strain expressing *SEC16* from the *GAL1* promoter was viable in medium containing 2% glucose, but did not grow in 2% galactose medium. Truncations of *SEC16* were used to map the portion of the protein responsible for the toxicity on

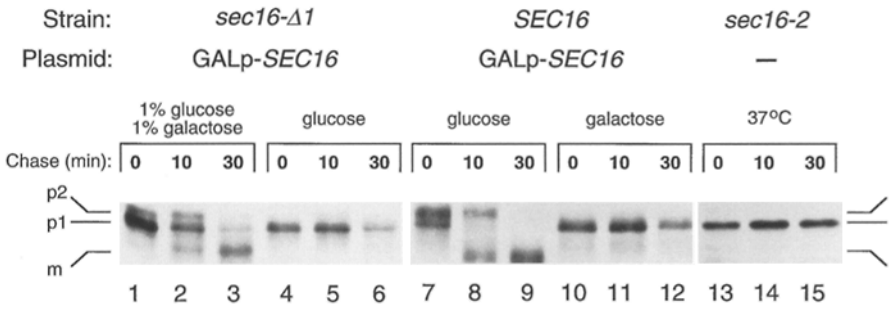

Figure 3. Depletion or overexpression of *SEC16* causes a block in ER to Golgi transport. A Gal$^+$, *sec16-Δ1::TRP1* strain carrying a plasmid with *SEC16* expressed from the *GAL1* promoter (CKY247) was grown in 1% glucose and 1% galactose (lanes *1–3*) or glucose only for 15 h to deplete cells of Sec16p (lanes *4–6*). A Gal$^+$ strain with a plasmid expressing *SEC16* from the *GAL1* promoter (CKY232) was grown in glucose (lanes *7–9*) or induced in galactose for 10 h (lanes *10–12*). CKY50, a *sec16-2* ts strain, was grown at 37°C for 1 h to express a secretion block (lanes *13–15*). Cultures were labeled with ^{35}S-translabel for 10 min and chased by the addition of excess unlabeled methionine and cysteine for 10 and 30 min. CPY was immunoprecipitated from labeled extracts and resolved by SDS-PAGE. The three forms of CPY are labeled p1 (ER), p2 (Golgi), and m (vacuole).

overproduction. Fig. 5 shows the growth on galactose medium of a strain expressing truncations of *SEC16* from the *GAL1* promoter. Strains expressing the full-length protein (*pPE4*) did not grow on galactose. Removal of amino acids 1–564 allowed slow growth on galactose. A more extensive deletion of the NH_2-terminus to beyond the first proline-rich region allowed for full growth on galactose and placed the toxic domain in the first half of the protein (Fig. 5, *pPE46*). Deletions from the COOH-terminus using *SEC16–SUC2* fusions also showed that the toxic portion of the protein lay in the NH_2-terminal domain and did not include the region where the point mutations were located (Fig. 5). Taken together, the behavior of the truncated proteins suggested that the first proline-rich region was the principle cause of lethality on overexpression. However, a test of the toxicity of this proline-rich region alone gave weak growth on galactose, suggesting that flanking regions contribute to either the toxicity or the conformational stability of this protein domain.

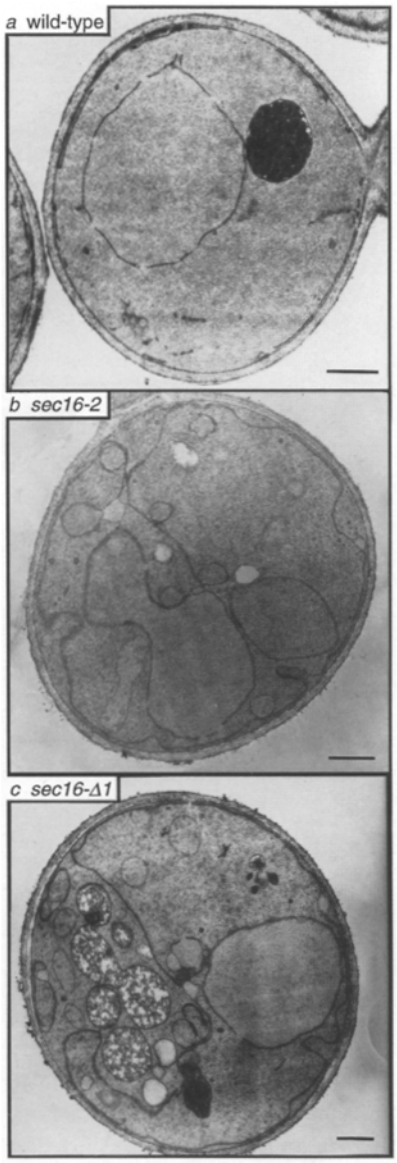

Figure 4. SEC16 depletion causes accumulation of ER membranes, but not vesicles. (*a*) Wild-type (CKY8) grown at 25°C and shifted to 37°C for 1 h. (*b*) A *sec16-2* ts mutant (CKY50) grown at 25°C and shifted to 37°C for 1 h. (*c*) A strain deleted for *SEC16* (*sec16-Δ1::TRP1*) carrying a plasmid with *SEC16* expressed from the *GAL1* promoter (CKY237), grown in glucose for 16 h at 30°C to deplete cells of Sec16p. Cells were fixed and stained with $KMnO_4$. Bars, 500 nm.

The effect of *SEC16* overexpression on ER to Golgi transport was examined by following the maturation of CPY. After induction of *GAL1* expression of *SEC16*, CPY maturation did not progress beyond the p1 form indicating a block in transport to the Golgi apparatus (Fig. 3, lanes *10–12*). This block was similar to that produced by either depletion of Sec16p or a *sec16-2* mutation (Fig. 3, lanes *4–6* and *13–15*).

Since either depletion or overexpression of Sec16p blocked ER to Golgi transport, the *sec16* ts mutations could act either by reduction or hyperactivation of *SEC16* function. Reduction of function seemed the more likely possibility since the ts alleles were recessive. We performed an additional explicit test of the mode of action of the ts mutations. Each of the four *sec16* ts alleles was overexpressed from the *GAL1* promoter and growth was examined at different temperatures. The presence of point mutations decreased the toxicity of *SEC16*, demonstrating that the mutations act by reducing, not hyperactivating, *SEC16* function (data not shown).

Sec16p Detection

To study the *SEC16* gene product, we generated antiserum to a protein containing a 460–amino acid segment internal to the *SEC16* coding sequence fused to protein A. Antibodies specific for Sec16p were affinity purified with the internal fragment of Sec16p fused to β-galactosidase. Initial attempts to identify Sec16p on Western blots failed because of two unusual properties of the protein. The protein was extremely labile in vitro and was completely degraded when cell extracts were prepared by boiling in 2% SDS. This proteolysis was eliminated either by lysing the cells with strong alkali or by preparing the extracts in the presence of protease inhibitors. Once stabilized against degradation, the full-length protein did not migrate through the stacking portion of a Laemmli gel (Laemmli, 1970), but we found that the protein could be resolved on a 6% SDS–polyacrylamide gel without a stacking layer. Anti-Sec16p antibody recognized a protein that migrated above the 190-kD molecular mass marker band (Fig. 6, lane *1*). The identity of Sec16p was confirmed by showing overproduction of this protein in a strain expressing Sec16p from the *GAL1* promoter (Fig. 6, lane *2*).

A second method for Sec16p detection used an epitope tag. Three tandem copies of the 10–amino acid epitope derived from the influenza HA protein were inserted at amino acid 1892 to yield *SEC16-HA* (Kolodziej and Young, 1991). *SEC16-HA* was shown to be fully functional by its ability to complement a null allele (Fig. 2, *pPE26*). The 12CA5 mAb recognized a protein in strains expressing *SEC16-HA* (Fig. 6, lane *4*) with the same mobility as the protein detected by Sec16p antiserum.

The extreme lability of Sec16p in extracts prompted us to examine turnover of Sec16p in vivo. Cells expressing *SEC16-HA* were radiolabeled for 10 min with [^{35}S]methionine and cysteine, and extracts were prepared at times af-

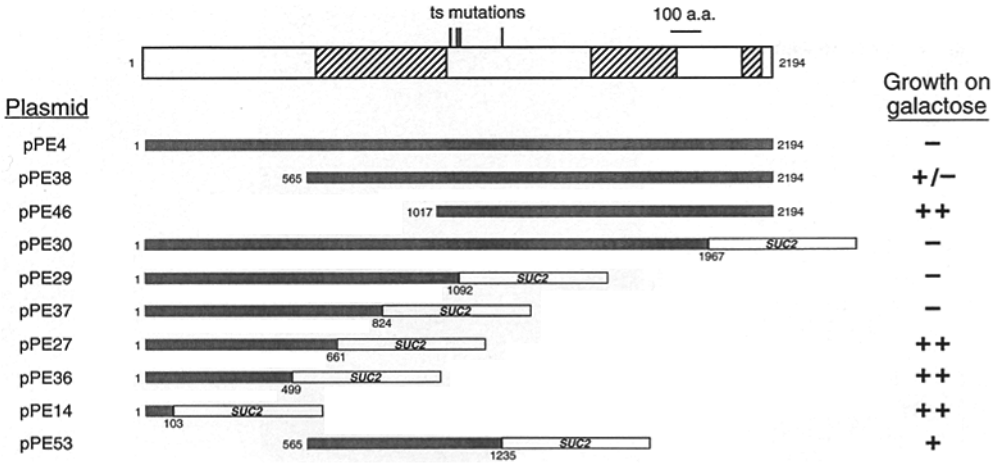

Figure 5. The NH_2-terminal domain is lethal when overexpressed. CKY96, a Gal^+ strain, was tested for the ability to grow when expressing different *SEC16* gene fragments from the *GAL1* promoter on galactose medium at 30°C. ++ indicates growth indistinguishable from CKY96 carrying vector only, and − indicates no growth. The shaded regions and amino acid numbers indicate *SEC16* sequences present in truncations and the fusions to *SUC2*.

ter addition of unlabeled amino acids. There was no reduction in labeled Sec16p after a 30-min chase (data not shown) showing that the protein is quite stable in vivo.

The abundance of Sec16p in the cell was estimated by two independent means that gave similar results. First, the invertase activity produced in a strain expressing a *SEC16–SUC2* fusion protein was used to calculate the cellular content of fusion protein assuming full specific activity of invertase (Goldstein and Lampen, 1975). The gene fusion contained the *SEC16* promoter plus the first 103 codons of Sec16p carried on a centromere plasmid (pPE14) and was expected to be expressed at approximately the same level as endogenous Sec16p. Based on the invertase activity, we calculated that there were 10^4 molecules of the hybrid protein per cell. The second method compared the intensity of bands on a Western blot using a known amount of bacterially expressed *SEC16-lacZ* fusion protein as a standard. This estimate gave 4×10^3 Sec16p molecules per cell by assuming that the full-length protein and fusion protein were electrophoretically transferred and detected with equal efficiency.

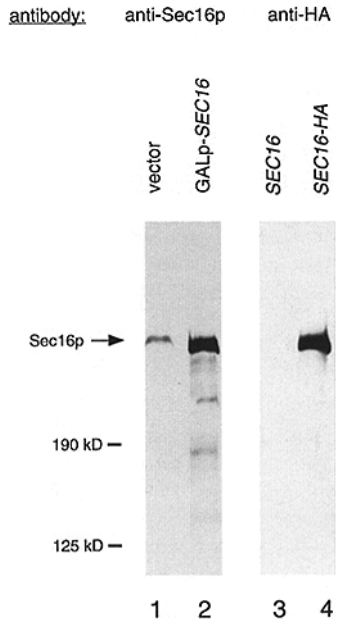

Figure 6. Antibody detection of Sec16p. CKY240 (lane *1*), a wild-type strain carrying empty vector, and CKY232 (lane *2*) expressing *SEC16* from the *GAL1* promoter, were induced in galactose medium for 4.5 h at 30°C. CKY238 (lane *3*), carrying *SEC16* on a low copy plasmid, and CKY239 (lane *4*), carrying *SEC16-HA* on a low copy plasmid, were grown in glucose at 30°C. Extracts were resolved on a 6% SDS–polyacrylamide gel without a stacking layer and Sec16p was visualized by Western blotting. Lanes *1* and *2* were probed using affinity-purified anti-Sec16p antibody. Lanes *3* and *4* were probed with the 12CA5 mAb recognizing the hemagglutinin epitope.

Sec16p Localization

The antibody to Sec16p was used to examine the intracellular distribution of the protein. A cell extract from gently lysed spheroplasts was successively centrifuged at 500, 10,000, and 150,000 *g*. Sec16p detected by immunoblotting was mostly in the 500-*g* pellet and the remainder was in the 10,000-*g* pellet (Fig. 7 *a*). As a control for cell lysis, the cytosolic form of invertase was shown to be present in the supernatant after sedimentation at 150,000 *g* (Carlson and Botstein, 1982). Conditions for release of Sec16p from the particulate fractions were tested by chemical treatments of the cell lysate followed by centrifugation at 150,000 *g* to test for protein released into the soluble fraction (Fig. 7 *b*). Sec16p was not solubilized by nonionic detergents or 2.5 M urea, but was partially released into the soluble fraction by 0.5 M NaCl or sodium carbonate (pH 11) (Fig. 7 *b*). This fractionation behavior was consistent with Sec16p being associated with a membrane or the cytoskeleton (Fujiki et al., 1982; Luna and Hitt, 1992). As a control for the efficacy of chemical extraction, the fractionation of the integral membrane protein Sec12p and the peripheral membrane protein Sec23p were also followed. Sec12p was solubilized by Triton X-100, but not by the other treatments, and Sec23p was extracted from the particulate fraction by treatment with sodium carbonate (pH 11), 2.5 M urea, and 0.5 M NaCl (data not shown) (Hicke and Schekman, 1989; Nishikawa and Nakano, 1991).

The intracellular location of Sec16p was further examined by immunofluorescence microscopy. Wild-type cells stained with affinity-purified anti-Sec16p antibody gave very weak staining. Conditions that gave clear Sec16p staining with a minimum of overproduction were found by examining a diploid strain expressing *SEC16* from the *GAL1* promoter (CKY241) 2 h after induction. Many, but not all, cells showed concentrated staining at the periphery of the DAPI-stained nucleus (Fig. 8, *a* and *b*). Since perinuclear staining is typical of ER proteins (Rose et al., 1989; Deshaies and Schekman, 1990), the pattern of Sec16p staining was consistent with some of the protein being peripherally associated with the membrane of the ER. Other cells showed punctate staining dispersed through-

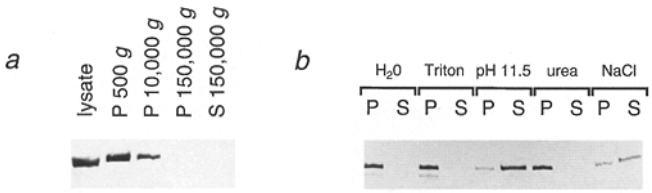

Figure 7. Sec16p is in the large particulate fraction of cell extracts and is solubilized by high salt, but not by detergent. (*a*) A cell lysate of a wild-type strain, CKY10, was subjected to a series of centrifugation steps, resulting in 500-, 10,000-, and 150,000-*g* pellets (*P*) and a 150,000-*g* supernatant (*S*). An equal number of cell equivalents was loaded in each lane. (*b*) Cell lysates were separated into pellet (*P*) and supernatant (*S*) fractions by centrifugation at 150,000 *g* after treatment with 1% Triton X-100, 0.1 M sodium carbonate (pH 11.5), 2.5 M urea, or 0.5 M NaCl. Protein samples in both *a* and *b* were analyzed by SDS-PAGE and Western blotting using anti-Sec16p antibody.

out the cell body suggesting that Sec16p was also located at sites other than the ER. The observed staining was specific for Sec16p because antibody against the HA epitope gave similar results with a strain overexpressing *SEC16-HA*, while no staining was seen in a strain overexpressing untagged *SEC16*.

Sec16p Copurifies with ER to Golgi Transport Vesicles

Since some Sec16p appeared to be on the ER, we tested directly for Sec16p on budded ER to Golgi transport vesicles produced in vitro. Transport vesicles will bud from isolated ER membranes in the presence of GTP and cytosol at 20°C (Groesch et al., 1990; Rexach and Schekman, 1991; Barlowe et al., 1994). To test for association of Sec16p with vesicles formed in vitro, partially purified ER microsomes isolated from a strain expressing *SEC16-HA* were incubated with GTP and cytosol at 20°C. Sec16p-HA behaved identically to Sec16p in all cell fractionation experiments, and therefore Sec16p-HA was used to facilitate the detection of Sec16p (data not shown). Because Sec16p-HA was insoluble, the only source of Sec16p-HA in this reaction was the microsomal fraction. A crude vesicle fraction was obtained by removing donor membranes from the reaction by centrifugation at 12,000 *g* and then collecting vesicles by centrifugation at 100,000 *g*. As expected for a vesicle protein, Sec16p-HA entered the vesicle fraction under conditions that promote vesicle formation from the ER; Sec16p-HA was in the vesicle fraction when incubated with GTP and cytosol at 20°C, but Sec16p-HA in this fraction was greatly reduced when the incubation was carried out at 4°C, with apyrase, or without cytosol (Fig. 9 *a*). Sec22p, an integral membrane protein that resides in ER to Golgi transport vesicles, served as a marker for this organelle (Newman et al., 1990; Lian and Ferro-Novick, 1993; Barlowe et al., 1994). The conditions for release of Sec16p-HA into the vesicle fraction paralleled those for Sec22p (Fig. 9 *a*). Fragmentation of the ER was ruled out as a possible source of Sec16p-containing membranes since the resident ER protein Sec61p was not present in this fraction (Fig. 9 *a*) (Rexach et al., 1994).

A more definitive test for association of Sec16p-HA with ER to Golgi transport vesicles is to examine whether

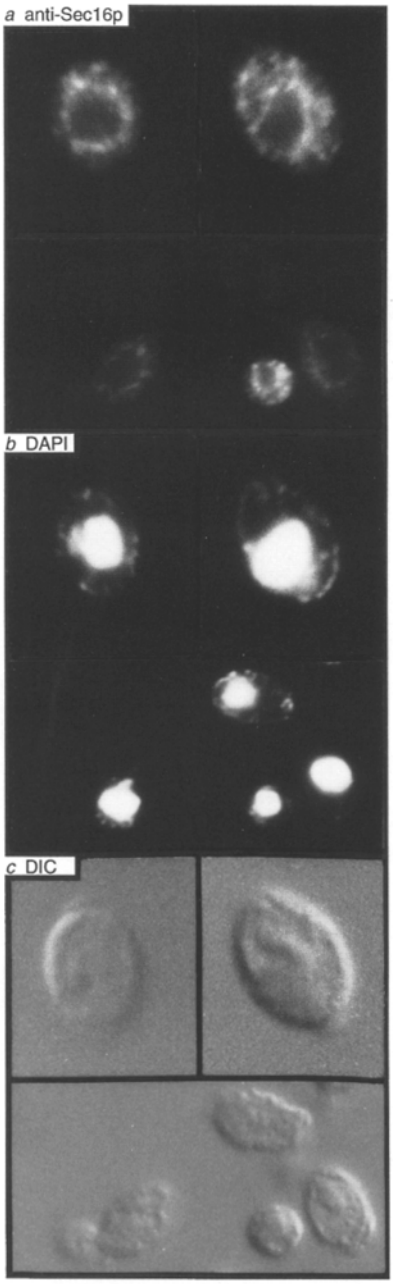

Figure 8. Immunolocalization of Sec16p. A wild-type diploid strain, CKY241, expressing *SEC16* from the *GAL1* promoter was grown in galactose for 2 h and examined by indirect immunofluorescence using affinity-purified rabbit anti-Sec16p antibody and a fluorescein-conjugated anti–rabbit secondary antibody. (*a*) Fluorescein-stained Sec16p. (*b*) DAPI-stained nuclear DNA. (*c*) Cell bodies visualized by differential interference contrast (DIC). Magnification in top panels: 2,800×; bottom panel, 1,750×.

the vesicles that contain Sec16p-HA behave similarly on gel filtration as Sec22p-containing vesicles. It was shown previously that vesicles formed in the presence of GMP-PNP retain their coat of peripheral membrane proteins, whereas those formed in the presence of GTP do not (Barlowe et al., 1994). In our assay, GMP-PNP and GTP gave similar levels of vesicle formation (Fig. 9 *a*, *Sec22p*). We chose to use GMP-PNP to produce a population of vesicles that were all coated and therefore would display uni-

form characteristics on fractionation. Vesicles synthesized in a reaction using GMP-PNP were separated by gel filtration on a Sephacryl 1000 column. Fractions were sedimented at high speed to collect vesicle pellets which were examined for the presence of Sec16p-HA and Sec22p by Western blotting. Sec22p-containing vesicles eluted as a single peak in the included volume before the elution of most of the total protein (Fig. 9 *b*). The elution profile for Sec16p-HA was identical to that for Sec22p. Thus, Sec16p-HA was either associated with the same ER-derived vesicle population as Sec22p or was associated with other vesicles that form under the same conditions and were of the same size.

COOH Terminus of Sec16p Binds Sec23p

The copurification of Sec16p with ER to Golgi transport vesicles prompted us to investigate potential protein–protein interactions between Sec16p and other recognized components of the vesicle coat, using the two-hybrid interaction assay (Fields and Song, 1989; Gyuris et al., 1993). Three overlapping fragments of *SEC16* (amino acids 1–824, 447–1737, and 1645–2194) were tested independently by fusion to a *lexA* DNA binding domain. *SEC23* and *SAR1* each were fused to an acidic activation domain and interaction with each of the *SEC16* fragments was tested by assaying activation of transcription of a *lacZ* reporter gene. Strong induction of β-galactosidase activity was observed only when the COOH terminus of Sec16p (amino acids 1645–2194) was combined with Sec23p (Table III). This interaction was shown to depend on a functional COOH-terminal domain of *SEC16* since a parallel experiment conducted with a derivative of the COOH-terminal domain that had the last 30 amino acids removed gave no interaction (not shown). These results indicated that the COOH terminus of Sec16p binds to Sec23p.

As an independent test for this interaction, binding of the COOH terminus of Sec16p to Sec23p was examined in yeast cell extracts. The coding sequence of *SEC23* was fused to GST expressed from the *GAL1* promoter (Smith and Johnson, 1988). The COOH terminus of Sec16p (amino acids 1638–2194) containing three copies of the hemagglutinin epitope tag (*SEC16C-HA*) was also expressed from the *GAL1* promoter. We found that, unlike the full-length Sec16p, a large fraction of the COOH-terminal domain was soluble in cell extracts, thus allowing solution binding studies to be performed. Extracts were prepared from strains overexpressing Sec16Cp-HA and either GST-Sec23p or GST only. Proteins bound to GST-Sec23p were isolated on glutathione Sepharose beads, and the presence of Sec16Cp-HA was tested by Western blotting with the 12CA5 mAb. Sec16Cp-HA associated with GST-Sec23p, but not GST, demonstrating dependence on Sec23p (Fig. 10, lanes *1–4*). These experiments demonstrated that the COOH terminus of Sec16p can form a complex with Sec23p in the soluble fraction of cell extracts.

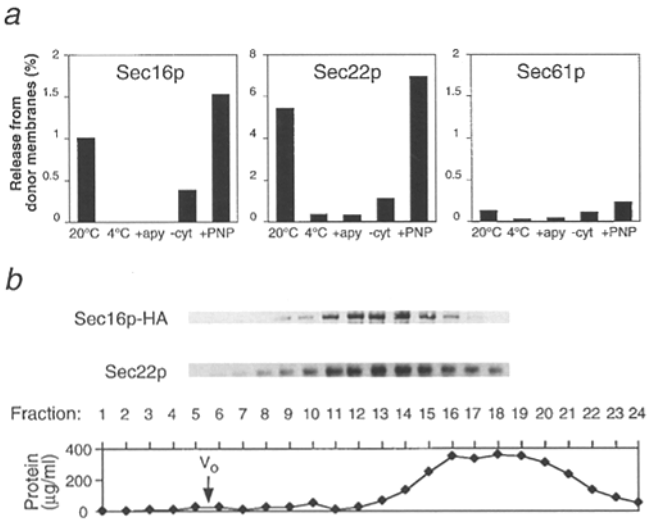

Figure 9. Sec16p-HA cofractionates with ER-derived vesicles. (*a*) Vesicle budding reactions were performed under the following conditions: complete reaction at 20°C, complete reaction at 4°C, reaction with apyrase added, reaction without cytosol, and reaction with GMP-PNP instead of GTP. The amount of Sec16p-HA, Sec22p, or Sec61p released from the donor membranes into the crude vesicle fraction was quantitated by Western blotting and is expressed as a percentage of that protein present in the total reaction. Data shown are the average of two experiments. (*b*) A crude vesicle fraction from a budding reaction performed in the presence of GMP-PNP was resolved by gel filtration on Sephacryl 1000. Membrane pellets were collected from column fractions by centrifugation and were examined by SDS-PAGE and Western blotting. The elution profile of total protein and the estimated void (V_o) fractions are shown.

Discussion

The *SEC16* gene had previously been shown to be one of the genes required for the formation of ER transport vesicles in vivo (Kaiser and Schekman, 1990). *SEC16* interacts genetically with *SEC23*, *SEC13*, and *SAR1*, genes whose products are part of a cytosolic protein coat, termed COPII, that encapsulates vesicles assembled from ER membranes in vitro (Barlowe et al., 1994). Taken together, these results suggested that *SEC16* might take part in the formation of the COPII vesicle coat. In this report, Sec16p finds its place as a constituent of COPII-coated vesicles. This conclusion rests on two findings. First, Sec16p appears to be associated with ER-derived vesicles produced in an in vitro budding reaction. When membranes bearing Sec16p are incubated with cytosol, some of the Sec16p is released into a slowly sedimenting fraction in a tempera-

Table III. Sec23p Interacts with the COOH Terminus of Sec16p by Two-Hybrid Assay

	β-galactosidase activity	
	SEC23	*no fusion*
SEC16(aa 1645–2194)	952.9 ± 39.0	6.2 ± 2.1
SEC16(aa 447–1737)	85.4 ± 14.1	12.2 ± 3.3
SEC16(aa 1–824)	10.1 ± 9.4	6.8 ± 1.8
no fusion	32.9 ± 12.6	118.9 ± 128.6*

EGY40 cells transformed by plasmids encoding a *lexA* fusion protein (pEG202, pPE58, pPE59, or pPE74), an activator domain fusion protein (pJG4-5 or pPE81), and a reporter plasmid (pSH18-34) were grown in galactose for 10 h before the assay to induce expression of fusion proteins. Units of β-galactosidase activity (nmol/mg × min) were calculated as (optical density at 420 nm × vol of assay)/(0.0045 × concentration of protein in extract assayed × time). Activities shown were the mean ± SD for three independent transformants.

*The three values from this experiment were 20.6, 71.6, and 264.4 units.

ture- and nucleotide-dependent reaction. Release of Sec16p occurs under conditions that closely parallel the behavior of Sec22p, an integral membrane protein marker for ER-derived transport vesicles. When the material released from ER membranes is subjected to gel filtration, Sec16p cofractionates with the Sec22p-containing vesicles. These results strongly suggest that Sec16p is coating ER-derived vesicles. We cannot rigorously rule out the possibility that Sec16p is associated with vesicles derived from another source, such as the Golgi apparatus. However, if this is the case, these other vesicles must form under the same conditions and have the same gel filtration properties as vesicles derived from the ER. The principal difficulty in establishing this point is that we have not yet found conditions whereby ER-derived vesicles can be affinity purified with the COPII coats intact.

The second finding that indicates Sec16p is part of the COPII coat is that Sec16p binds to the COPII protein Sec23p. Initially, this interaction was detected using the two-hybrid transcription assay when testing for interactions between *SEC16* and the COPII genes, *SEC23* and *SAR1*. A strong interaction was only detected for *SEC23* in combination with the COOH-terminal domain of *SEC16*. Binding of these proteins was confirmed by showing that the COOH-terminal domain of *SEC16* expressed in yeast cells is found in protein complexes affinity isolated using a GST-Sec23p fusion. The full-length Sec16p was not used in these tests because it is so tightly bound to intracellular membranes that the protein is not available in cell extracts for affinity isolation steps. The simplest interpretation of these results is that Sec23p is in physical contact with the COOH terminus of Sec16p, however, direct protein–protein contact has not been established. It is possible that a third protein present in the cytoplasm (and in the nucleus for the two-hybrid assay) binds to both Sec16p and Sec23p, providing a link between the two proteins.

ER membranes that have been stripped of peripheral proteins by washing with urea are absolutely dependent on cytosolic proteins for the formation of transport vesicles in vitro (Baker et al., 1988; Salama et al., 1993). The five purified COPII proteins, Sec23p/Sec24p, Sec13p/Sec31p, and Sar1p, satisfy the cytosolic requirement, and

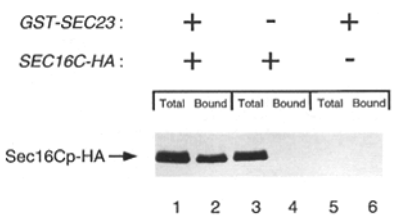

Figure 10. Complexes formed between Sec23p and the COOH terminus of Sec16p. Lanes *1* and *2*: CKY282, with both *GST-SEC23* (pPE119) and *SEC16C-HA* (pPE86). Lanes *3* and *4*: CKY282, with GST (pRD56) and *SEC16C-HA* (pPE86). Lanes *5* and *6*: CKY282, with *GST-SEC23* (pPE119) and the empty vector (pRS315). Expression of proteins from the *GAL1* promoter was induced by growth in galactose for 4 h. Lysates from 10^7 cells were cleared by centrifugation and proteins bound to glutathione Sepharose beads were isolated. Proteins bound to the glutathione beads are in lanes *2*, *4*, and *6*. One-tenth of the total lysate was loaded in the extract lanes *1*, *3*, and *5*. Sec16Cp-HA was detected by SDS-PAGE and Western blot analysis using the 12CA5 mAb.

these proteins become components of a protein coat on the transport vesicles formed in this reaction (Barlowe et al., 1994). No requirement for exogenously added Sec16p has been detected for the vesicle budding reaction, but a reason for this is now clear. Whereas other peripheral ER proteins, such as Sec13p and Sec23p (Hicke and Schekman, 1989; Pryer et al., 1993), are removed from ER membranes by washing with urea, we have found that Sec16p is not extracted from membranes by this procedure. Thus, to the extent that Sec16p is needed for budding in vitro, the requirement is presumably satisfied by Sec16p introduced into the budding reaction as a component of the donor ER membranes.

Models for transport vesicle budding have emphasized the role of vesicle coat proteins partitioned between soluble and membrane-bound states. The idea is that vesicle formation is driven by the assembly of soluble coat proteins onto the membrane (Pearse and Robinson, 1990; Rothman and Orci, 1992). If Sec16p is such a coat protein, the time that it spends off the membrane must be very brief since there is virtually no soluble Sec16p detected in cell extracts. When Sec16p is localized using cell fractionation and immunofluorescence, some of the protein appears to be associated with the ER. A simple explanation for this behavior is that Sec16p forms a permanent, peripheral membrane coat. The Sec16p that resides on the surface of the ER may be required to recruit other vesicle coat components, such as Sec23p, to the sites of vesicle budding.

The finding that overexpression of *SEC16* on a 2μ plasmid or from the *GAL1* promoter is lethal and blocks ER to Golgi transport suggested that Sec16p could be an inhibitor of vesicle formation. According to this hypothesis, the ts alleles could exert their effect by hyperactivating the inhibitory function. Two genetic experiments argue strongly against this possibility. First, depletion of Sec16p by shutting off regulated expression from the *GAL1* promoter is lethal and causes a secretion block. Thus, *SEC16* performs a positive function in transport and cannot simply be an inhibitor. Second, we tested the effect of mutations on the toxicity of overexpressed *SEC16*; if the point mutations are hyperactivating, then overexpression of the mutant alleles at high temperature should inhibit growth more than wild-type *SEC16*. We found that the overexpressed ts alleles are less restrictive than wild-type, and therefore conclude that the ts alleles antagonize the function of *SEC16* that causes lethality on overproduction. Thus, there is a critical stoichiometry for Sec16p, and more or less protein causes a lethal secretion defect. This behavior points to a structural role for Sec16p in an assembly whose subunit composition is critical for function and too much or too little Sec16p leads to the assembly of inactive complexes.

The *SEC16* sequence contains alternating regions that are either rich in charged amino acids or rich in proline and uncharged residues. Clusters of proline residues have been observed in synaptic vesicle proteins and have been proposed to serve as structural spacers between functional domains (Linial, 1994). Interestingly, our results indicate that Sec16p is a multifunctional protein, suggesting that the proline-rich regions may serve to connect globular domains that carry out different functions.

Genetic analysis of *SEC16* identifies three functionally separable domains that roughly correspond to the central region, NH_2-, and the COOH termini of the protein. The five ts mutations are clustered in a 250-bp region of the gene suggesting that the mutations may affect a single function. Internal fragments of *SEC16* that contain the central portion of the protein can complement these mutations. This intragenic complementation behavior defines the central region of the protein as an independent functional unit.

We were able to map roughly the portion of the protein that causes a lethal secretion block on overexpression by testing truncated versions of Sec16p for this lethal effect. The critical portion of the protein lies in the NH_2-terminal region (amino acids 1–824). This part of the protein may bind to and thereby deplete another factor necessary for vesicle formation. This second domain, defined by overexpression lethality, extends to the middle of the first proline-rich region, but does not overlap with the region containing the point mutations.

Biochemical and genetic experiments identify the COOH terminus as a third functional domain of Sec16p. The COOH terminus of Sec16p (amino acids 1643–2194) and Sec23p interact in the two-hybrid assay and by binding experiments in yeast cell extracts. In the accompanying paper, we show that the cytosolic domain of the ER protein Sed4p also binds to the COOH-terminal domain of Sec16p. Complementation experiments demonstrate that the COOH terminus of *SEC16* is essential for the growth of a strain deleted for *SEC16*. This requirement for the COOH terminus may reflect the need for this domain in binding to Sec23p, Sed4p, and possibly additional proteins.

Collectively, *SEC16* functional studies indicate that Sec16p is composed of a number of different functional units. We are now in a position to identify other transport factors that bind to the different regions of *SEC16* by affinity purification, genetic suppression screens, or two-hybrid screens. Since Sec16p appears to be part of the vesicle coat, sandwiched between membrane proteins and the cytosolic coat proteins, many of the significant subunit interactions in the vesicle coat structure may be revealed by studying proteins associated with *SEC16*.

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